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RESEARCH ARTICLE

Isolation of the Tomato *AGAMOUS* Gene *TAG1* and Analysis of Its Homeotic Role in Transgenic Plants

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To understand the details of the homeotic systems that govern flower development in tomato and to establish the ground rules for the judicious manipulation of this floral system, we have isolated the tomato *AGAMOUS* gene, designated *TAG1*, and examined its developmental role in antisense and sense transgenic plants. The *AGAMOUS* gene of Arabidopsis is necessary for the proper development of stamens and carpels and the prevention of indeterminate growth of the floral meristem. Early in flower development, *TAG1* RNA accumulates uniformly in the cells fated to differentiate into stamens and carpels and later becomes restricted to specific cell types within these organs. Transgenic plants that express *TAG1* antisense RNA display homeotic conversion of third whorl stamens into petaloid organs and the replacement of fourth whorl carpels with pseudocarpels bearing indeterminate floral meristems with nested perianth flowers. A complementary phenotype was observed in transgenic plants expressing the *TAG1* sense RNA in that first whorl sepals were converted into mature pericarpic leaves and sterile stamens replaced the second whorl petals.

INTRODUCTION

Genetic analysis and molecular isolation of several floral homeotic genes from Antirrhinum and Arabidopsis culminated in the formulation of a combinatorial model for flower organ identity genes (Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991b; Coen and Meyerowitz, 1991). Alone or in combination, genes of three groups regulate the formation of unique organs in each of the four whorls of dicot flowers. The activity of group A genes in whorl one, or of group C genes in whorl four, leads to the formation of sepals and carpels, respectively. The combined activities of group A and B genes in whorl two and of group B and group C genes in whorl three lead to the specification of petals and stamens, respectively.

Several homeotic genes of group B (*DEFICIENS A*, *GLOBOSA*, and *APETALA3* [Sommer et al., 1990; Jack et al., 1992; Tröbner et al., 1992]) and group C (*AGAMOUS* [*AG*] and *PLENA* [Yanofsky et al., 1990; Bradley et al., 1993]) from Antirrhinum and Arabidopsis were isolated and shown to encode putative transcription factors of the MADS box gene family (Schwarz-Sommer et al., 1990). The isolation and sequence analysis of additional MADS box genes from Arabidopsis and tomato led to the suggestion that the divergence of MADS box genes, as well as their selection as important regulatory factors of flower development, preceded the divergence of dicot plant species (Ma et al., 1991; Pnueli et al., 1991).

The combinatorial model provides an excellent reference point for a more detailed analysis as it contains elements of the genetic and molecular components that will be needed to understand the evolution of the basic floral structures and the vast variation that is observed among plant species. Such variations are most likely due to modulated spatial expression of the major combinatorial genes, to subtle changes in their temporal expression, and to the multiplicity of flower-specific MADS box genes, which number up to two dozen per species. Given that at least some of their gene products are known to interact (Tröbner et al., 1992), such a multiplicity probably plays a major role in the fine regulation of the floral programs.

Although cognate homologs for many of the floral homeotic genes have been isolated and characterized from a number of plant species, they clearly play somewhat different roles in different plants. For example, mutations in the petunia group B gene *GREEN PETALS* (van der Krol et al., 1993) affect predominantly the second whorl organs in contrast to *DEFICIENS A*, *GLOBOSA*, *APETALA3*, and *PISTILLATA* mutations, which alter organs in both second and third whorls of Antirrhinum and Arabidopsis flowers (Coen and Meyerowitz, 1991). Similarly, mutations in floral meristem identity genes *SQUAMOSA* and *FLORICAULA* from Antirrhinum and cognate homologs

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APETALA1 and *LEAFY* from Arabidopsis lead to related but distinct phenotypes (Coen et al., 1990; Huijser et al., 1992; Mandel et al., 1992b; Weigel et al., 1992).

Tomato is one of several important crop plants in which genetic analysis of flower development is hindered by the lack of appropriate mutations and by the time-consuming effort needed to collect such mutations in the absence of a gene-tagging system. Currently, a rational means of studying flower development in such genetically handicapped species is to produce, by means of reverse genetic techniques, a graded series of phenotypic variations that will mimic gene mutations. To this end, we have isolated several members of the MADS box gene family from tomato (Pnueli et al., 1991) and are studying the consequences of gene inhibition and ectopic or gain-of-function situations by generating transgenic plants bearing antisense and sense gene constructs.

A pivotal role in the combinatorial gene model is played by the group C gene *AG* (Yanofsky et al., 1990; Bowman et al., 1991b; Bradley et al., 1993). Mutations in the *AG* gene of Arabidopsis and its homolog *PLENA* of Antirrhinum result in the conversion of stamens into petals and in the indeterminate formation of nested flowers in the fourth whorl, with each internal flower consisting of only perianth organs. Consistent with the genetic model, ectopic expression of the *AG* gene in the first and second whorls results in the homeotic alterations of sepals to carpels and petals to stamens (Mandel et al., 1992a; Mizukami and Ma, 1992; Bradley et al., 1993).

The well-characterized morphogenetic alterations that are expected from inhibition or overexpression of the *AG* gene are ideal for the establishment of the ground rules for further analysis of species in which such studies depend primarily or exclusively on the reverse genetic approach. Once consistent results are obtained, the mutated plants can be subjected to further molecular genetic manipulations. This is especially attractive in species like tomato, which can be maintained indefinitely by vegetative propagation.

We have isolated the cognate homolog of the *AG* gene from tomato, which we have designated *TAG1*. We have determined the domains of its developmental expression and the morphological consequences of its inhibition and ectopic expression. The results of this study permitted the analysis of the functionally anonymous MADS box gene from tomato, *TM5*, which is expressed in three, rather than two, whorls (see the companion paper, Pnueli et al., 1994).

RESULTS

Molecular Cloning and Sequence Analysis of *TAG1*

As a start toward identifying and characterizing the tomato homolog of the Arabidopsis floral homeotic gene *AG*, a cDNA library prepared from RNA isolated from tomato pistils was screened under reduced stringency with an *AG*-specific probe. One class of isolated cDNA was selected for subsequent analyses based on its high degree of sequence similarity to the Arabidopsis *AG* cDNA. The resulting gene, designated *TAG1* (Tomato *AGAMOUS1*), encodes a putative 28.7-kD protein with 248 amino acids and a pI of 9.60 (Figure 1A). A comparison of the deduced *TAG1* protein sequence with that of other putative *AG* homologs from other plant species is shown in Figure 1B. Overall, TAG1 is most similar to NAG1 from tobacco; they share greater than 90% sequence identity over their entire lengths.

Located in the main terminal region of each deduced protein is the 56-residue MADS domain (see Introduction), which is remarkably conserved among the *AG* gene products from the various plant species. TAG1 shares greater than 96% sequence identity to all of the previously characterized AG homologs within this MADS domain. In addition, there are several other regions of these putative proteins that share striking sequence conservation; this suggests that these regions may define important functional domains of the AG protein.

