Conservation of B-class floral homeotic gene function between
maize and Arabidopsis

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Summary

The ABC model of flower development, established through studies in eudicot model species, proposes that petal and stamen identity are under the control of B-class genes. Analysis of B- and C-class genes in the grass species rice and maize suggests that the C- and B-class functions are conserved between monocots and eudicots, with B-class genes controlling stamen and lodicule development. We have undertaken a further analysis of the maize B-class genes Silky1, the putative AP3 ortholog, and Zmm16, a putative PI ortholog, in order to compare their function with the Arabidopsis B-class genes. Our results show that maize B-class proteins interact in vitro to bind DNA as an obligate heterodimer, as do Arabidopsis B-class proteins. The maize proteins also interact with the appropriate Arabidopsis B-class partner proteins to bind DNA. Furthermore, we show that maize B-class genes are capable of rescuing the corresponding Arabidopsis B-class mutant phenotypes. This demonstrates B-class activity of the maize gene Zmm16, and provides compelling evidence that B-class gene function is conserved between monocots and eudicots.

Key words: Silky1, Zmm16, B-class, Maize, MADS-box, Petal evolution

Introduction

Significant progress in elucidating the genetic control of floral patterning has come from research in the model eudicot species Antirrhinum and Arabidopsis. Most flowers consist of four concentric whorls of distinct organs. The first, outermost, whorl comprises sepals, the second petals, the third stamens, and the fourth, central, whorl carpels. In the well-known ABC model of flower development, three classes of genes act alone or in combination to specify the floral organ identity in each whorl (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). A-class genes alone specify sepal identity, A- and B-class genes combine to specify petals, B- and C-class genes combine to specify stamens, and C-class genes alone specify carpels (Fig. 1). The genes of each class have been cloned, and most belong to the MADS-box family of transcription factors. A group of D-class MADS-box genes that appear to play a key role in ovule development have also been proposed (Angenent et al., 1995; Colombo et al., 1995). More recently, the SEPALLATA1, 2 and 3 MADS-box genes in Arabidopsis have been shown to act redundantly as an ‘E’-class, required in combination with B- and C-class activity for the specification of whorls two through four (i.e. petals, stamens and carpels) (Pelaz et al., 2000). Analysis of other higher eudicot species suggests that most aspects of the ABC model are highly conserved (reviewed by Kramer et al., 1998). Similarly, research in the model grass species maize (Ambrose et al., 2000; Mena et al., 1996) and rice (Kang et al., 1998; Kyozuka and Shimamoto, 2002; Nagasawa et al., 2003) suggests that B- and C-class gene activities and patterns of expression are also largely conserved between eudicots and monocots, in spite of at least 150 million years of divergence since their last common ancestor (Wikstrom et al., 2001).

However, other research indicates that some aspects of the model may not be strictly conserved. Analysis of the expression patterns of B-class genes in the petals of a variety of species basal to the higher eudicots shows a striking variability relative to the fixed pattern seen in eudicots (Kramer and Irish, 1999; Kramer and Irish, 2000). Considering the proposal that petals have evolved independently in various angiosperm lineages (Takhtajan, 1991), B-class control of petal identity may not be strictly conserved across all flowering plants, and each independent evolution of petals could have evolved its own mechanism of petal specification (Kramer and Irish, 2000). Consequently, the conservation or divergence of B-class function in the specification of petal identity across the angiosperms is of particular evolutionary and developmental interest.

Loss of B-class function results in the homeotic transformation of whorls two and three, such that petals are converted to sepals, and stamens are converted into carpels, which then fuse with the central gynoecium. In Arabidopsis, two genes have been shown to control B-class function: APETELA3 (AP3) and PISTILLATA (PI) (Goto and Meyerowitz, 1994; Jack et al., 1992). Similarly, in Antirrhinum, the B-class function is controlled by the
orthologous genes: DEFICIENS (DEF) and GLOBOSA (GLO), respectively (Sommer et al., 1990; Trobner et al., 1992). In both organisms, knockouts in either gene have nearly identical phenotypes, and both genes are expressed in whorls two and three of developing flowers. It has also been shown that the AP3 and PI proteins function as an obligate heterodimer to bind DNA in vitro (Riechmann et al., 1996), and to regulate their own transcription in vivo (Goto and Meyerowitz, 1994; Jack et al., 1994), as do DEF and GLO (Schwarz-Sommer et al., 1992). Nuclear localization of the AP3 and PI gene products requires their simultaneous expression (McGonigle et al., 1996). These results suggest that obligate heterodimerization, and simultaneous expression in petals and stamens, are conserved features of higher eudicot B-class function. However, it is not yet clear that the B-class function described for Arabidopsis and Antirrhinum is identical in more basal groups, such as the monocots.

