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RESEARCH ARTICLE

Identification and Molecular Characterization of *ZAG1*, the Maize Homolog of the Arabidopsis Floral Homeotic Gene *AGAMOUS*

Robert J. Schmidt,[a,1] Bruce Veit,[b] M. Alejandra Mandel,[a] Montaña Mena,[a] Sarah Hake,[b] and Martin F. Yanofsky[a]

[a] Department of Biology and Center for Molecular Genetics, University of California at San Diego, La Jolla, California 92093-0116

[b] United States Department of Agriculture–Agricultural Research Station Plant Gene Expression Center, 800 Buchanan Street, Albany, California 94710

Recent genetic and molecular studies in Arabidopsis and Antirrhinum suggest that mechanisms controlling floral development are well conserved among dicotyledonous species. To assess whether similar mechanisms also operate in more distantly related monocotyledonous species, we have begun to clone homologs of Arabidopsis floral genes from maize. Here we report the characterization of two genes, designated *ZAG1* and *ZAG2* (for *Zea AG*), that were cloned from a maize inflorescence cDNA library by low stringency hybridization with the *AGAMOUS* (*AG*) cDNA from Arabidopsis. *ZAG1* encodes a putative polypeptide of 286 amino acids having 61% identity with the AGAMOUS (AG) protein. Through a stretch of 56 amino acids, constituting the MADS domain, the two proteins are identical except for two conservative amino acid substitutions. The ZAG2 protein is less similar to AG, with 49% identity overall and substantially less similarity than ZAG1 outside the well-conserved MADS domain. Like *AG*, *ZAG1* RNA accumulates early in stamen and carpel primordia. In contrast, *ZAG2* expression begins later and is restricted to developing carpels. Hybridization to genomic DNA with the full-length *ZAG1* cDNA under moderately stringent conditions indicated the presence of a large family of related genes. Mapping data using maize recombinant inbreds placed *ZAG1* and *ZAG2* near two loci that are known to affect maize flower development, *Polytypic ear* (*Pt*) and *Tassel seed4* (*Ts4*), respectively. The ZAG1 protein from in vitro translations binds to a consensus target site that is recognized by the AG protein. These data suggest that maize contains a homolog of the Arabidopsis floral identity gene *AG* and that this gene is conserved in sequence and function.

INTRODUCTION

One of the most striking examples of morphogenesis in plants is the transition from vegetative growth to flowering. The morphology of flower development has been described in detail for many plant species, but the analyses of specific molecular events leading to floral differentiation and development have only recently begun. Much progress toward describing mechanisms controlling floral development has been made through the analysis of mutants in Arabidopsis and Antirrhinum (reviewed by Coen and Meyerowitz, 1991). Some of these mutants may be termed homeotic because they convert one organ type to that of another (Haughn and Somerville, 1988; Komaki et al., 1988; Bowman et al., 1989, 1991; Coen et al., 1990; Sommer et al., 1990). The genes affected by these mutations can, in general, be placed into two distinct classes; for example, in Arabidopsis, "early acting" genes, such as *LEAFY* (Weigel et al., 1992) and *APETALA1* (*AP1*) (Irish and Sussex, 1990; Mandel et al., 1992), cause a partial loss of the transition of inflorescence meristems into floral meristems (Weigel et al., 1992); "late-acting" genes, such as *APETALA2* (*AP2*), *AGAMOUS* (*AG*), *APETALA3* (*AP3*), and *PISTILLATA*, act alone or in combination to specify organ identity in a position-dependent fashion (Bowman et al., 1991b; Coen and Meyerowitz, 1991).

The cloning of these genes has provided insight into the possible mechanism by which they affect floral development. A number of these genes, including *AP1*, *AP3*, and *AG* (Yanofsky et al., 1990; Jack et al., 1992; Mandel et al., 1992), encode proteins with sequence similarity to the transcription factors MCM1 in yeast (Passmore et al., 1988) and the serum response factor in humans (Norman et al., 1988). This sequence similarity encompasses a highly conserved stretch of more than 50 amino acids, the "MADS domain," which has been shown to be a DNA binding motif (Hayes et al., 1988; Norman et al., 1988; Schwarz-Sommer et al., 1990). More recent studies

[1] To whom correspondence should be addressed.

have shown the products of the Arabidopsis *AG* gene and the Antirrhinum *DEFICIENS* gene (another MADS-box gene affecting floral organ identity) to be sequence-specific DNA binding proteins (Mueller and Nordheim, 1991; Schwarz-Sommer et al., 1992). These observations strongly suggest that these proteins affect floral development by regulating patterns of gene transcription. A variety of genetic and molecular studies indicate that the mechanisms controlling floral development in Arabidopsis and Antirrhinum are similar, with homologous genes performing similar functions in each species (reviewed by Coen and Meyerowitz, 1991).

We are interested in establishing whether less closely related species, especially those with distinct patterns of floral development, such as that exemplified by gramineous species, rely on mechanisms of floral morphogenesis similar to Arabidopsis and Antirrhinum. To explore this question, we have begun to analyze the process of maize floral development. Maize produces flowers typical of most grasses in which the perianth is highly reduced or absent. Maize flowers are further differentiated according to sex; staminate flowers develop on the terminally positioned inflorescence, the tassel, while pistillate flowers form on axillary inflorescences known as ears (Mangelsdorf, 1945; Cutler and Cutler, 1948; Kiesselbach, 1949). The unisexual flower types of the tassel and ear are both derived from an initially bisexual state through the respective abortion of pistil and stamen primordia (Bonnett, 1940, 1948; Cheng et al., 1983).