The *TAG1* cDNA was placed on the restriction fragment length polymorphism map between markers TG14 and TG308 on chromosome 2. There are no known morphological mutants at this location that would correspond to mutations in the *TAG1* gene. A detailed map of chromosome 2 is given in Tanksley et al. (1992).

TAG1 Is Expressed in Stamens and Carpels

Monitoring spatial and temporal expression of the *TAG1* gene by means of RNA tissue in situ hybridization revealed that it is expressed only in the primordia and developing organs of whorls three and four. These expression territories correspond to the expression domains of its cognate genes in Arabidopsis and Antirrhinum (Yanofsky et al., 1990; Drews et al., 1991; Bradley et al., 1993). Early in flower development, *TAG1* RNA is detected in all cells of the stamen primordia and the central region of the floral meristem (Figures 2A and 2B). Although *TAG1* expression is uniform in cells fated to develop into the two inner whorls early in flower development, at later developmental stages the distribution of *TAG1* transcripts becomes progressively confined to specific cell types (Figures 2C to 2F). *TAG1* expression levels remain high in the walls of the developing anthers (3-mm-long floral bud, one-quarter of final length, Figure 2C) through the circumference of the lobes, and in a later stage after pollen mother cells have completed differentiation (Figure 2D). The solitary vascular bundles of the filaments and all parenchyma cells of the connective exhibit high signals, but endothecial cells, tapetum, and pollen mother cells show only background level. *TAG1* is also highly expressed in the vascular bundles of the ovary and style (Figures 2E and F). *TAG1* transcripts, like those of *AG* in Arabidopsis, are extremely abundant in the ovules, presumably in the integuments (Bowman et al., 1991a), as well as in the conducting tissue of the style.

A

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CATTATATACTAATTTGCAACCAAAAAACTTAAAAATCTTCTCTTTCCTTTCCTTACAAGGTGAAGTA
ATG GAC TTC CAA AGT GAT CTA ACC AGA GAG ATC TCA CCA CAA AGG AAA   48
 M  D  F  Q  S  D  L  T  R  E  I  S  P  Q  R  K                 16

CTA GGA AGG GGG AAA ATT GAG ATC AAA AGG ATC GAA AAC ACG ACG AAT   96
 L  G  R  G  K  I  E  I  K  R  I  E  N  T  T  N                 32

CGA CAA GTA ACA TTC TGC AAG AGG CGC AAT GGT TTG CTT AAA AAG GCT  144
 R  Q  V  T  F  C  K  R  R  N  G  L  L  K  K  A                 48

TAT GAA TTG TCT GTG CTC TGT GAT GCT GAG GTT GCT TTG GTT GTC TTC  192
 Y  E  L  S  V  L  C  D  A  E  V  A  L  V  V  F                 64

TCA AAC AGA GGC AGA CTC TAT GAG TAT GCC AAC AAC AGT GTG AAA GCA  240
 S  N  R  G  R  L  Y  E  Y  A  N  N  S  V  K  A                 80

ACA ATC GAG AGG TAC AAG AAA GCA TGC TCA GAT TCC TCA AAC ACT GGT  288
 T  I  E  R  Y  K  K  A  C  S  D  S  S  N  T  G                 96

TCA GTA TCC GAG GCC AAT GCT CAG TAT TAC CAG CAA GAA GCC TCC AAA  336
 S  V  S  E  A  N  A  Q  Y  Y  Q  Q  E  A  S  K                112

CTG CGC GCA CAA ATT GGA AAT CTG ATG AAC CAA AAC AGG AAC ATG ATG  384
 L  R  A  Q  I  G  N  L  M  N  Q  N  R  N  M  M                128

GGT GAA GCT CTT GCT GGA ATG AAA CTC AAA GAA CTG AAG AAT CTG GAG  432
 G  E  A  L  A  G  M  K  L  K  E  L  K  N  L  E                144

CAA AGA ATT GAA AAA GGG ATT AGC AAA ATC CGA TCC AAA AAG AAT GAG  480
 Q  R  I  E  K  G  I  S  K  I  R  S  K  K  N  E                160

CTG TTG TTT GCT GAA ATT GAG TAT ATG CAG AAG AGG GAA GTT GAT TTA  528
 L  L  F  A  E  I  E  Y  M  Q  K  R  E  V  D  L                176

CAC AAC AAC AAT CAG TAC CTG AGA GCA AAG ATT GCT GAA ACT GAG AGA  576
 H  N  N  N  Q  Y  L  R  A  K  I  A  E  T  E  R                192

GCT CAG CAT CAG CAT CAG CAG ATG AAC TTG ATG CCA GGG AGT TCA TCA  624
 A  Q  H  Q  H  Q  Q  M  N  L  M  P  G  S  S  S                208

AAC TAT CAT GAG CTT GTG CCT CCA CCT CAG CAA TTC GAT ACT CGA AAC  672
 N  Y  H  E  L  V  P  P  P  Q  Q  F  D  T  R  N                224

TAT CTA CAA GTT AAT GGA TTG CAA ACC AAC AAC CAT TAC CCT AGA CAA  720
 Y  L  Q  V  N  G  L  Q  T  N  N  H  Y  P  R  Q                240

GAC CAA CCC CCT ATT CAA CTA GTC TAA                             747
 D  Q  P  P  I  Q  L  V  *                                      248

TGTGCTTGAGAGATTGTCTTCTTTCTGATGTGATCTACATATAATCCACAAAAGAAGACTGCTATTAA
GCTTGAAGATTAATGGAACTTAAGTATGTATACCATATTATTAATTACTTGTTGCATGAGCTAAGACT
CTTGAATATTGTATGTTAATTAAGTGGACATGTATTTTTTTTGCTTGGTGTACTTCCTTTTCTGCCAG
TGAACTATGAAGAATTAAGCTTGTTATATTAAGTTCACAACTGTTTTTCTGTCCAAAAAAAAAAAA
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B

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1  TAG1     MDFQSDLTRE ISPQRKLGRG KIEIKRIENT TNRQVTFCKR
1  NAG1     ********** ******V*** ********** **********
1  PLENA    *E*PNQD--- SESL**N*** *********I **********
35 AGAMOUS  TAY**E*GGD S**L**S*** ********** **********
1  BAG1     *AY*ME*GG* S*****A*** ********** **********
37 ZAG1     GSASVAGSAA ERNNGGR*K* *T******** **********

RNGLLKKAYE LSVLCDAEVA LVVFSNRGRL YEYANNSVKA TIERYKKACS
********** ********** *I***S**** ********** **********
********** ********** *****S**** ********R* ********SA
********** ********** *I***S**** ***S*****G ********I*
********** ********** *I***S**** ***S*****G ********I*
********** ********** *I***S**** *********G ********T*

DSS-NTGSVS EANAQYYQQE ASKLRAQIGN LMNQNRNMMG EALAGMKLKE
***-****I* ********** ********** *Q******L* *S**ALS*RD
***-*SV*T* ***T*F**** *N***R**RE IQTS**Q*L* *GVSN*A**D
*N*-*****A *I********* SA***Q**IS IQ*S**QL** *TIGS*SP**
*N*-*****A *I********* SA***Q**IS IQ*S**QL** *TIGS*SP**
*N*SAA*TIA *VTI*H*K** SAR**Q**V* *Q*S**ALI* DSITT*SH**