Analysis of B-class function in the grasses is complicated by their unique morphology. The grass flower (floret) is highly derived relative to the eudicot flower. Whereas the first two whorls of the eudicot flower contain sepals and petals, the grass floret comprises a palea and a lemma followed by lodicules (all grass-specific organs), then stamens and carpels as in eudicot flowers (Fig. 1B). The evolutionary relationship of lodicules, palea and lemma to the sterile organs of other flowers has been historically controversial. However, analysis of B-class genes identified and characterized in the grasses maize and rice has suggested a possible interpretation of these structures. In both species, there appears to be only one AP3 ortholog (Ambrose et al., 2000; Moon et al., 1999). Loss of function of the maize AP3 ortholog Silky1 (SI1), results in homeotic transformation of stamens into carpels, and lodicules into palea/lemma-like organs (Ambrose et al., 2000). A nearly identical phenotype was observed in a recent report of the knockout of the rice AP3 ortholog SUPERWOMAN1 (SWP1) (Nagasawa et al., 2003). There are at least three PI-like genes in maize, Zmm16, Zmm18 and Zmm29 (Münster et al., 2001), and two in rice, OsMADS4 and OsMADS2 (Chung et al., 1995). Reduction of OsMADS4 transcript levels by antisense expression in transgenic rice gives a similar phenotype to SI1 and spw1, with a partial conversion of stamens to carpels and lodicules to palea/lemma (Kang et al., 1998). Taken together, these results suggest an interpretation of palea/lemma as sepal homologs, and lodicules as homologous to petals (Ambrose et al., 2000).

In order to more completely characterize the maize B-class function and its relationship to the Arabidopsis B-class function, we have undertaken a further functional analysis of two maize B-class genes: SI1 and Zmm16. We show that SI1 and ZMM16 interact to bind DNA as a heterodimer, and that each protein is capable of interacting with its distantly related Arabidopsis partner to bind DNA. Furthermore, we show that this in vitro binding activity is also present in vivo, as both maize genes can rescue stamen and petal identity in their corresponding Arabidopsis mutants when expressed from the AP3 promoter.

Materials and methods

**cDNA isolation**

A previously characterized RFLP probe designated ucsd72D (Mena et al., 1995) was used to isolate and clone a corresponding genomic fragment of 4.5 kb. Sequence analysis indicated homology to PI and GLO genes, and a subclone was used to probe a cDNA library derived from immature ear inflorescences (Mena et al., 1996) to identify the corresponding cDNA. Several clones were identified as products of the same gene by restriction analysis, and the longest was sequenced from its entirety. The deduced amino acid sequence indicated the highest sequence identity with the PI protein of Arabidopsis and was subsequently designated Zmm16 (Münster et al., 2001). A single cDNA with restriction sites distinct from the others was sequenced and mapped using the recombinant inbred lines (Burr et al., 1988). This cDNA was an apparent duplicate of Zmm16 and was subsequently designated Zmm29.

**In situ hybridization**

A 300 bp PCR product of Zmm16 cDNA, containing all of the I and K domains and part of the C domain, was used in all hybridizations. Probe labeling, tissue preparation and hybridization were performed as previously described (Ambrose et al., 2000).