Several features of maize should facilitate the description of molecular events which underlie inflorescence development. The sophisticated genetics of maize permits manipulation of a variety of mutants that affect various aspects of inflorescence development (Coe et al., 1988; Sheridan, 1988). Biochemical and morphological analyses are simplified by the relatively large size of the developing inflorescence and its reiterated pattern of organization. In addition, there exist several families of transposable elements that can be used for gene cloning by transposon tagging (Fedoroff and Baker, 1989; Walbot, 1992). However, the potential of this approach for the analysis of maize floral development and for the cloning of genes affecting this process is still largely unrealized.

In this report, we describe an alternative approach in which two floral-specific maize genes were cloned based on their sequence similarity to *AG*, a gene that is required for the determination of stamen and carpel identity in Arabidopsis. On the basis of several criteria, one of these genes appears to be the maize homolog to the *AG* gene of Arabidopsis. Our analyses also indicate that as in Arabidopsis (Ma et al., 1991) maize contains a large family of MADS-box genes.

RESULTS

We have used the *AG* cDNA clone from Arabidopsis to screen a maize cDNA library made from developing female inflorescences. cDNAs representing two different genes were isolated and sequenced. As shown in Figure 1, each contains a long

A

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                                                        CCCAGAGCTACCACCCCTCTTATCCCCACC
CCTCCTCCTCCTACCTTTTCTCCTTCAGACCTCAAAATCTGTGTGTCTCCTGCCGCGGCTAGCTGATAGGAACAAGAGC
ATG CAC ATC CGA GAA GAG GAG GCT ACA CCA TCC ACA GTA ACA GGC ATC ATG TCG ACC CTG    60
 M  H  I  R  E  E  E  A  T  P  S  T  V  T  G  I  M  S  T  L                      20

ACT TCG GCG GGG CAG CAG AAG CTG AAG GAG CCC ATA TCC CCT GGT GGC GGC TCC GCG TCG   120
 T  S  A  G  Q  Q  K  L  K  E  P  I  S  P  G  G  G  S  A  S                      40

GTC GCT GGG TCC GCT GCG GAG AGG AAC AAC GGC GGC AGG GGC AAG GCC AAG ACT GAG ATC   180
 V  A  G  S  A  A  E  R  N  N  G  G  R  G  K  G  K  T  E  I                      60

AAG CGC ATC GAG AAC ACG ACC AAC AGG CAG GTC ACC TTC TGC AAG CGC CGC AAC GGC CTC   240
 K  R  I  E  N  T  T  N  R  Q  V  T  F  C  K  R  R  N  G  L                      80

CTC AAG AAG GCG TAC GAG CTC TCC GTG CTC TGC GAC GCC GAG GTC GCG CTC ATC GTC TTC   300
 L  K  K  A  Y  E  L  S  V  L  C  D  A  E  V  A  L  I  V  F                     100

TCC AGC CGC GGC CGC CTC TAC GAG TAC GCC AAC AAC AGC GTG AAG GGC ACC ATT GAG AGG   360
 S  S  R  G  R  L  Y  E  Y  A  N  N  S  V  K  G  T  I  E  R                     120

TAC AAG AAG GCA ACC AGT GAC AAC TCC AGC GCA GCT GGT ACG ATT GCA GAG GTC ACC ATT   420
 Y  K  K  A  T  S  D  N  S  S  A  A  G  T  I  A  E  V  T  I                     140

CAG CAT TAC AAG CAG GAA TCT GCT AGG CTG AGG CAG CAG ATC GTT AAC TTG CAG AAC TCC   480
 Q  H  Y  K  Q  E  S  A  R  L  R  Q  Q  I  V  N  L  Q  N  S                     160

AAC AGG GCC CTG ATA GGT GAT TCT ATC ACA ACC ATG AGC CAC AAG GAA CTT AAG CAC TTG   540
 N  R  A  L  I  G  D  S  I  T  T  M  S  H  K  E  L  K  H  L                     180

GAG ACT AGG TTA GAC AAA GCT CTC GGA AAG ATT AGA GCA AAA AAG AAC GAT GTG CTG TGT   600
 E  T  R  L  D  K  A  L  G  K  I  R  A  K  K  N  D  V  L  C                     200

TCT GAA GTC GAG TAC ATG CAG AGA AGG GAA ATG GAG TTG CAG AAT GAC AAC TTG TAC TTA   660
 S  E  V  E  Y  M  Q  R  R  E  M  E  L  Q  N  D  N  L  Y  L                     220

AGG AGC CGG GTT GAT GAG AAT GAA AGG GCA CAA CAG ACA GCG AAC ATG ATG GGG GCA CCA   720
 R  S  R  V  D  E  N  E  R  A  Q  Q  T  A  N  M  M  G  A  P                     240

TCG ACA AGT GAG TAT CAG CAG CAC GGT TTT ACT CCT TAT GAT CCA ATA AGG AGC TTC CTG   780
 S  T  S  E  Y  Q  Q  H  G  F  T  P  Y  D  P  I  R  S  F  L                     260

CAG TTC AAC ATC GTG CAG CAG CCT CAG TTC TAT TCT CAG CAG GAG GAC CGG AAA GAC TTC   840
 Q  F  N  I  V  Q  Q  P  Q  F  Y  S  Q  Q  E  D  R  K  D  F                     280

AAC GAC CAA GGT GGA AGA TAA ATATTGGACCTCTCAAGCTTCAGTACTTATCCGTGATGATGCATGACGTAC   912
 N  D  Q  G  G  R  *                                                            286
TGCCAGTGAGAAACTGAGCTACATTACTGTGGAATTATATGTAAAGACTAGTACTACTGCTTCATATGTGCATGGCACA   991
CGCGCACGTAGTATGCACAATTTCATCCCACTATTATGGCTTGGCACCAACTATGTCTCCTTAATTATCATAGGAGAAA  1070
AATAAGTCACGCACAAAAAAATTCTAAAGATGAGGCGCAGTGTTGGATGACTGAAACTGAAAGATAGGATTCTCGTGGA  1149
TGGCGGTGGTCATATACGGCCTGTTTTGGTTGATGTACCCGAGCGCACCCGCGGAATCTTAAAGCAAGTTGTGTTTAAT  1228
TGACTGCACTTTATATACGGGTTGAACAGATGT(A)                                             1261
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B