LKNLEQRIEK GISKIRSKKN ELLFAEIEYM QKREVDLHNN NQYLRAKIAE
******K*** ********** ********** ****I***** **********
**ST*AKV** A**R****** ********H* ****LE***A *MF********
*R***G*L*R S*TR****** ****S**D** *********D **I*******
*R***G*LDR SVNR****** *******D** *********D **L*******
**H**T*LD* ALG***A*** DV*CS*V*** *R**ME*Q*D *L***SRVD*

TERA--QHQH QQMNLMPGSS SNYHELVPPP ----QQFDTR NYLQVNGLQT
****QQ*Q*Q ********** *-*-****** ----H***** **********
G***--*-*- -********D YQPMTS---- ----*SY*V* *F*PM*LMEP
N**-----NN PSIS****-G ***EQ*M*** QTQS*P**S* **F**AA**P
N**-----NN PS*S****G* NYEQIMR**- QTQP*P**S* **F**AA**P
N***--*QTA NM*--GAP*T *E*QQHGFT* ----YDP-I* SF**F*IV*Q

NNH-YPR--- Q-DQPPIQLV*
****-*T*--- *-***SL***.
NQQQ*S*--- H-**TAL***.
***H*SSAGR *-**TAL***.
***H*SSAGR E-**TAL***.
PQF-*SQQED RKDFNDQGGR.
```

TAG1 Antisense RNA Confers Homeotic Transformations of Floral Organs in the Third and Fourth Whorls

Although molecular homology and corresponding expression patterns are excellent criteria for assessing the role of a cloned gene, only functional tests can establish the developmental role of a given gene member of the MADS box family. Therefore, employing the antisense RNA procedure in tomato, we have tried to mimic the phenotypic alterations that are caused by mutations in the *AG* and *PLENA* genes of Arabidopsis and Antirrhinum, respectively.

Twenty independent kanamycin-resistant plants were generated. Fourteen plants displayed aberrant flowers of the type described below and were, in addition, male and female sterile. Six plants were completely normal and fertile. Of the 14 aberrant plants, two exhibited the most extreme alterations of floral organs in whorls three and four. In the other 12 affected T_1 plants, the morphogenetic alterations of whorl four were as severe as those exhibited by the two extreme plants, but they differed with respect to the alterations conferred in the third whorl.

The extreme phenotype is exemplified by the description of flowers of the transgenic antisense plant 2-40 (Figure 3). All vegetative parts of the plants as well as sepals and petals were identical to the progenitor plant in every respect. The regular wild-type stamens of the third whorl were replaced, however, by extremely short diminutive petaloid organs, one-tenth or less of the size of normal petals (Figure 3A). No normal stamens were formed, but adventitious additional petaloid structures were occasionally detected (see below and Figure 3E).

The most dramatic changes are observed in the floral structures that replace the pistillate organ. Outer, somewhat twisted, evergreen sepaloid leaves were connately fused to engulf the internal indeterminate succession of floral buds to form a pseudocarpel. Additional floral meristems developed in the positions normally occupied by placental tissue and ovules. Each of these primary floral buds developed only perianth organs, of which the outer one had the same anatomy and distribution of vascular bundles as the outer envelope of the pseudocarpel (see Figures 3E and 3F). Each of these lateral flowers then gave rise to an additional nested bud of identical organization.

Figure 1. Nucleotide and Deduced Amino Acid Sequence of the *TAG1* cDNA.

(A) The putative 5′ and 3′ untranslated regions are shown before and after the open reading frame. The underlined region denotes the MADS box. Amino acids are represented by one-letter code. The sequence reported here has been assigned GenBank accession number L26295.
(B) Alignment of the deduced amino acid sequences for the *TAG1* cDNA with *AG* cognates from Arabidopsis (AGAMOUS), Antirrhinum (PLENA), tobacco (NAG1) (Kempin et al.,1993), Brassica (BAG1), and maize (ZAG1) (Schmidt et al., 1993) is shown. Asterisks indicate identity with TAG1; a dash indicates a gap inserted to maximize alignment.

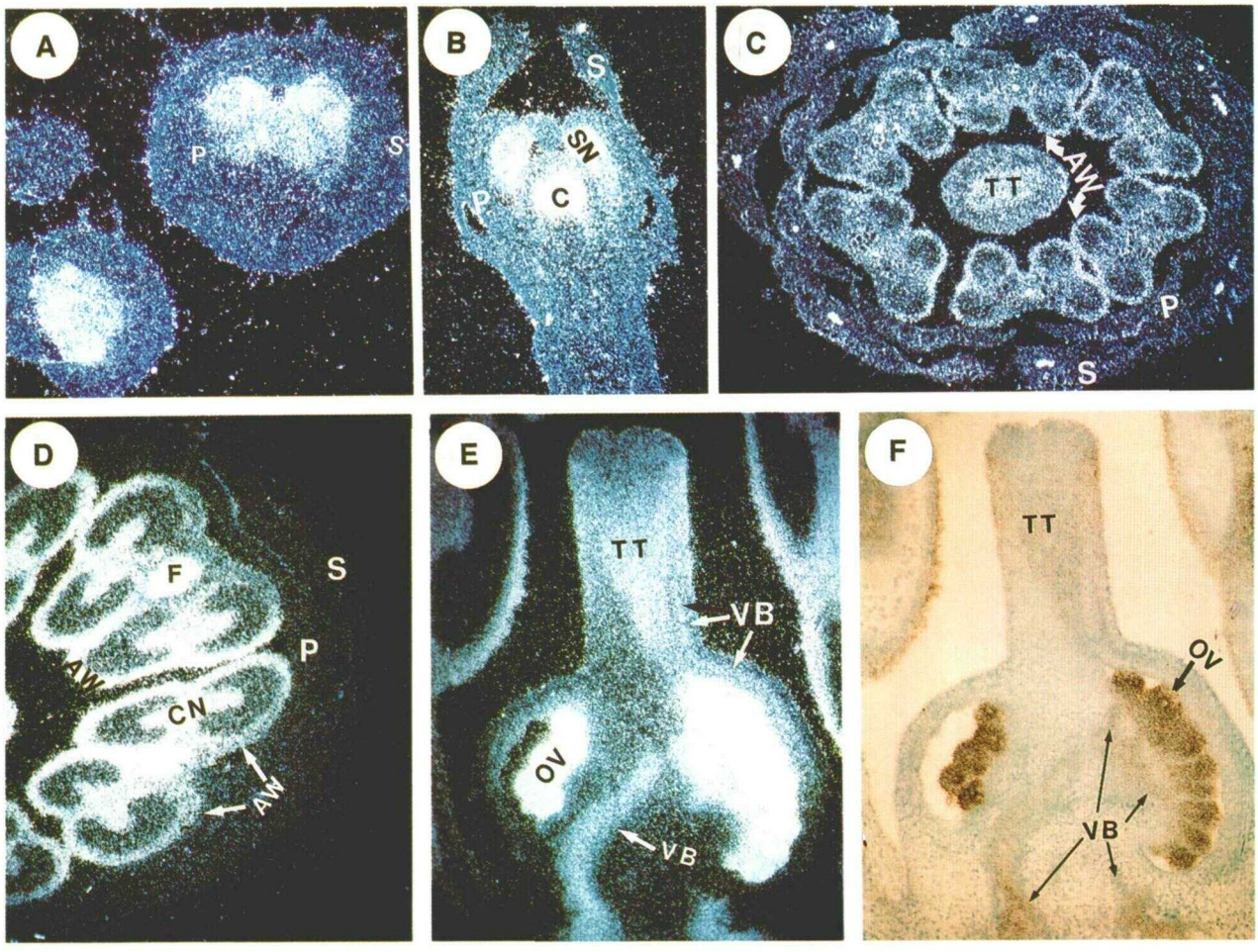

Figure 2. Localization of *TAG1* RNA Transcripts in Primordia and Floral Buds of Tomato Flowers by in Situ Hybridization.

(A) and **(B)** Early primordial buds. Tangential and longitudinal median sections in **(A)** and **(B)**, respectively, are shown. Signals are confined to cells fated to form stamen (SN) and carpels (C) only. Note the intensive signals in the central region of the floral meristem in **(B).** P, petals; S, sepals.
(C) Distribution of *TAG1* transcripts in a young (3 mm long) floral bud as shown in a cross-section. At this stage, all organs have been specified, have emerged, and have acquired their basic anatomical distinctions. Yet, pollen mother cells have not yet differentiated in the anther sacs. At this stage, the *TAG1* transcripts are concentrated mostly in the anther walls (AW) and the transmitting tissue (TT) of the style.
(D) A later stage of anther development. Pollen mother cells have differentiated and the floral bud is about 6 mm long, i.e., 60% of its final size. Intense hybridization signals are detected now in the vascular tissues of the filament (F) and the parenchyma cells of the connective (CN) in addition to the anther walls (AW). None of the tissues of the sepals (S) and petals (P) has signals above background level.