**Electrophoretic mobility shift assays (EMSA)**

AP3 and PI proteins were produced from clones derived from the in vitro transcription-translation vector pSPUTK (Stratagene), as previously described (Riechmann et al., 1996). The entire coding sequence of the SI1 cDNA was PCR-subcloned into the pSPUTK vector, using a primer at the 5′ end to create an NcoI site at the start codon and KpnI site at the 3′ end. Similarly, the Zmm16 cDNA was...
subcloned into pSPUTK, with a 5′ NcoI site and a 3′ EcoRI site. These clones were subsequently used to produce unlabelled protein for the DNA-binding experiments, using the TNT Coupled rabbit reticulocyte lysate in vitro transcription-translation system (Promega), according to the manufacturer’s protocol. Control TNT reactions were carried out, in parallel with the unlabelled ones, using 35S-labelled methionine, and the labeled proteins were analyzed on 15% SDS-PAGE to verify protein translation efficiency and quality. 32P-labelled, double-stranded oligonucleotide probes were derived from the CARG box sequences of the Arabidopsis AP3 and AGL5 promoters, and synthesized as described by Riechmann et al. (Riechmann et al., 1996). The AGL5 sequence is 5′-AATTGGAATTCAAAAAAGGAAGTT-3′. The AP3 sequence is 5′-TTAGGCAATACCTTTCCATTTTTAGTAAC-3′. The mutant CARG sequence is 5′-AA-TTGGATTAGGAAAAAACAAAGTT-3′ (CARG box sequences are underlined, mutations are in bold). DNA-binding reactions were carried out in 1× Binding Buffer (BB1X: 10 mM Hepes (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM DTT, 2 mg/ml BSA, 0.5 mg/ml fragmented salmon sperm DNA (as a non-specific competitor) and 10% Glyceral in a final volume of 25 μl. The average amount of TNT reaction (or ‘protein input’) used in one DNA-binding assay was about 5 μl. Reactions were incubated without probe for 30 minutes at room temperature. After the addition of the probe (1×106 cpm/ng of double reaction (or ‘protein input’) used in one DNA-binding assay was about 5 μl). Reactions were incubated without probe for 30 minutes at room temperature. After the addition of the probe (1×106 cpm/ng of double stranded oligo), the incubation was extended for 15 additional minutes at room temperature. Reactions were then loaded onto a 5% polyacrylamide gel (0.25×TBE) and run at 150 Volts constant for 1-2 hours in the cold room. The gel was then dried and exposed to Biomax film (Kodak).

**Arabidopsis transformation and genotyping**

A ~1.3 kb fragment of the AP3 promoter from –1312 to –16 relative to the start ATG (kind gift of the Weigel Laboratory) was fused to the coding region of the AP3, SII and Zmm16 cDNAs using standard subcloning methods (AP3pro:AP3, AP3pro:SII, and AP3pro:Zmm16, respectively). These fusion constructs were then further subcloned into the binary vector pMX202 and transformed into Agrobacterium tumefaciens by heat shock. Arabidopsis plants segregating the ap3-3 mutation were transformed with AP3pro:AP3 and AP3pro:SII, and plants segregating the pi-1 mutation were transformed with AP3pro:Zmm16 by the floral-dip method (Clough and Bent, 1998). Kanamycin-resistant seedlings were selected and genotyped by PCR with transgene specific primers, and confirmed by Southern blot for the presence of the transgene.

Homologous ap3-3 transformants were isolated using a dCAPS marker designed using the dCAPS finder program (Neff et al., 1998). The forward primer (AAGAGGATAGGAAACCCAGACAAAGAG) introduces a BsmAI site into the wild-type sequence (introduced mutation in bold) but not the ap3-3 sequence. PCR performed with this primer and the reverse primer (CAAGAATCCAAAAAAAGTG-AGTGG) creates a 257 bp product which, when digested with BsmAI, results in a 20 bp polymorphism between wild-type and ap3-3 products. Homologous pi-1 plants were identified using a CAPS marker, in which FokI cuts the wild-type sequence, but the site is abolished by the pi-1 mutation.

**Expression analysis**

Total RNA was extracted from the inflorescences of wild-type plants and strongly rescued plants of ap3-3 homoyzogotes carrying the Ap3pro:SII transgene, as well as pi-1 homoyzogotes carrying the Ap3pro:Zmm16 transgene. RNA levels were quantified by analysis of gel electrophoresis of the samples and confirmed by verifying equal AGAMOUS levels on a dot blot. Dot-blot analysis of expression levels was performed by spotting equal quantities of RNA from each sample onto nylon membranes. Membranes were then probed with a 32P-dATP-labeled 280-bp fragment from a corresponding 3′ region of the Silky1, Zmm16, AP3, PL and AG cDNAs. Hybridizations were performed at 42°C, as previously described (Ambrose et al., 2000). Blots were washed and exposed to Kodak Biomax film, and a phosphoimagier screen. Quantification of levels was performed using the Scanner Control SI software by subtracting background and averaging the total intensity of three or four replicate dots for each sample.

**SEM analysis**

All tissues were fixed, dried and coated as described previously (Mena et al., 1996). Scanning electron microscopy was performed on a Quanta 600 environmental scanning electron microscope (ESEM) with an accelerating voltage of 15 kV.