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ATC GAG AAC AAC ACG AGC CGG CAG GTC ACC TTC TGC AAG CGC CGC AAT GGG CTC CTC AAG    60
 I  E  N  N  T  S  R  Q  V  T  F  C  K  R  R  N  G  L  L  K                      20

AAG GCG TAC GAG CTC TCC GTC CTC TGC GAC GCT GAG GTG GCC CTC ATC GTC TTC TCT AGC   120
 K  A  Y  E  L  S  V  L  C  D  A  E  V  A  L  I  V  F  S  S                      40

CGT GGT CGC CTC TAC GAG TAT GCC AAC AAC AGT GTC AAG GCT ACT GTT GAG AGG TAC AAG   180
 R  G  R  L  Y  E  Y  A  N  N  S  V  K  A  T  V  E  R  Y  K                      60

AAG GCA CAC ACC GTT GGC TCT TCC TCT GGG CCC CCG CTC CTA GAG CAC AAT GCC CAG CAA   240
 K  A  H  T  V  G  S  S  S  G  P  P  L  L  E  H  N  A  Q  Q                      80

TTC TAC CAG CAA GAA TCA GCA AAA CTG CGC AAC CAG ATC CAG ATG CTG CAA AAC ACT AAC   300
 F  Y  Q  Q  E  S  A  K  L  R  N  Q  I  Q  M  L  Q  N  T  N                     100

AGG CAC TTG GTT GGT GAC TCC GTG GGA AAC CTG TCA CTC AAG GAG CTG AAG CAG CTG GAG   360
 R  H  L  V  G  D  S  V  G  N  L  S  L  K  E  L  K  Q  L  E                     120

AGC CGC CTT GAG AAA GGC ATC TCT AAG ATC AGG GCC AGG AAG AGT GAG CTG CTG GCT GCG   420
 S  R  L  E  K  G  I  S  K  I  R  A  R  K  S  E  L  L  A  A                     140

GAG ATC AGT TAC ATG GCC AAA AGG GAG ACT GAG CTT CAG AAT GAC CAC ATG ACC CTC AGG   480
 E  I  S  Y  M  A  K  R  E  T  E  L  Q  N  D  H  M  T  L  R                     160

ACC AAG ATT GAG GAG GGA GAG CAA CAG CTG CAG CAG GTG ACC GTG GCA CGG TCA GTT GCA   540
 T  K  I  E  E  G  E  Q  Q  L  Q  Q  V  T  V  A  R  S  V  A                     180

GCA GCA GCA GCT GCC ACC AAC TTG GAG CTG AAC CCA TTC TTG GAG ATG GAT ACC AAA TGC   600
 A  A  A  A  A  T  N  L  E  L  N  P  F  L  E  M  D  T  K  C                     200

TTC TTC ACT GGC GGC CCC TTC GCG ACG CTG GAC ATG AAG TGC TTT CTC CCC GGC AGC TTG   660
 F  F  T  G  G  P  F  A  T  L  D  M  K  C  F  L  P  G  S  L                     220

CAG CAG ATG CTG GAG GCA CAG CAG CGC CAG ATG CTC GCC ACC GAG CTG AAC CTC GGC TAC   720
 Q  Q  M  L  E  A  Q  Q  R  Q  M  L  A  T  E  L  N  L  G  Y                     240

CAA CTG GCG CCG CCT GGT TCT GAC GCT GCC AAC AAT AAC CCT CAT CAT CAG TTC TAA ACT   780
 Q  L  A  P  P  G  S  D  A  A  N  N  N  P  H  H  Q  F  *                        258
GGATGCATGTACGAGAAGCTTTTCAAGCGAACTGTGAACCGTGGAAGCTGGCTGTTGCCTCTAGATGTTCTTGGCTTCC  859
ATGACCTAGCTTATTACGGTGTGTATTGTTGTTGCCTTTTGTGTGTGATTCTCCTTGTGCTACTCTACCTGATGGAGAA  938
CTAAACCTAATCGATTGGACATAATGATCAAATGCTACAGATCCTGTGAGGCTGTGATCAGGTTAAGGCAAAACAAGTA 1017
TACTGATTGCTTAAATGGGTTTACGAGTGACGACAATGCTATTTGTTGCTTCTATT(A)                     1073
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Figure 1. Nucleotide and Deduced Amino Acid Sequence of *ZAG1* and *ZAG2*.

(A) Sequence of *ZAG1*. The MADS domain is underlined. The sequence shown is a composite from two independent cDNA clones, one having a longer 5′ end but shorter 3′ end, and the other having a longer 3′ end but shorter 5′ end. (GenBank accession number L18924).
(B) The sequence of the longest *ZAG2* cDNA clone (GenBank accession number L18925).
Numerals along the right of each sequence indicate nucleotide and amino acid positions with the initiation codon providing the start.

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ZAG1   GKGKTEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEY
AG     *G**I**************************************************
AGL1   *G**I*************************************VI**T*******
AGL5   *G**I*************************************VI**T*******
AGL3   *G**V*L*****KI******A*******************I**LI**N**K***F
AGL2   *G*RV*L*****KI******A*********************I**N**K***F
AGL4   *G*RV*L*****KI******A*********************S*****N**K***F
AGL6   *G*RV*M*****KI******S*********************I*****K***F
AP3    *G**IQ******Q******YS******F***H**T*****R*SI*M***SNK*H**
AP1    *G*RVQL*****KI******S***A******H*I**********V***HK*K*F**
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Figure 2. A Comparison of the Deduced Amino Acid Sequence through the MADS Domain.