(E) and **(F)** Localization of *TAG1* transcripts in the tissues of the pistil. Dark-field **(E)** and bright-field **(F)** photographs of longitudinal sections are shown. The stage of development is comparable with that shown in **(D).** Arrows point to regions of high signal in ovules (OV), vascular bundles (VB), and transmitting tissue (TT).

Figures 3A to 3F follow the development of one such multifloral pseudocarpel in live flowers and histological sections. As the flower opens, most pseudocarpels are composed of three or four loculate compartments and a short, hollow, style-like structure with no stigmatic tissues at the top (Figure 3A). Continuous lateral emergence of flowers on the axial indeterminate meristem results in the giant (now 2-in diameter) multiloculate organ that is shown in Figure 3B. At a slightly later stage, the primary outer green envelope succumbs to the internal pressure (Figure 3C), and yellow petals of the nested flowers emerge. A cross-section of such pseudocarpels is shown in Figure 3D, and a stained cross-section of such an organ at the early stage, similar to that shown in Figure 3A, is illustrated in Figure 3E. An enlarged picture of an arrangement in one nested perianth flower is shown in Figure 3F, and this section was also stained with antibody for the threonine

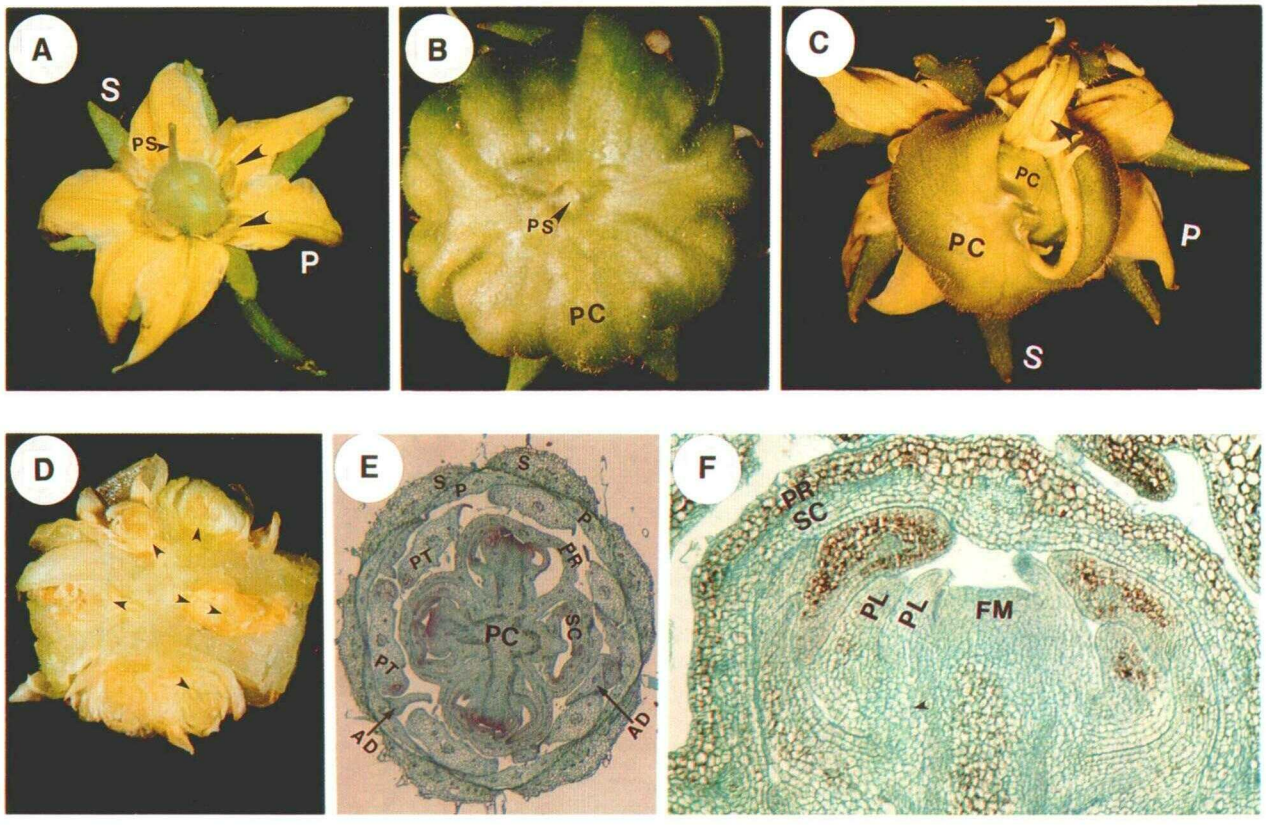

Figure 3. Homeotic Alterations of Tomato Flowers Conferred by the *TAG1* Antisense RNA.

(A) A mature, open, and defective flower of plant 2-40. Diminutive petaloid organs replace normal stamens at the third whorl (arrowheads), and the pistil is replaced by a locular pseudocarpel with a short, hollow style (PS). Petals (P) and sepals (S) are indistinguishable from their normal counterparts.

(B) Pseudo, multilocular carpel (PC), 3 weeks after flower opening. Pseudo style (PS) remained at the same size as shown in **(A)** and is, therefore, barely visible. Note the contours of fused leaves. The organ is now 5 cm in diameter.

(C) A pseudocarpel following a forced opening as a result of internal pressures. Emerging petals are pointed.

(D) A cross-section through a multilocular, multifloral pseudocarpel at a stage similar to or somewhat earlier than that shown in **(B).** Arrowheads show nested floral buds.

(E) A cross-section through a homeotic flower shortly before opening. The locular organization of the pseudocarpel and the lateral emergence of floral meristems are shown. The anatomical organization of the petaloid stamens (PT) in the third whorl and adventitious perianth organs (AD) is demonstrated. PR and SC designate primary and secondary outer envelops, respectively, of the nested flowers.

(F) A higher magnification of a nested flower in which an additional perianth flower is emerging. The anatomy of the outer envelope of the nested flower is similar to that which engulfs the whole structure (compare with **[E]**). Inner perianth leaves (PL) and new floral meristems (FM) are also shown. The fixed and embedded paraffin section was also counterstained with antibody for the threonine deaminase gene to facilitate the recognition of the developing organs.

deaminase gene (Samach et al., 1991) to facilitate the identification of floral structures. A cross-section of a wild-type carpel is shown for comparison in Figure 5A.

Under no circumstances were ovules or stigmatic tissues found in any of the 14 aberrant transgenic plants, and it is only the internal architecture of the pistil that was maintained in the transformed fourth whorl. The green envelopes engulfing the superstructure, as well as each of the nested flowers, were sepaloid by various criteria. They remained green throughout and developed glandular trichomes typical of leaves, sepals, and petals; the carotenoid system was not activated (see the analysis of overexpressed transgenes), and they did not develop the fleshy succulent texture of pericarps.

As mentioned previously, the multifloral structure that replaces carpels was surprisingly similar in all 14 aberrant T_1 plants. The homeotic reactions, however, varied considerably in the third whorl. Some plants developed sterile stamens with apparently normal morphology, while others gave rise to stamenoid petals, some of which are shown in the cross-section of a complete flower in Figure 3E, in which adventitious petaloid

appendages are also indicated by arrows. In no cases were stamenlike structures observed in flowers of the fourth whorl, irrespective of the severity of the changes in the third whorl.

Ectopic Expression of *TAG1* Alters Identity of Organs in Two Outer Whorls and Disrupts Normal Differentiation in Anthers and Gynoecia