**Results**

**In situ localization of Zmm16**

Although the activity of the AP3 ortholog SII has been functionally defined in maize, comparatively little is known about the activity of the maize PI orthologs. To gain further insight into the role of PI-like genes in maize, we cloned two PI-like genes that were subsequently designated Zmm16 and Zmm29 (Münster et al., 2001). Our Zmm29 clone contained a frameshift causing a premature stop codon and a truncated protein. Thus, it was not analyzed further, and we focused on the expression and functional characterization of the full-length Zmm16 clone. In Arabidopsis and Antirrhinum, the MADS-box B-class genes are expressed in a defined region of the floral meristem, and as the flower develops, expression is detected mainly in developing petal and stamen primordia, where it is maintained throughout the development of these organs (Goto and Meyerowitz, 1994; Jack et al., 1992; Schwarz-Sommer et al., 1992; Trobner et al., 1992). Similarly, the maize B-class gene SII is initially expressed throughout the floral meristem and is subsequently restricted to developing stamen and lodicule primordia (Ambrose et al., 2000). We examined expression of the maize PI-like gene Zmm16 in developing male spikelets by in situ hybridization and found a very similar pattern to that previously reported (Münster et al., 2001); however, there were some informative differences, and so we report our results here to confirm their findings and to provide a more detailed analysis.

As the maize tassel develops, Zmm16 expression is first observed throughout the upper floret meristem, just before the stage when palea and lemma primordia begin to emerge (Fig. 2A). Later, as lemma and palea begin to form, Zmm16 is strongly expressed in the region that will give rise to the stamen and lodicule primordia, but is only weakly expressed in the center of the meristem where the carpel primordia will emerge (Fig. 2B,C). As the floret develops, Zmm16 expression is seen in the stamens and the lodicules, and is maintained at a high level throughout the development of these organs (Fig. 2D,F), but it eventually becomes completely absent from the developing carpel of the male spikelet (Fig. 2F). Development of the lower floret is retarded relative to the upper floret, and Zmm16 expression in the lower floret is consequently delayed yet mimics that in the upper floret, with initial expression throughout the meristem (Fig. 2D), and subsequent restriction to the region of the meristem that will give rise to stamen and lodicules (Fig. 2E). Zmm16 expression was never observed in glumes, palea, lemma or any other organ, with the exception of the developing endosperm and embryo (data not shown).
In vitro DNA-binding activity of SI1, ZMM16, AP3 and PI

A key feature of the B-class function in higher eudicots is the obligate heterodimerization of AP3-like proteins with PI-like proteins (Riechmann et al., 1996; Schwarz-Sommer et al., 1992). If B-class function were conserved between monocots and eudicots, it would be expected that the maize B-class proteins SI1 and ZMM16 would act as an obligate heterodimer. In order to assess the conservation of heterodimer specificity of maize B-class genes, we tested their ability to bind a defined CArG-box DNA sequence, a well-characterized target of many MADS proteins (reviewed by Riechmann and Meyerowitz, 1997).

SI1 and ZMM16 proteins were synthesized in vitro, then analyzed in an electrophoretic mobility shift assay for their ability to bind a radioactively labeled CArG-box probe, either alone or in combination with other B-class proteins from maize and Arabidopsis. Fig. 3A shows that neither SI1 nor ZMM16 alone are capable of binding the CArG-box derived from the promoter of the Arabidopsis AGL5 gene (Savidge et al., 1995); however, SI1 and ZMM16 together bind this DNA sequence, suggesting that the formation of a SI1-ZMM16 heterodimer is necessary for DNA binding. The specificity of this binding is shown by a mutation of the CArG-box, which abolishes binding (Fig. 2B). We also tested DNA binding of SI1 and ZMM16, alone and together, using a CArG-box probe derived from the AP3 promoter, and obtained identical results to those from the binding assays performed using the AGL5 CArG-box probe (data not shown).

It is possible that the domains of SI1 and ZMM16 necessary for their specific heterodimerization have evolved independently from those promoting heterodimerization of the eudicot AP3 and PI proteins, in which case the maize B-class proteins would not necessarily be expected to bind DNA with their corresponding Arabidopsis partners. Consequently, we tested the ability of SI1 and ZMM16 to interact with their corresponding Arabidopsis partners to bind DNA. Fig. 3C shows that ZMM16 is capable of binding DNA only in the

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Fig. 2. Zmm16 RNA in situ hybridization of maize developing male spikelets. (A) Two young male spikelets with Zmm16 expression throughout the upper floral meristem (Uf) and absent from the emerging lower floral meristem (Lf). (B) Developing spikelet surrounded by inner (Gi) and outer (Go) glumes. In this section, Zmm16 expression appears throughout the upper floral meristem, but is absent from emerging lemma (Le) and palea (Pa) primordia. (C) Subsequent section of the same spikelet shown in B, showing absence of Zmm16 in the center of the floral meristem. (D) Zmm16 expression in the developing stamen (St), lodicule (Lo) and lower floral meristem. (E) Later stage spikelet with lower floret, reiterating the pattern of expression seen in the upper floret, shown in C. (F) Transverse section showing expression in stamens, but not in the aborting carpel (Ca).