The deduced amino acid sequence of the MADS domain of ZAG1 is compared with previously published sequence information from AG, a number of AG-like proteins (AGLs), and the related putative proteins AP1 and AP3. Asterisks indicate identity with amino acids in ZAG1.

open reading frame encoding a putative protein with a MADS domain. One of these, *ZAG1* (for *Zea AG*), encodes a polypeptide of 286 amino acids having high similarity to the AG protein. Beginning at the MADS domain and extending 220 amino acids through the end of ZAG1, AG and ZAG1 share 61% identity (Figure 1A). We could not accurately compare N-terminal homology of the MADS domain because the translation start in AG is not known. The other clone, designated *ZAG2*, is an incomplete cDNA that (through the 230 amino acids that can be compared) specifies a protein with 49% identity with AG (Figure 1B). A number of additional *ZAG2* cDNA clones were isolated, but none was any longer than the one reported here. Through a stretch of 56 amino acids constituting the MADS domain, ZAG1 and AG are identical except for two conservative amino acid substitutions. As indicated in Figure 2, this level of sequence conservation is higher than the degree of similarity between AG and any of the AG-like (AGL) proteins from Arabidopsis (Ma et al., 1991), suggesting that *ZAG1* is the cognate homolog of *AG*. ZAG2, by contrast, shows less similarity to AG (49%), especially for sequence outside the well-conserved MADS domain.

An initial characterization of the expression patterns of the *ZAG1* and *ZAG2* genes by RNA gel blot analysis indicated that both genes are expressed in a floral-specific manner. Figure 3A shows that *ZAG1* expression is restricted to RNAs of developing male and female inflorescences. For all tissues examined, *ZAG2* expression qualitatively mimicked the pattern observed for *ZAG1*, with the exception that *ZAG2* message was not detected in male inflorescences (Figure 3A).

We first localized *ZAG1* and *ZAG2* expression in developing inflorescences by examining RNA from glumes and stamens of tassels and from carpels of mature ears. RNA gel blot analysis was performed with these three RNAs using a 3′-specific fragment of the *ZAG1* clone as a probe to avoid cross-hybridization with the MADS box. As seen in Figure 3B, *ZAG1* RNA is not detected in glumes but is present in the stamens and carpels. Because *AG* is known to be expressed during stamen and carpel development in dicot flowers (Drews et al., 1991), these results are consistent with a similar function for *ZAG1* in maize flower development. In contrast, *ZAG2* expression

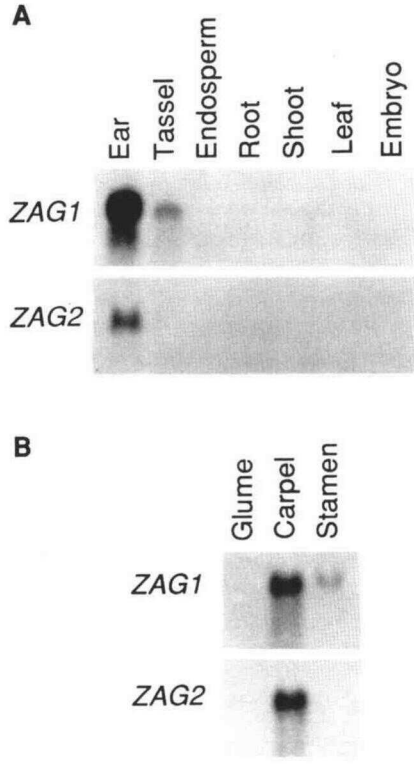

Figure 3. Analysis of *ZAG1* and *ZAG2* Expression in Different Organs of Maize.

(A) RNA gel blot analysis of *ZAG1* and *ZAG2* expression in developing ears, tassels, endosperms, roots, shoots, leaves, and embryos. The blots contain 10 μg of total RNA loaded in each lane, except for embryos where 1 μg of poly(A)$^+$ RNA was loaded. The blots were hybridized with 3′ specific probes of *ZAG1* and *ZAG2*. The lengths of the *ZAG1* and *ZAG2* messages are estimated at 1.7 and 1.6 kb, respectively.

(B) RNA gel blot analysis of *ZAG1* and *ZAG2* expression in floral organs. The blots contain 12 μg of total RNA from glumes and stamens of developing tassels and carpels of developing ears.

All blots werre subsequently hybridized with a tubulin probe as a loading control (data not shown).

was apparent only in the RNA from mature carpels. This restricted pattern of *ZAG2* expression is consistent with its lack of expression in the tassel (Figure 3A), given the early abortion of carpels in this inflorescence type.

In another analysis, using poly(A)$^+$-selected RNAs from root, shoot, leaf, and embryo, both *ZAG1* and *ZAG2* failed to produce a detectable signal on RNA gel blots (data not shown). However, expression of *ZAG1* and *ZAG2* was detected among poly(A)$^+$-selected message from 18-day-old endosperm and, as shown for *ZAG1* in Figure 4, in total endosperm RNA isolated early in development. *ZAG1* expression appeared highest in unfertilized ovules and at 5 days postpollination. The message decreased during endosperm development and became undetectable by 21 days after pollination. A similar pattern of expression was observed for *ZAG2* (data not shown).

To more precisely determine the localization of *ZAG1* and *ZAG2* expression in developing flowers, the patterns of *ZAG1* and *ZAG2* mRNA localization were determined by in situ hybridization of digoxigenin-labeled RNA probes to longitudinal and transverse sections of immature inflorescences. Figure 5 shows a comparison of *ZAG1* and *ZAG2* expression, indicating highly localized and distinct patterns that are limited to developing floral structures. In longitudinal sections of immature ears, *ZAG1* transcripts are seen in a wide range of developmental stages (Figure 5A), beginning with distally located dome-shaped floret primordia (Figure 5C) and extending into more mature basal florets in which anther and pistil primordia are well defined (Figure 5E). Throughout development, *ZAG1* expression is limited to stamens and pistils or their precursors. The pattern of hybridization in transverse sections (Figure 5G) indicates that *ZAG1* is expressed throughout the developing stamen and pistil primordia.