Judicious manipulation of the tomato floral system requires detailed knowledge of its response to inhibition, overexpression, and ectopic expression of regulatory genes. Thus, parallel to the experiments described previously, the *TAG1* cDNA clone under the control of the Ω enhancer and the cauliflower mosaic virus (CaMV) 35S promoter (Odell et al., 1985) was introduced into tomato plants. Of the four T_1 kanamycin-resistant plants that were obtained, three developed aberrant flowers and one was normal. Of the three abnormal plants, two displayed extreme phenotypes (3-01, 3-04) and one displayed a more modest alteration (3-02). All three plants exhibited a similar alteration in the first whorl, but the plant designated 3-02 showed a less severe response in the second whorl. Flowers of all three plants set fruits, but occasional seeds were found only in fruits of the plant with the less severe alterations of the petals (3-02). Five such seeds of one fruit from plant 3-02 germinated and were all found to form flowers with the most severe phenotype. Analysis of seven seeds from an additional fruit gave two plants with wild-type flowers, two with flowers similar to the 3-02 parent, and three T_2 plants formed flowers with the extreme phenotype. Analysis of genomic DNA indicated that none of the T_1 plants harbors more than one insert, and we do not have an explanation for the aberrant segregations.

The homeotic alterations of the first and second whorls shared by all extreme plants, including T_2 progeny of 3-02, are illustrated in Figure 4. Basically, the five typical slender sepals that are normally separated from one another during maturation acquired instead a bell-like shape, were connately joined, and had short apical tips (Figure 4A). They reached only half the height of regular sepals and remained proximally fused throughout development. This morphogenetic alteration was accompanied by the induction of gene systems normally confined to pericarps. Chlorophylls were completely degraded, concomitant expression of all genes essential for the biosynthesis of the pericarp carotenoids ensued, and the fused leaves turned fleshy and acquired the typical succulent texture of true pericarp (Figures 4C and 4D). All of these events were independent of the presence of any of the other three whorls and continued unimpaired following their removal. However, no carpelloid placenta, ovules, or stigmatic tissues were observed, and, furthermore, the pericarpic leaves did not elongate or fuse to form stylelike structures. The three T_1 transgenic plants and T_2 progeny of 3-02 were indistinguishable with respect to conversion of sepals to pericarpic leaves.

The development of ectopic stamens in whorl two is documented in Figures 4B, 4E, and 4F. Morphologically normal, but otherwise sterile, stamens developed in flowers of plants 3-01, 3-04, and in progeny of 3-02 in the sites reserved normally for petals. No additional whorls of petals developed, but infrequently lateral expansion of the ectopic stamens occurred with the resultant formation of petaloid stamens, as shown in Figures 4F and 4G. Similarly, the petals of 3-02 itself were only partially converted in that they were slender and elongated; hollow locules were only occasionally produced, but even then they were not full length and numbered only one per organ. A section through mature wild-type anthers and styles is shown in Figure 5B.

Because ectopic expression of the *TAG1* gene is driven by the CaMV 35S promoter, overexpression is expected in tissues of the two inner whorls. In such circumstances, the normal balance of *TAG1* transcripts in the organ is disturbed; indeed, overexpression results in both male and female sterility and defective anatomy of anthers and ovaries.

Antisense RNA of the *TAG1* Gene Abolishes All Homologous Sense Transcripts

Although the mechanism by which antisense RNA inhibits homologous gene expression in plants is not understood and correlations between level of antisense RNA and phenotypic changes are not always informative, it is useful to follow the level of sense RNA in affected plants. Thus, RNA samples from 5-mm-long flowers were probed on blots with labeled sense and antisense RNA probes. At this stage of wild-type flowers, all organs have been specified and have emerged, but the three inner whorls are still engulfed by the fused calyx. Petals and stamens are completely green and only pollen mother cells are found in anthers. The results obtained with a sample of sense- and antisense-bearing plants are shown in Figures 6A to 6D. Sense RNA of the *TAG1* gene was not detected in antisense-bearing plants (Figure 6B), irrespective of the severity of the morphogenetic effect, but antisense RNA was found in flowers of all such plants (Figure 6D). It is possible, of course, that only residual sense RNA in a fraction of early cells is sufficient to explain the variability of the response. Likewise, as is expected from the transgene constructs, the plants bearing the coded orientation of *TAG1* driven by the CaMV 35S promoter overexpressed the *TAG1* transcripts (Figure 6A) but not the antisense RNA (Figure 6C).

As shown in Figure 7, the mRNA levels of two other members of the MADS box family, *TM5/AS16* and *TM5/AS6* genes (Pnueli et al., 1994), remained unaffected in flowers expressing *TAG1* antisense (Figures 7B and 7D) or sense (Figures 7A and 7C) RNA. Such results are presented here to demonstrate the specificity of the molecular mechanisms by which the *TAG1* antisense RNA is operating and are not intended to reach conclusions about the regulation of one gene by the other.