Fig. 3. In vitro DNA-binding assay of maize and Arabidopsis B-class proteins. (A) Autoradiogram of in vitro transcribed and translated SI1 and ZMM16 proteins incubated with labeled AGL5 CArG-box probe. Neither SI1 nor ZMM16 alone are capable of binding the AGL5 CArG-box, but together (SI1+ZMM16 lane) they can, as indicated by the mobility shift of labeled AGL5 CArG probe. Control lane consists of TNT lysate (without added plasmid DNA) incubated with probe. FP designates the lane loaded only with free probe. Arrows indicate background bands in the negative control caused by nonspecific binding of lysate proteins to the probe. (B) As in A, but the probe contains mutations in the AGL5 CArG-box that abolishes SI1-ZMM16 heterodimer binding (see Materials and methods for details). (C) As in A, but includes in vitro transcribed and translated AP3 and PI proteins. Weak binding to the probe in lanes containing PI (AP3+PI and PI+SI1) is due to poor in vitro expression of the PI template, as demonstrated by 35S-labelled TNT control reactions (data not shown).
presence of SI1 and the Arabidopsis ortholog of SI1, AP3, but not in the presence of the Arabidopsis PI protein. Similarly, SI1 is capable of interacting with ZMM16 and PI to bind DNA, but not of interacting with AP3. Taken together, these results suggest that the heterodimer specificity of B-class proteins is conserved between maize and Arabidopsis.

**Complementation of Arabidopsis B-class mutants with orthologous maize genes**

To test the relevance of the SI1-PI and ZMM16-AP3 interaction observed in the in vitro DNA-binding assays, and to determine whether maize B-class genes are capable of interacting with AP3. Taken together, these results suggest that the heterodimer specificity of B-class proteins is conserved between maize and Arabidopsis.

Fig. 4. Complementation of Arabidopsis B-class mutants ap3 and pi by their maize orthologs. (A-E) Arabidopsis flowers of (A) wild type, (B) ap3-3 mutant, (C) ap3-3 with AP3pro:SI1 transgene, (D) pi-1 mutant with AP3pro:Zmm16 transgene (note white sepal margins), and (E) ap3-3 pi-1 double mutant with both AP3pro:SI1 and AP3pro:Zmm16 transgenes (note white petals and sepals). (F-J) Scanning electron microscopy (SEM) of flowers from the same plants from which the flowers shown in A-E were obtained, in the same order. (K-O) SEM of abaxial petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. (K-O) SEM of abaxial petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. (K-O) SEM of abaxial petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order. Cells of wild-type petal or second whorl organ epidermis from flowers shown in F-J, in the same order.

Table 1. Transgenic rescue of Arabidopsis B-class mutants

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<tr>
<th>AP3pro:AP3 in ap3-3</th>
<th>4 (44%)</th>
<th>2 (22%)</th>
<th>1 (11%)</th>
<th>2 (22%)</th>
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<tr>
<td>AP3pro:SI1 in ap3-3</td>
<td>0 (0%)</td>
<td>4 (33%)</td>
<td>4 (33%)</td>
<td>4 (33%)</td>
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<tr>
<td>AP3pro:Zmm16 in pi-1</td>
<td>0 (0%)</td>
<td>6 (50%)</td>
<td>2 (17%)</td>
<td>4 (33%)</td>
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*Full rescue: indistinguishable from wild-type Arabidopsis flowers.
†Strong rescue: petals white but often short, stamens also often short with occasional papilla on tips.
‡Medium rescue: greenish sepaloid petals, and carpeloid stamens.
§Weak rescue: little to no rescue of petal or stamen identity.
pollen, and petals that were slightly green and small. Later flowers were more similar to wild type, with stamens that were often fully extended and which produced fertile pollen, and white petals that had the shape of wild-type petals but were smaller (Fig. 4C,H). These later flowers were self-fertile and all progeny had an identical phenotype. A closer analysis of the epidermis of rescued petals (Fig. 4M) in these plants showed that their cell morphology was intermediate between the elongated cells of second whorl ap3-3 ‘sepalas’ (Fig. 4L) and the characteristic rounded petal cells of wild type (Fig. 4K). The rescued petals also contained occasional stomata, which are found in Arabidopsis sepalas but not petals. Elongated stamens on later flowers also had some slight morphological differences from wild type, such as pointed tips (Fig. 4G) that occasionally had papillae characteristic of carpels (not shown).