ZAG2 transcripts, although also distributed over a wide range of developmental stages (Figure 5B), begin to accumulate later in a more restricted pattern. Expression is first detected in carpel primordia of florets in which stamen primordia have just emerged (Figure 5D) and persists into mature stages in which the ovule and surrounding carpels are well differentiated (Figure 5F). Transcripts appear most concentrated within the developing ovule but are also distributed in cells lining the inner faces of carpels, continuing in a threadlike pattern along the extensions that form the silk (Figures 5F and 5H).

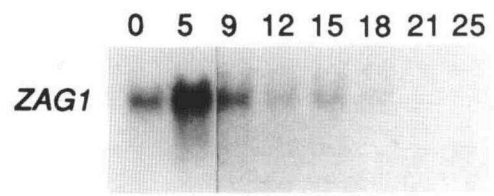

Figure 4. Expression of *ZAG1* during Endosperm Development.

RNA was isolated from unfertilized ovules (0) and developing endosperm. The number above each lane refers to the age of the endosperm expressed as the number of days after pollination. The blot contains 10 μg of total RNA loaded in each lane. The lanes 9 to 25 were exposed to film four times longer than lanes 0 and 5.

To map *ZAG1* and *ZAG2*, we utilized a set of maize recombinant inbreds (Burr et al., 1988) to follow the segregation of restriction fragment length polymorphisms detected by the *ZAG1* and *ZAG2* probes. A number of hybridizing bands were apparent when full-length *ZAG1* and *ZAG2* were used as hybridization probes. An example of a DNA gel blot hybridized with *ZAG1* is shown in Figure 6. The most strongly hybridizing restriction fragments on this blot correspond to the gene for *ZAG1*, as determined by reprobing the DNA blot with a 3′-specific fragment of *ZAG1* (data not shown). A different pattern of hybridization to these DNA blots was obtained using *ZAG2* as the probe (data not shown). This suggests that there exists in maize a large family of related genes. It is likely that many of the hybridizing bands detected with the full-length probe share similarity with the MADS box of *ZAG1*.

The DNAs from the recombinant inbreds were digested with *HindIII*, blotted to Nytran membranes, and hybridized with either *ZAG1* or *ZAG2*. The results of the mapping data place *ZAG1* on chromosome 6L at position 58. Intriguingly, the *Polytypic ear* mutation (*Pt*), which is known to affect maize flower development (Nelson and Postlethwait, 1954; Postlethwait and Nelson, 1964), has also been mapped near this position. This dominant mutation results in a proliferation of pistil-like structures in spikelets of both ears and tassels. The phenotype of *Pt*, including the dominant nature of the mutation, is consistent with what might be expected for ectopic expression of a gene encoding an AG-like function. This similarity, along with the correlative map positions, makes *Pt* a candidate for a gene encoding ZAG1. The gene encoding ZAG2 was mapped in a similar fashion using a 3′-specific fragment of the cDNA. The results from the mapping data place *ZAG2* at position 68 on 3L, which is near *Tassel seed4* (*Ts4*) at position 73. Like *Pt*, *Ts4* conditions the production of supernumerary pistil-like structures in the tassel and to a lesser extent in the ears.

The AG protein is known to bind to a consensus site consisting of the sequence 5′-CC(A/T)$_6$GG-3′ (Mueller and Nordheim, 1991; M. A. Mandel and M. F. Yanofsky, unpublished data). To assess the capacity of ZAG1 to recognize this same binding site, we performed mobility shift assays using the ZAG1 protein made in vitro and labeled wild-type (5′-TTACCAAAAAAGGAAA-3′) or mutant (5′-TTAGGAAAAAACCAAA-3′) binding site oligonucleotides. The data shown in Figure 7 indicate that, like AG, ZAG1 can recognize the MADS binding site but not the mutant site. This result is consistent with the high identity observed between the MADS DNA binding domain of AG and ZAG1.

DISCUSSION

Although studies in *Arabidopsis* and *Antirrhinum* suggest that the function of genes controlling floral development is in some