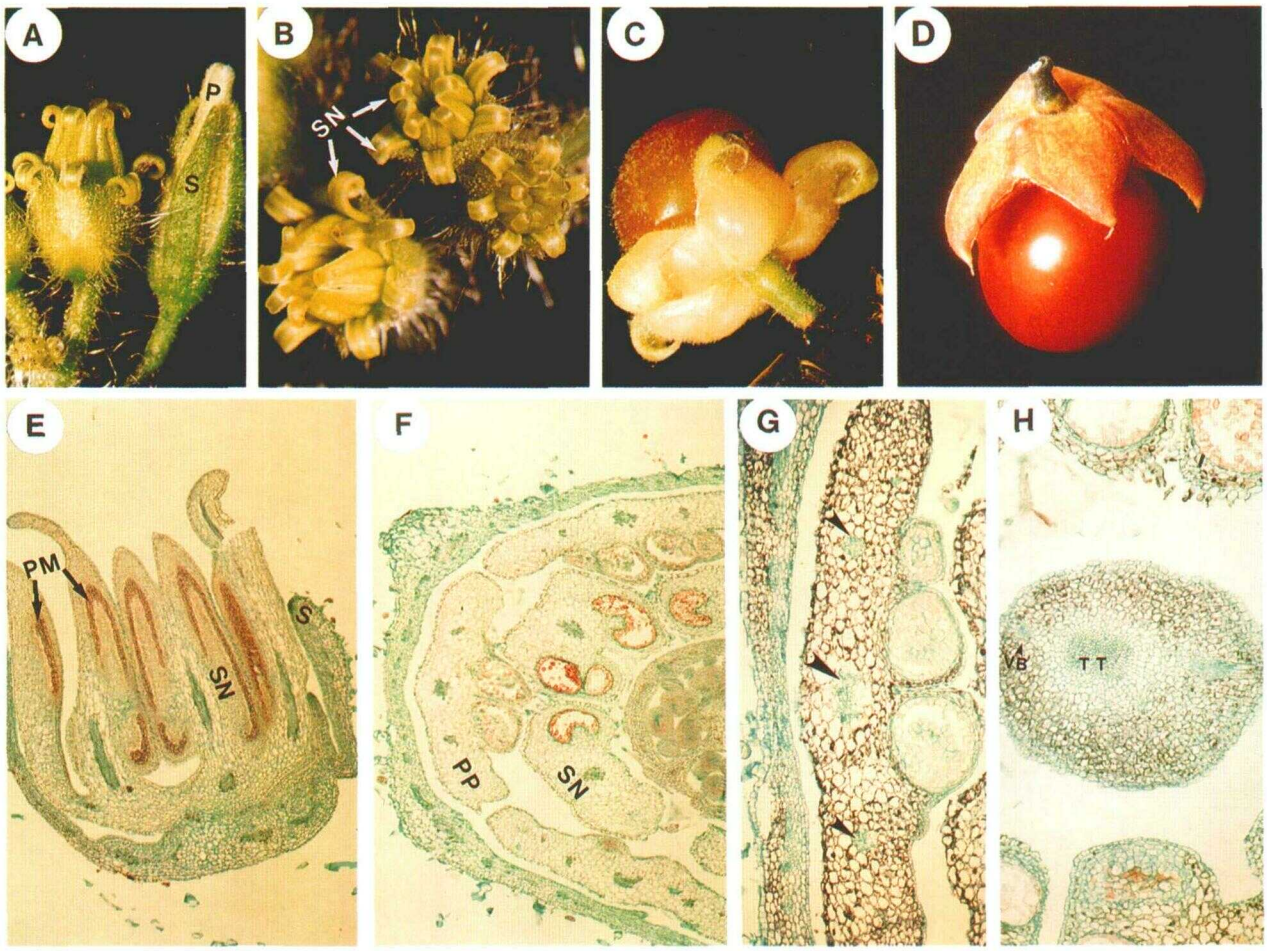

Figure 4. Homeotic Alterations in Flowers Expressing the *TAG1* Gene in All Four Whorls.

(A) Homeotic (left) and normal (right) flower at about the same developmental stage. The first whorl organs of the homeotic flower are short, connately fused, and have pointed tips. At the same time, normal sepals (S) are already separated. Also note the precocious opening of the green immature anthers of the second and third whorls. At this or even a later stage, the wild-type petals (P) and stamens are still connected in a cone-shaped structure.

(B) Homeotic flowers at a later stage showing correct alternating sites of organs in two stamenoid whorls (SN).

(C) The final stage of pericarp development of the outer whorl in the affected homeotic flowers. Morphologically normal fruits are also formed.

(D) A later stage of that shown in **(C)**.

(E) Section through the homeotic floral bud at a stage shown in **(A)**, demonstrating the homeotic conversion on the tissue level. PM, pollen mother cells; S, sepals; SN, stamens.

(F) Deformations of the third whorl anthers (SN) and partial transformation of petals to stamens in the second whorl (PP) of an altered flower.

(G) High magnification of a petaloid stamen in the second whorl of flowers with incomplete transformation. The organ is laterally expanded, there are several vascular bundles (arrowheads), and the anther locules are at a proximal abaxial position.

(H) Cross-section of a deformed style of flowers overexpressing the *TAG1* gene. Mislocation of poorly developed vascular tissues (VB), constructed and irregular transmitting tissue (TT), and expanding files of unidentified cells are shown. For comparison with wild-type flowers, refer to Shahar et al. (1992) and Pri Hadash et al. (1992).

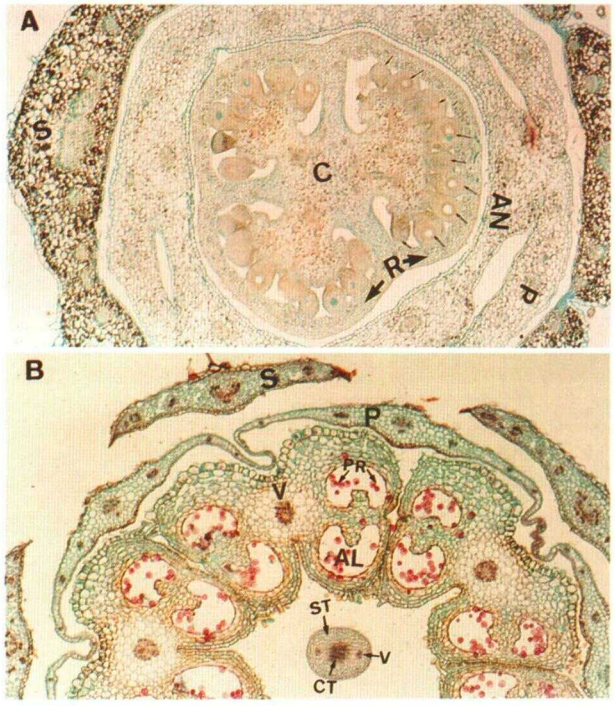

Figure 5. Wild-Type Flowers.

(A) and **(B)** Cross-sections of paraffin-embedded flowers showing the normal organization of carpel **(A)** and perianth and androecium **(B)**. In **(A)**, perianth organs and stamens are still fused at their bottom and the flower is 6 mm long (60% final length). The mature flower 2 days before anthesis is shown in **(B)**.

AL, anther locule; AN, stamens; C, carpel; CT, conducting tissue; P, petal; PR, pollen grain; R, ovary wall, future pericarp; S, sepals; ST, style; V, vascular bundles. Arrows in **(A)** point to ovules.

DISCUSSION

Molecular Characterization of *TAG1*

Positive identification of the tomato *AG* gene was accomplished using both molecular and functional criteria. The comparison of coding sequences of cognate genes from six plant species, including closely and remotely related dicot species as well as a monocot gene, revealed sequence features common to all members of this MADS subfamily. These cognate homologs are characterized by encoding an N-terminal polypeptide (17 to 53 residues) separating the translation initiation site and the MADS box domain. Most other MADS box genes do not encode such N-terminal additions, and the MADS box domain constitutes the N-terminal region. Such an N-terminal addition was also found in the *TAG1* gene. The role of this conserved N-terminal addition, if any, is not clear. The MADS box domain of *AG* cognates is highly conserved, and these gene products are also characterized by conserved features outside the DNA binding domains. The central domain (residues 60 to 175) includes putative positively or negatively charged amphipathic helices (Pnueli et al., 1991), as well as keratin-like regions (Ma et al., 1991). In this region, the evolutionary relationships between species are apparent because the tomato and tobacco proteins are more closely related to one another than to any of the Brassicaceae genes, and *PLENA* of Antirrhinum is more closely related to the Solanaceae proteins than to the proteins from Brassicaceae genes. The C-terminal region of the characterized MADS box gene products is always more variable than the preceding domains, yet the dicot *AG* cognates are closely related to one another. Particularly intriguing is the QLV tripeptide at the extreme C terminus of all *AG* cognate dicot proteins.

The sequence analyses clearly indicated that *TAG1* is the cognate homolog of *AG*. This conclusion was supported by analysis of *TAG1* RNA accumulation. These data show that *TAG1* RNA accumulates from the outset in all cells of primordia fated to form the stamens and carpels and not at all in petals and sepals. Like its Arabidopsis counterpart, *TAG1* is expressed

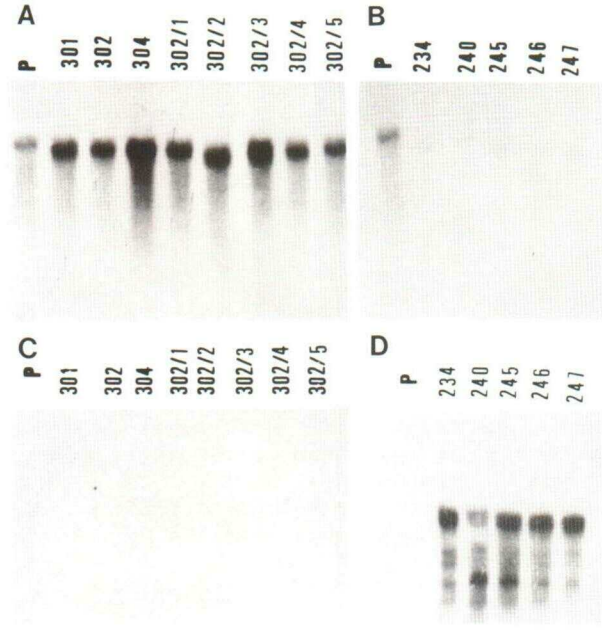

Figure 6. RNA Blot Analysis of *TAG1* Sense and Antisense Transcripts in Flowers of Transgenic Plants.

(A) and **(B)** RNA blot of sense and antisense flowers probed with labeled antisense *TAG1* RNA.

(A) P, progenitor plant; 301, 302, 304, T_1 plants bearing the 35S:*TAG1* sense gene construct; 302/1 to 302/5, T_2 progeny of the primary transformant 302.