Unlike the AP3pro:Si1 plants, early flowers of the AP3pro:Zmm16 plants were more wild type in appearance (Fig. 4D,I). These early flowers had elongated stamens, which shed fertile pollen. The second-whorl organs were white and generally petal-like in appearance although they were smaller than wild type, and typically more involuted than the comparatively flat petals of wild-type flowers. Epidermal cells of these rescued petals showed a similar level of rescue to the AP3pro:Si1 flowers, with small round cells and occasional stomata. Interestingly, sepalas of the AP3pro:Zmm16 plants had a partial transformation to petal identity (Fig. 4N). Generally petal-like in appearance although they were smaller (Fig. 4C,H). These later flowers were self-fertile. The sepals of these plants showed even more dramatic petaloid characteristics than the AP3pro:Zmm16 plants, and were almost completely white (Fig. 4E). The epidermal cells of the rescued double mutant showed partial rescue similar to the individually rescued plants (Fig. 4O).

In order to determine the approximate insert copy number for these rescued plants, Southern blot analysis was carried out with a transgene specific probe. The AP3pro:Si1 line contained 7-11 copies of the transgene, whereas the AP3pro:Zmm16 line contained 4-8 copies (data not shown). The large number of inserts may indicate increased levels of transgene expression relative to their native Arabidopsis orthologs. In order to assess expression levels, we performed an RNA dot blot with total RNA isolated from the inflorescences of the rescued lines and wild type, and hybridized with gene-specific AP3, Si1 and Zmm16 probes (see Materials and methods). The AP3pro:Si1 transgene was expressed at approximately five times the level of the wild-type AP3, whereas AP3pro:Zmm16 was expressed at twice the wild-type AP3 levels (Fig. 5). These results indicate that although capable of rescuing the mutant phenotype, the maize B-function orthologs may require higher levels of expression than are exhibited by the wild-type Arabidopsis B-class genes.

**Discussion**

**Zmm16 functions as a maize B-class gene**

Based on sequence and expression similarities with the eudicot PI and GLO genes, Zmm16, along with two closely related PI/GLO-like genes (Zmm18 and Zmm29), have been proposed to be potential PI/GLO orthologs (Münster et al., 2001). Unfortunately, a loss-of-function phenotype for these genes is likely to be difficult to obtain owing to a probable redundancy in function. Our results provide functional evidence that, at least one of the PI/GLO-like genes of maize acts as a B-class member. ZMM16 is capable of interacting in vitro with Si1, as
well as with the orthologous Arabidopsis AP3, to bind target DNA sequence as an obligate heterodimer. Furthermore, Zmm16 under the control of the Arabidopsis AP3 promoter can rescue a null Arabidopsis pi mutant. Taken together, these data strongly support a B-class function for Zmm16.

A careful analysis of Zmm16 expression in developing male spikelets shows that Zmm16 expression mimics that of the previously characterized Sil, with the exception of a slightly higher level of expression in the emerging carpel primordia. A similar expression pattern was reported previously (Münster et al., 2001). However, our in situ results also show a downregulation in the center of the floral meristem prior to organogenesis. A similar downregulation is seen with Sil, and suggests that the maize B-class genes are tightly co-regulated.

That Sil and Zmm16 expression domains overlap in the stamen and the lodicule primordia suggests that Sil1 and ZMM16 proteins interact there to promote stamen and lodicule identity, as do AP3 and PI in promoting stamen and petal identity. Further evidence for this interaction is provided by the DNA-binding assays, which show that neither SI1 nor ZMM16 can bind DNA alone; instead each requires the presence of the other. The similar expression patterns of Sil and Zmm16, together with their mutual dependence for DNA binding, indicate that an obligate heterodimer pair performs the B-class function of promoting stamens and lodicules in maize just as AP3/PI and DEF/GLO promote petal and stamen identity in the eudicot species Arabidopsis and Antirrhinum, respectively. Our observation of obligate heterodimerization is further corroborated by, and is consistent with, the silky1 mutant phenotype. Unlike many closely related MADS-box genes that show genetic redundancy in Arabidopsis (e.g. SEPALATA1, 2 and 3), AP3 and PI show no apparent redundancy, a probable result of obligate heterodimerization. It is clear from the sil phenotype that ZMM16 alone is not capable of promoting either stamen or lodicule identity, suggesting that a SI1-ZMM16 heterodimer is necessary for B-class function in maize.