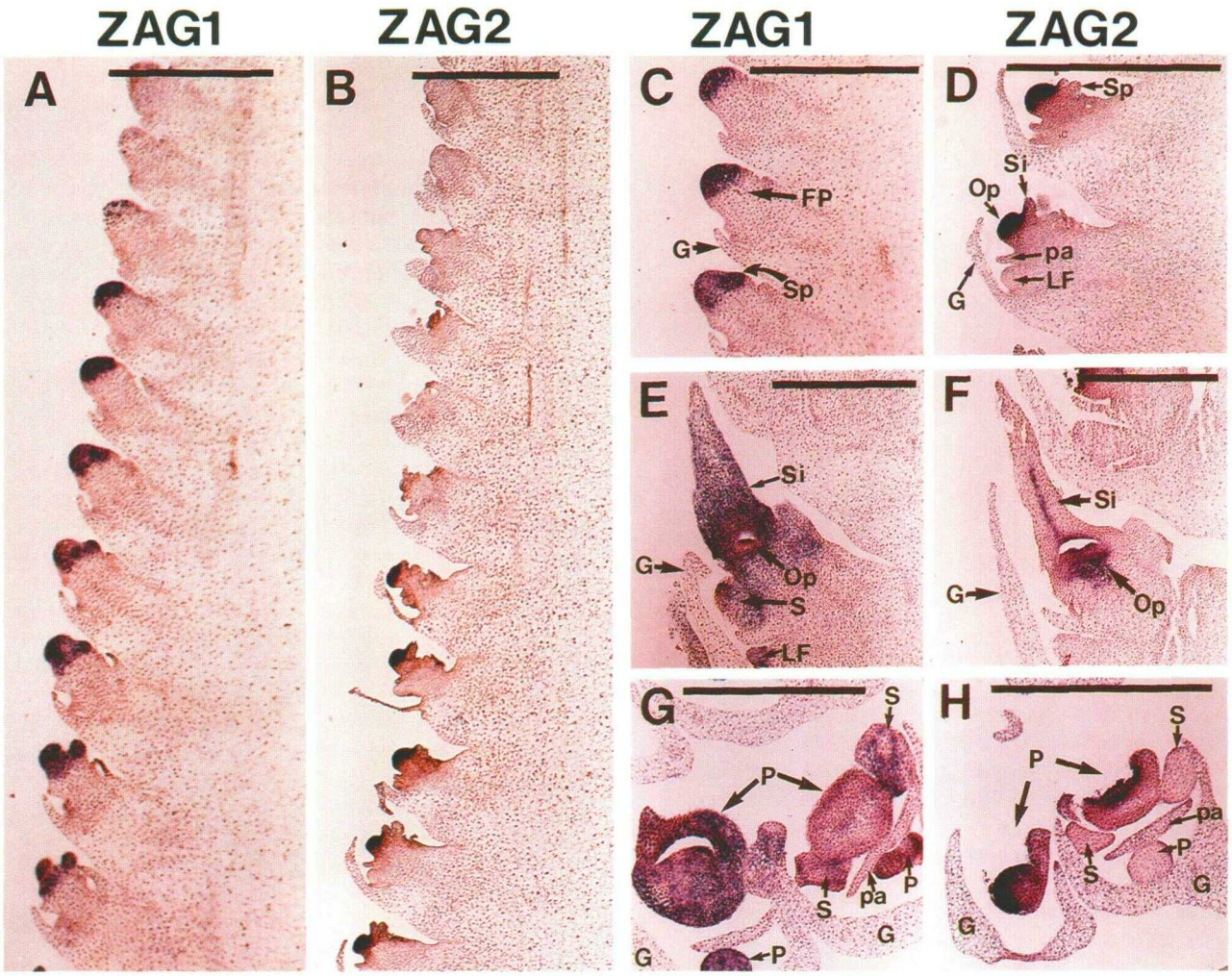

Figure 5. RNA in Situ Hybridization Analysis with *ZAG1* and *ZAG2* on Developing Female Inflorescences.

(A) and **(B)** Median longitudinal sections of an immature ear hybridized to gene-specific probes as indicated.
(C) Close-up of median longitudinal section of three undifferentiated ear spikelet primordia.
(D) As in **(C)**, but showing only two spikelets.
(E) and **(F)** Close-ups of median longitudinal sections of partially differentiated ear spikelets hybridized as indicated.
(G) and **(H)** Transverse cross-sections of a differentiated ear spikelet pair hybridized as indicated.
FP, floret primordium; G, glume primordium; LF, lower floret; Op, ovule primordium; P, pistil primordium; pa, palea; S, stamen; Si, silk primordium; Sp, stamen primordium. Bars = 0.5 mm.

cases highly conserved, it is unclear how such genes function in plants with distinctly different patterns of floral development. As a first step toward addressing this issue, we have cloned and characterized two genes from maize, *ZAG1* and *ZAG2*, whose sequence and pattern of expression are similar to the Arabidopsis floral gene *AG*.

Several observations suggest that *ZAG1* represents the maize homolog of *AG*. The amino acid sequence encoded by *ZAG1* is highly similar to that of *AG* both within and outside the well-conserved MADS motif. The ZAG1 protein produced in vitro binds to putative AG binding sites. Finally, the expression pattern of *ZAG1* is similar to that observed for *AG* (Yanofsky et al., 1990; Bowman et al., 1991a; Drews et al., 1991). Like *AG*, *ZAG1* expression is restricted to developing stamen and carpel primordia. In situ hybridizations also show that *ZAG1* RNA does not appear until well after the transition of the inflorescence meristem into the floral meristem. This *AG*-like pattern of expression suggests that, like *AG*, *ZAG1* may play an important role in determining development of maize stamens and carpels.

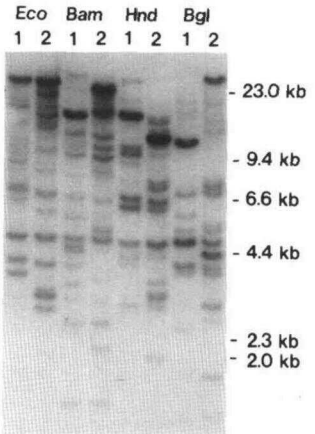

Figure 6. DNA Gel Blot Analysis Using *ZAG1* as a Hybridization Probe.

DNA isolated from the two inbred lines CM37 (lanes 1) and T232 (lanes 2) were digested with either EcoRI (Eco), BamHI (Bam), HindIII (Hnd), or BglII (Bgl) and hybridized with the entire *ZAG1* cDNA under moderate stringencies. Each lane contains 2 μg of digested genomic DNA.

ZAG2, although less similar to *AG* than *ZAG1*, is also expressed in a floral-specific pattern. The pattern of *ZAG2* expression differs, however, in several respects from that observed for *ZAG1*. *ZAG2* appears in the floral primordia at a time in development that is later than *ZAG1* expression. In contrast to *ZAG1*, which is expressed in both stamen and carpel primordia, *ZAG2* expression is largely restricted to the developing ovules and the inner carpel faces. Depending on the angle of the section, a threadlike pattern of hybridization is apparent in the central domain of developing silks. The significance of this expression in the silks is unclear. The apparent restriction of *ZAG2* expression to ovules of developing flowers explains the failure to detect its expression in RNA from tassels and stamens. Scanning electron microscopy studies of developing tassels indicate that abortion of pistil primordia in the tassel begins prior to the stage at which *ZAG2* expression is initiated (Cheng et al., 1983).