(B) The lanes labeled 234 to 247 represent RNA from five primary transformants (T_1) bearing the 35S:*TAG1* antisense gene construct.

(C) and **(D)** Same RNA samples as shown in **(A)** and **(B)** but probed with labeled *TAG1* sense RNA. Total RNA (6 μg) extracted from flowers 5 days before anthesis (2/3 of the final size) was electrophoresed in each lane.

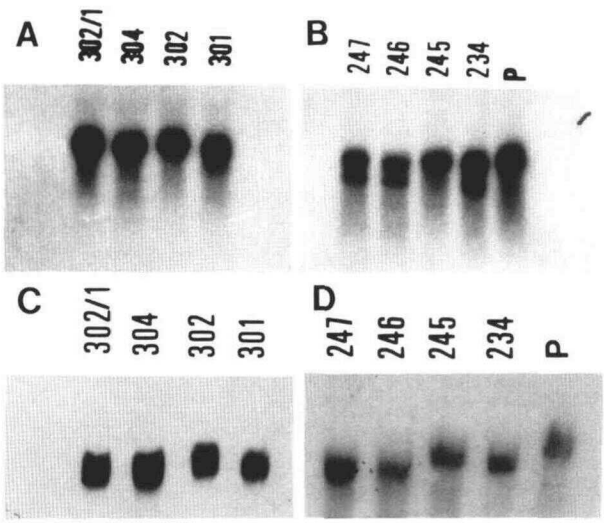

Figure 7. Expression of the MADS Box Genes *TM16* and *TM6* in *TAG1* Sense and Antisense Transgenic Plants.

In wild-type plants, *TM16* is expressed in all four whorls, whereas *TM6* is expressed in the three inner whorls only (see Figure 6 in Pnueli et al., 1994).

(A) and **(B)** The level of *TM16* RNA remains unchanged in the sense **(A)** or antisense **(B)** *TAG1* gene constructs.

(C) and **(D)** Similar to **(A)** and **(B)**, the level of *TM6* RNA is unchanged in plants expressing the sense **(C)** or antisense **(D)** *TAG1* gene constructs.

early on in the central pith cells of the future carpels. Later in flower development, *TAG1* transcripts are progressively restricted to specific tissues of the developing anthers and ovaries. For example, as the anthers begin to differentiate, *TAG1* RNA is observed primarily in the anther walls. As the anther differentiates further, expression of *TAG1* is observed at high levels in the filaments and connective tissues. The progressive restriction of expression domains is also observed with group B genes in Antirrhinum and Arabidopsis (Tröbner et al., 1992; Jack et al., 1992) as well as by MADS box genes of tomato that are expressed in three, rather than two, whorls; in *TM5*, however, a progressive wave of gene expression follows the differentiation of the sporogenic tissues and is not limited to anther walls, filaments, and connectives (see accompanying manuscript, Pnueli et al., 1994). It is also interesting to note that all floral homeotic genes studied thus far are highly active in the vascular tissues of organs in which they are expressed.

Functional Analyses of *TAG1*

Having established similarities in the molecular organization and expression patterns, we next sought proof that similar developmental functions are performed by the *TAG1* and *AG* genes. We chose to inhibit gene function by means of antisense RNA (Cannon et al., 1990; Mol et al., 1990) and in parallel to overexpress the gene in ectopic organs. Inhibition of MADS box genes by antisense RNA has not yet been reported, but Angenent et al. (1993) obtained two plants in which cosuppression of the petunia gene *FBP1* resulted in a phenocopy of mutations in B group genes. van der Krol et al. (1993) have also employed cosuppression in petunia to help identify the *pMADS1* gene as responsible for the green petal mutation.

Inhibition of the *TAG1* gene by antisense RNA is expected to specifically affect organ development in the two inner whorls, resulting in the conversion of stamens into petals and carpels into indeterminate nested sets of perianth flowers. Both predictions were satisfied. In all transgenic plants analyzed, the first and second whorl organs were not affected by the *TAG1* antisense construct. The most prominent fact is that the reduction or loss of *TAG1* transcripts was sufficient to convert third whorl organs into petaloid organs and to replace the determinate fourth whorl carpels with an indeterminate production of nested perianth flowers. In the most extreme plants, diminutive petaloid organs rather than the expected full-size petals appeared in the third whorl. In more modest cases, intermediate organs were found, some tissues accompanied by additional (adventitious) structures, whereas in the least extreme cases, the stamens were anatomically normal but completely sterile.

The outer envelope of the pseudocarpels as well as the envelopes of the inner nested flowers remained green and tiny, the carotenoid system was not activated, chlorophylls were not degraded, and pericarpic tissue was not formed. Yet some overall architectural features of carpel leaves were retained. The leaves were connately fused and appeared to begin to form styles, and the inner arrangement of locules, in which flowers ectopically emerge, was maintained at least in the primary design. The inability of the pseudocarpel leaves of the fourth whorl in the antisense flowers to form pericarps is contrasted sharply with the development of pericarpic leaves in the first whorl upon ectopic expression of *TAG1* (see below). Furthermore, no ovulated organs were found within the fourth whorl flowers even if, as mentioned before, the arrangement of the ectopic floral meristem resembled that of the placenta tissue in the normal carpels. Because the pseudocarpels retained some organizational features of the normal carpels, it is possible that transformation was not complete. In tomato, the placental tissue, and thus the seeds, are borne on extensions of the inner wall of the pericarp. Observations of *TAG1* antisense flowers indicated that the nested floral meristems that proliferate develop from the same regions of the pseudocarpels in wild type that would develop into placental tissue (compare Figures 3E and 3F with Figure 5A). Whether this is unique to tomato is not known.