A study of the DNA-binding properties of B-class genes from Gnetum gnomon (a gymnosperm) and Lilium regale (lily, a monocot) has suggested that obligate heterodimerization of AP3-like proteins with PI-like proteins evolved from homodimerization (Winter et al., 2002b). Interestingly, that study shows that the two lily PI-like proteins, LRGLOA and LRGLOB, are both capable of binding DNA as a homodimer, whereas the lily AP3-like protein, LRDEF, requires a PI-like partner. Similar results were found for B-class proteins in Tulipa, a genus closely related to Lilium (Kanno et al., 2003). These data appear to be inconsistent with our observation that ZMM16 cannot bind DNA as a homodimer, but requires SI1 as a partner. However, a clear interpretation of these findings is complicated by the existence of various PI-like gene duplications in monocots and insufficient analysis of this character among those lineages. Consequently, it is not clear whether obligate heterodimerization is an ancestral state that was lost in the LRGLOA/B lineage, or whether homodimerization is ancestral and obligate heterodimerization evolved independently in the lineage leading to Zmm16. Although the ability of the gymnosperm B-class protein GGM2 to bind DNA as a homodimer might suggest that homodimerization is ancestral, it is potentially problematic to draw inferences from such a distant outgroup that diverged before the duplication that created the AP3 and PI lineages. A more phylogenetically representative analysis of the dimerization specificity of monocot and other PI-like proteins, including the two other maize duplicates of ZMM16 (ZMM18 and ZMM29), would help to elucidate these issues. This is especially important in the light of recent evidence suggesting divergent roles for the rice PI orthologs OsMADS2 and OsMADS4 (Prasad and Vijayraghavan, 2003).

Evidence for conservation of B-Class gene function between Arabidopsis and maize

Our observation that both Sil and Zmm16 are sufficient to rescue their corresponding Arabidopsis mutants also provides compelling evidence for the conservation of B-class function. It would be expected, if B-class genes had evolved significantly different roles in either maize or Arabidopsis, that the maize genes would not be sufficient to functionally replace the Arabidopsis genes. However, our results indicate that the maize genes, either in combination with their respective Arabidopsis B-class protein partners, or together in Arabidopsis ap3 pi double mutants, are capable of correctly regulating the downstream targets necessary for stamen and petal development, even though in maize, B-class activity is essential for promoting stamen and lodicule development.

It is important to note that the rescue seen in the two strong lines examined was correlated with higher levels of expression than that of the Arabidopsis orthologs. Consequently, it may be necessary to have higher levels of the maize proteins in order to rescue the Arabidopsis mutants. Considering there is an estimated 150 million years of evolution separating monocots and eudicots, and that there is an overall amino acid sequence identity of only 48% in the case of Sil and AP3, and 51% for Zmm16 and PI, higher amounts of the maize proteins may be required to drive interactions with other Arabidopsis proteins on target gene promoters. Furthermore, Sil is a member of the paleoAP3 lineage, whereas AP3 is a member of the higher eudicot euAP3 lineage, created by a gene duplication event and a subsequent translational frameshift that resulted in distinct C-terminal motifs characteristic of each lineage (Kramer et al., 1998; Vandenbussche et al., 2003). In the light of this divergence, it is perhaps not surprising that increased levels of the maize genes are necessary to strongly rescue the Arabidopsis B-class mutants. When the eudicot Antirrhinum DEF gene was used to rescue the Arabidopsis ap3-3 mutant, the rescue was not complete (Irish and Yamamoto, 1995). Thus, even a closely related AP3 homolog of the euAP3 lineage is not sufficient to fully rescue the strong ap3-3 mutant.

If it is granted that lodicules represent a modified petal, then our complementation results make it intriguing to speculate on the difference between a petal and lodicule. The extreme morphological differences between mature lodicules and petals suggest that many of the genes controlling their respective morphogenesis would either be different, or have evolved different transcriptional or biochemical roles. Contrary to this expectation, our results show that the major regulators of lodicule identity in maize are capable of correctly identifying most of the immediate downstream targets needed to correctly specify petal identity in Arabidopsis, suggesting that many of the immediate B-class gene targets may be similar in maize and Arabidopsis. It is possible, then, that the differences...
between petals and lodicules are largely due to the differential activity of genes downstream of the initial targets of B-class proteins. However, a microarray analysis designed to identify the downstream targets of Arabidopsis AP3 and PI proteins suggests that they regulate very few transcription factors, and thus directly control the basic biochemical genes involved in petal and stamen morphogenesis (Zik and Irish, 2003). In the light of that study, another possibility is that the unique morphogenesis of lodicules requires an ancestral petal-promoting activity that is still associated with the maize B-class genes, in addition to an important novel transcriptional activity necessary for lodicule specification.