AGL1 and *AGL5* are the two *AGL* genes that are most similar in sequence to *AG* (Figure 2). The expression of these two *AGL* genes is flower specific and restricted to developing carpels. *ZAG2*, with its high sequence similarity to *ZAG1* and its carpel-specific pattern of expression, may be the cognate homolog of either of these Arabidopsis genes. The timing of *ZAG2* expression is most similar to *AGL5* (B. Savidge and M. F. Yanofsky, unpublished data) because the onset of *AGL1* expression occurs during considerably later stages of ovule development (Ma et al., 1991).

For both *ZAG1* and *ZAG2*, expression was detected in endosperm tissue as well as in flowers. A blot containing samples of RNA isolated from endosperms harvested between 5 and 25 days postpollination showed the highest levels of expression occurring in the earliest time point, with undetectable levels after 18 days (Figure 4). This could mean that *ZAG1* and/or *ZAG2* play a regulatory role early in endosperm development. Alternatively, the signal we obtain from these endosperm samples may come from the maternal nucellar tissue that surrounds the endosperm early in development and not from endosperm tissue itself (Kiesselbach, 1949). Based on the expression pattern of *AG* in mature carpels (Bowman et al., 1991b), it seems likely that the nucellus of maize could contain *ZAG1* message. The strong signal we observed in unfertilized ovules and at 5 days postpollination (where endosperm, embryo, and nucellar tissue cannot be manually separated), followed by a diminution of signal strength approaching 22 days after pollination, would also be consistent with the time course for the disappearance of nucellar tissue (Kiesselbach, 1949). Resolution of where in developing seeds *ZAG1* and *ZAG2* are expressed will have to be determined by RNA in situ hybridization or immunolocalization of the protein.

Mapping of *ZAG1* and *ZAG2* using the maize recombinant inbred populations has allowed us to place these genes on the restriction fragment length polymorphism map. The position of *ZAG1* correlates well with the genetic map position of the dominant flower mutation that is known as *Pt*. We have examined *Pt* mutant flowers for alterations in *ZAG1* expression. *ZAG1* message is present in mutant male and female flowers, but its appearance is delayed relative to the wild type in ears, and levels of *ZAG1* expression are dramatically elevated in mature tassels (M. Mena and R. J. Schmidt, unpublished results). Although *Pt* clearly affects *ZAG1* expression, we have not determined if this is a direct effect of the mutation. With scanning electron microscopy, comparison of ear development in the wild type and *Pt* mutants (B. Veit, unpublished data) may indicate that the altered expression of *ZAG1* in *Pt* plants is an indirect effect. In *Pt* mutants, the inflorescence meristem initiates normally, but where the floral meristems should arise, an

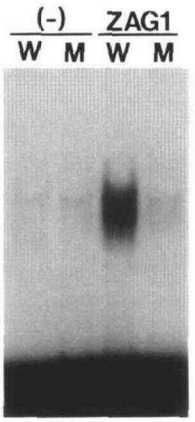

Figure 7. DNA Mobility Shift Assay with ZAG1.

DNA fragments containing the AG consensus binding site sequence (W) or a mutated site (M) were end labeled and incubated with 8 μL of in vitro translation reaction product programmed with *ZAG1* RNA (ZAG1) or lacking *ZAG1* RNA (–).

apparent reiteration of an inflorescence-like meristem occurs, causing a delay in the formation of floral primordia. We are performing further analyses to determine if *Pt* and *ZAG1* are the same locus.

ZAG2 shows linkage with *Ts4*, a mutation resulting in a mutant that produces supernumerary silks in the tassel and to a lesser extent in the ear. However, by RNA in situ analysis, *ZAG2* expression appears largely unaffected in normally developing flowers of *Ts4* ears (B. Veit, unpublished results). *ZAG2* expression is also associated with the silks that develop in the tassel of *Ts4*. Expression of *ZAG2* in *Ts4* mutants might still be consistent with their correspondence if the mutation affected post-transcriptional stages of gene expression.

The 120 million years that separate grass flowers from dicot flowers has allowed for the evolution of a distinctly different reproductive structure. What both flowers have in common is the presence of an inner whorl of carpel(s) adjacent to a whorl of stamens. Our data suggest that a key regulatory protein important for Arabidopsis stamen and carpel development has been conserved in structure and function in a distantly related grass species, such as maize. It will be interesting to see if those regulatory genes important for sepal and petal formation have functional homologs among grasses in which such structures do not now exist but instead have been replaced by structures that distinguish grass flowers from the dicot flowers. One such regulatory gene from Arabidopsis is *AP1*, which is important for sepal and petal development. We have recently isolated the putative *AP1* homolog from maize and are characterizing its pattern of expression. Our initial successes with using clones of dicot floral homeotic genes to identify maize homologs demonstrate that this approach can be one means of obtaining genes that may be important for maize flower development.

METHODS

Construction of cDNA Library

A cDNA library was constructed from poly(A)$^+$ mRNA isolated from wild-type immature ears that were between 10 and 15 mm in length using a λZAPII cDNA cloning kit (Stratagene). These ears encompass a relatively complete set of floral developmental stages, ranging from preemergence of the floret to complete differentiation of floral organs.

Library Screening

Approximately 300,000 plaques were screened with a gel-purified radiolabeled DNA probe specific for the *Arabidopsis thaliana AGAMOUS* (*AG*) cDNA (pCIT565) (Yanofsky et al., 1990). The DNA probes were labeled using the random primed DNA labeling kit from Boehringer Mannheim according to the manufacturer's recommendations. Hybridizations were done at 52°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, which was followed by washes in 5 × SSPE, 0.1% SDS once at room temperature and three times at 52°C. Filters were exposed to Kodak XAR-5 film for 7 days.