To provide further evidence that *TAG1* in tomato functions in a manner that is similar to the *AG* gene in other dicots and to determine whether certain aspects of the A, B, and C genetic model also hold true for tomato, we characterized transgenic plants that ectopically express the *TAG1* gene in the sense orientation. As predicted, stamens possessing all of the basic anatomical features of wild-type third whorl organs replaced

petals in the right position and number. This was also observed following ectopic expression of the *BAG1* gene in tobacco (Mandel et al., 1992a). In tobacco, Arabidopsis, and Antirrhinum (Mandel et al., 1992a; Mizukami and Ma, 1992; Bradley et al., 1993), ectopic expression of the *AG* cognates in the first whorl results in the formation of leaves bearing stigmatic papillae and in many instances also ovules. In tomato, their action was restricted to full differentiation of pericarpic leaves, complete degradation of chlorophylls, activation of the carotenoid system, and all that is needed for fruit maturation. Interestingly, a partial phenocopy of this phenomenon was reported by Ishida (1992) in tissue culture of mature calyces in which red, succulent sepals developed. Such cultures were initiated from mature flowers and it is not known whether fused pericarps will develop following early cultures. Perhaps these tissue culture–induced alterations are due to ectopic *TAG1* activity.

The functional significance of progressively restricting *TAG1* expression to specific cell types of developing anthers and carpels and of restricting expression of other MADS box genes throughout development remains unclear. The simplest interpretation is that each gene product, being a putative transcription factor, participates in several independent developmental programs. During the early primordial stages, it is activated to provide the whorls with their proper identity. Subsequently, it is down-regulated in a fraction of the cells but remains active in cells fated to execute a specific differentiation pathway. The loss of expression in certain cell types late in development may be important, because ectopic expression of *TAG1*, for example, prevents normal development of pollen.

It is possible, however, that in addition to the above-mentioned role, the major organ identity genes are continuously expressed to maintain a particular state of differentiation in a given organ. We do not know in which tissues and at what level the homeotic genes are expressed to maintain the identity of all cells of the organ, but the observation that all homeotic genes studied thus far are expressed in the floral vascular tissues of mature organs is, to our view, suggestive.

As expected, there was no detectable effect of antisense *TAG1* RNA on vegetative organs. However, in all T_1 plants overexpressing the sense *TAG1* RNA as well as in the T_2 progeny of the semifertile plant (number 3-02), we observed a consistent change of leaf shape. Leaves became rounded and curled along their entire margins. Whether this is the result of ectopic activation in leaves of floral targets of *TAG1* remains to be determined.

We have shown that *TAG1* is structurally and functionally related to its cognate homologs *AG* and *PLENA* from Arabidopsis and Antirrhinum. Indeed, the *TAG1* antisense plants result in flowers whose phenotypes closely resemble loss-of-function mutations in the cognate genes of Arabidopsis and Antirrhinum. These results support the notion that antisense technology can be a useful means of assessing the function of gene members of the homeotic MADS box family in the absence of existing mutations in a given cloned gene. In an accompanying manuscript (Pnueli et al., 1994), we use this approach to assess the role of a functionally anonymous gene, *TM5*.

METHODS

Isolation of the *TAG1* cDNA

Approximately 200,000 plaques were screened with a gel-purified radiolabeled DNA probe specific for the Arabidopsis *AGAMOUS* cDNA (pCIT565) (Yanofsky et al., 1990). The DNA probes were labeled using the random-primed labeling kit from Boehringer Mannheim according to the manufacturer's recommendations. Hybridizations were done at 55°C in 5 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 0.5% SDS, 25 mg/L salmon sperm DNA for 48 hr, followed by one room temperature and three 55°C washes in 5 × SSPE, 0.1% SDS. The tomato (*Lycopersicon esculentum*) cultivar VF36 cDNA library is in λgt10 and represents mature pistil RNA (Gasser et al., 1989). cDNAs were subcloned into the vector pGEM7Zf(+) (Promega) for sequencing. Double-stranded sequencing was performed using the (U.S. Biochemicals) Sequenase Version 2.0 kit according to the manufacturer's protocol.

RNA Analyses and Transgenic Plant Analyses

All RNA analyses and transgenic plant studies are as described by Pnueli et al. (1994).

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