To our knowledge, our results represent the first time an orthologous maize regulatory gene has been successful at rescuing an Arabidopsis developmental mutant, when expressed from the orthologous promoter. It seems likely, therefore, that many maize genes are capable of complementing Arabidopsis developmental mutants. A study in which the R gene (a maize bHLH transcription factor that regulates anthocyanin biosynthesis) was constitutively expressed in Arabidopsis showed that it could rescue the transparent testa glabrous (ttg) mutant (Lloyd et al., 1992). However, TTG was subsequently shown to be caused by a mutation in a gene encoding a WD40 repeat protein, and was clearly not orthologous to R (Walker et al., 1999). Consequently, care must be taken when interpreting rescue as sufficient evidence of functional equivalence without further genetic or biochemical evidence. In the case of Sl1 and Zmm16, we feel that there is a strong case for functional equivalence when one considers the similarity in mutant phenotypes of Sl1 plants with those of ap3 or pi, together with the similarity in DNA-binding activity of the maize and Arabidopsis B-class genes, and the ability of the maize genes to largely complement their corresponding Arabidopsis mutants when expressed under an appropriate B-class promoter.

Implications for the evolution of angiosperm petals

Some interesting issues raised by the ability of maize B-class genes to functionally replace Arabidopsis homologs concern the relationship of lodicules to petals, and the history of petal evolution in angiosperms. The classical view of flower evolution in angiosperms holds that the reproductive structures (stamens and carpels) evolved just once, whereas the petals and sepals independently evolved many times (Takhtajan, 1991). It is generally accepted that B-class genes promote stamen identity across the angiosperms. Such a role is likely to be derived from an ancestral role, maintained in gymnosperms, of specifying male cone identity (Fukui et al., 2001; Mouradov et al., 1999; Sundstrom et al., 1999; Sundstrom and Engstrom, 2002; Winter et al., 1999). However, a conserved B-class role in specifying petal identity in all angiosperms is more controversial, as B-class gene expression is often highly variable, even among the basal eudicots (Kramer and Irish, 1999; Kramer and Irish, 2000). More recently, Lamb and Irish (Lamb and Irish, 2003) have shown that the C terminus from a basal eudicot AP3-like protein, when fused to the Arabidopsis AP3, is capable of rescuing stamen but not petal development in an ap3 mutant. In contrast to these findings, our results show that the more distantly related, full-length grass B-class proteins are capable of identifying and properly regulating the genes necessary for proper petal and stamen development in a eudicot flower. Thus, the lack of petal rescue observed by Lamb and Irish (Lamb and Irish, 2003) may represent a derived state of this lineage of basal eudicot AP3 genes resulting from gene duplication and divergence. Alternatively, amino acid differences in the C-terminal region of this AP3 ortholog may require compensatory changes in other domains of the protein in order to promote both stamen and petal development. Their derived fusion construct between AP3 and the C terminus of this paleoAP3 would not have contained such compensatory changes.

We feel that the striking rescue of petal identity in Arabidopsis by maize B-class genes is further evidence supporting the homology between petals and grass lodicules. Although compelling, this evidence cannot exclude the possibility that B-class genes were recruited independently to specify lodicules in grasses. Furthermore, the petal rescue demonstrated by Sl1 and Zmm16 could be interpreted as non-specific, and simply the result of expressing a related gene family member. However, we think this is an unlikely explanation, as a Gnetum gnemon B-class gene is totally incapable of promoting petal identity in the second whorl of an Arabidopsis ap3-3 mutant (Winter et al., 2002a). Nevertheless, a more rigorous demonstration of homology would need to involve loss of B-class gene function in a range of species, including monocots that have more obvious petaloid organs. One such mutant possibly exists in the viridiflora cultivar of tulip, which shows homeotic transformations similar to eudicot B-class mutants (van Tunen et al., 1993). However, it is not yet known whether a mutant B-class MADS-box gene is involved. It is interesting to note that the Joinvilleaceae, a sister group to the grasses (Kellogg, 2000), has a differentiated whorl of sepals and petals, and Streptochaeta, a basal grass genus, contains three foliar organs in the position of lodicules (Mathews et al., 2000; Page, 1951). Analysis of B-class gene expression in these species will provide evidence that may help resolve these questions regarding the relationship of lodicules and petals.

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References