Cloning and Sequence Analyses

cDNAs were subcloned into the vector pGEM7Zf(+) (Promega) for sequencing. Double-stranded sequencing was performed using the Sequenase Version 2.0 kit (U.S. Biochemicals) according to the manufacturer's protocol. Both strands were sequenced for each cDNA subclone. DNA and putative protein sequences were analyzed using the MacVector program (International Biotechnologies, Inc., New Haven, CT).

RNA Gel Blot Analysis and in Situ Hybridization

RNA was obtained from developing ears at ~2 cm in size, developing tassels (first- and second-order spikes) at several days preemergence, endosperm and embryos at 22 and 18 days postpollination, respectively, shoots and roots of germinated seedlings, and leaves from 2- to 3-week-old plants. Total RNA was also isolated from glumes and stamens of tassels at the same stage of development as stated above and from carpels of ears obtained at the first day of silk emergence from the husk leaves. Endosperm samples were isolated from developing seeds by cutting off the top of the seed coat, extracting the seed contents with a small spatula, and removing the embryo from the endosperm. The embryo could not be separated from endosperm at time points earlier than 9 days postpollination. Unfertilized ovules were isolated at the time of silk emergence from husk leaves. All tissue samples were frozen in liquid nitrogen upon isolation and stored at −80°C. RNA was extracted as previously described (Cone et al., 1986).

Total RNA samples (10 μg each) were separated by electrophoresis on a 1% agarose gel containing 7.4% formaldehyde and 1 × Mops solution (20 mM 3-[*N*-morpholino]propanesulfonic acid [Mops], 5 mM sodium acetate, 1 mM EDTA). The gel was stained in a solution of 0.125 mg/mL acridine orange, equilibrated in 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 30 min followed by 20 × SSC for 30 min, and then transferred to Hybond-N membranes (Amersham International) using standard blotting techniques (Sambrook et al., 1989) for incubation overnight. Conditions for prehybridization and hybridization were as described previously (Schmidt et al., 1987), except that hybridizations were performed at 42°C in 50% formamide. The random primer–labeled probes used in the RNA gel blot analyses were a 500-bp HpaI-NdeI fragment from the 3′ end of *ZAG1* (for *Zea AG*) or a HindIII-XhoI fragment from the 3′ end of *ZAG2*. A tubulin probe, a subclone of pUCα-1 (Brunke et al., 1982), was used as a loading control. Although equivalent amounts of total RNA were loaded in each lane, the tubulin signal varied between samples, with the leaf and glume RNAs producing the weakest signal.

RNA in situ analyses were performed on tissue sections obtained from fixed ears between 10 and 15 mm in length. Pretreatments were performed on 8-μm-thick sections cut from embedded tissue, as described by Jackson (1991), except that after the initial rehydration steps, tissue was treated with 0.2 M HCl for 20 min at room temperature, followed by rinsing with water for 5 min, 2 × SSC for 20 min, and then rinsing with water for 5 min. Proteinase K was used at 1 μg/mL for 30 min at 37°C instead of pronase. Digoxigenin-labeled RNA probes were prepared according to the manufacturer's instructions (Boehringer Mannheim). The *ZAG1* sense and antisense probes were transcribed

from a linearized template in which sequences 5′ to the HpaI site (containing the MADS box) were deleted. *ZAG2* sense and antisense probes were transcribed from a linearized template in which the MADS box had been deleted by removing sequences 5′ to the PstI site. Probes were denatured for 2 min at 80°C, and then applied to dehydrated sections in a hybridization buffer consisting of 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris-HCl, pH 6.8, 10 mM $NaPO_4$, pH 6.8, 5 mM EDTA, 0.4 μg/mL tRNA, and 0.25 μg/mL poly(A)$^+$ RNA. Probes were used at a concentration of 0.1 μg/mL per kb of probe complexity. Hybridization was conducted for 12 to 18 hr at 50°C. Slides were washed according to the procedures of Langdale et al. (1987). Antibody binding and substrate reaction steps were performed as described by Coen et al. (1990). Unless indicated otherwise, treatments were performed at room temperature.

DNA Gel Blot Analysis and Mapping

Conditions for DNA gel blot hybridizations were performed as reported previously (Evola et al., 1986). Hybridizations were performed at either 42°C in 50% formamide (moderate stringency) or 55°C in 50% formamide (high stringency). Washes were performed three times for 15 min each in 0.1 × SSC, 0.1% SDS at 55°C. The genome map locations of the isolated clones were obtained by DNA gel blot analysis using the 3′-specific fragments described above as probes of restricted DNAs from the maize recombinant inbred family CM37 × T232 (Burr et al., 1988) that were blotted on Nytran membranes. Computer analysis of the results was furnished by Ben Burr (Brookhaven National Laboratory, Upton, NY).

Gel Shift Analysis

A linearized template was transcribed in vitro with T7 RNA polymerase, and an aliquot of the transcription product was used to program a wheat germ lysate translation system (Promega), according to the manufacturer's specifications. Part of each translation reaction product was labeled with ^{35}S-methionine and analyzed on 8% SDS–polyacrylamide gels. The unlabeled ZAG1 protein was assayed for DNA binding by equilibrating 8 μL of programmed lysate for 5 min at room temperature in a mobility shift buffer (10 mM Hepes, pH 7.8, 50 mM KCl, 1 mM EDTA, 10 mM DTT, 2 mg/mL BSA, 100 μg/mL salmon sperm DNA, 10% glycerol). Labeled binding site DNA (0.5 ng) was added, and the reaction (25 μL final volume) was incubated at room temperature for 20 min and loaded onto a 4% polyacrylamide gel cast in 0.25 × Tris-borate-EDTA. Electrophoresis was performed for 2 to 3 hr at 30 mA and was followed by drying of the gel and autoradiography. The wild-type and mutant binding site probes used in this experiment were generated by the fill-in reaction using the Klenow fragment of DNA polymerase I and ^{32}P-αdATP.

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