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* (*SPW1*) (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.

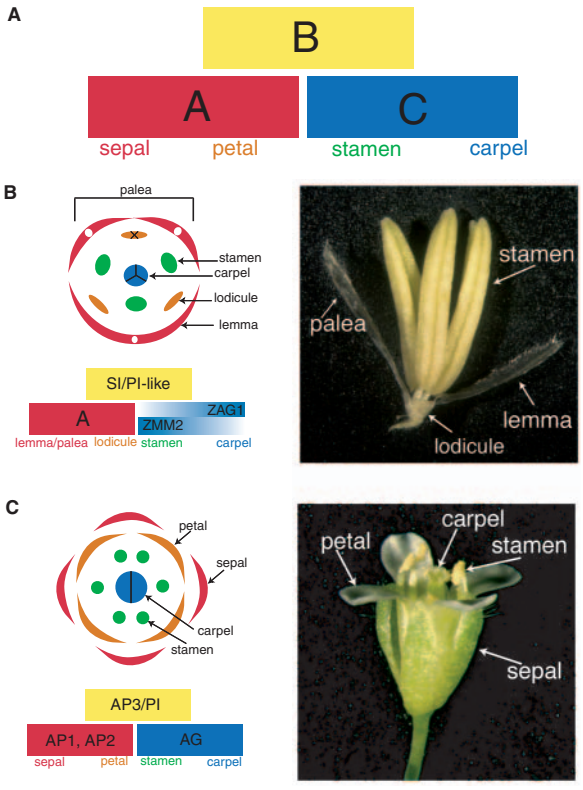

Fig. 1. ABC Model for patterning maize florets and *Arabidopsis* flowers. (A) Diagram of the traditional ABC model. (B) ABC model adapted for the maize floret (Ambrose et al., 2000). Maize genes are indicated in their appropriate domains (an aborted lodicule is indicated by X). A photo of a maize floret, with floral organs indicated, is shown on the right. (C) Floral diagram, ABC model and photo of *Arabidopsis* flower.

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 in 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 *Nco*I site at the start codon and *Kpn*I site at the 3′ end. Similarly, the *Zmm16* cDNA was

sublconed into pSPUTK, with a 5′ *Nco*I site and a 3′ *Eco*RI 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 ^{35}S-labelled methionine, and the labeled proteins were analyzed on 15% SDS-PAGE to verify protein translation efficiency and quality. ^{32}P-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′-AATTGGATTACCAAAAAAGGA-AAGTT-3′. The *AP3* sequence is 5′-TTAGGCAATACTTT-CCATTTTTAGTAACTC-3′. The mutant CArG sequence is 5′-AA-TTGGATTA**GG**AAAAAA**CC**AAAGTT-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% Glycerol] 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 (1×10^6 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*, *Si1* and *Zmm16* cDNAs using standard subcloning methods (*AP3*pro:*AP3*, *AP3*pro:*Si1*, and *AP3*pro:*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 *AP3*pro:*AP3* and *AP3*pro:*Si1*, and plants segregating the *pi-1* mutation were transformed with *AP3*pro:*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.

Homozygous *ap3-3* transformants were isolated using a dCAPS marker designed using the dCAPS finder program (Neff et al., 1998). The forward primer (AAGAGGATAGAGAACCAGACAAA**G**AGA) introduces a *Bsm*AI 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 (CAAAATCACCAAAAAAGT-AGTGG) creates a 257 bp product which, when digested with *Bsm*AI, results in a 20 bp polymorphism between wild-type and *ap3-3* products. Homozygous *pi-1* plants were identified using a CAPs marker, in which *Fok*I 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* homozygotes carrying the *Ap3*pro:*Si1* transgene, as well as *pi-1* homozygotes carrying the *Ap3*pro:*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 ^{32}P-dATP-labeled 280-bp fragment from a corresponding 3′ region of the *Silky1*, *Zmm16*, *AP3*, *PI* 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 phosphoimager 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 *Si1* 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 *Si1* 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).

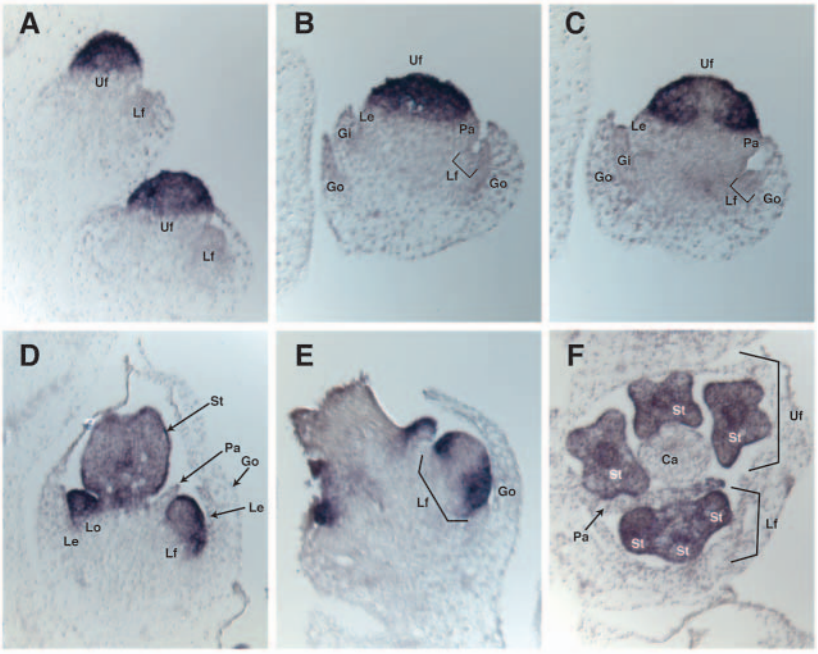

Fig. 2. *Zmm16* RNA in situ hybridization of maize developing male spikelets. (A) Two young male spikelets with *Zmm16* expression throughout the upper floral meristem (Uf) and absent from the emerging lower floral meristem (Lf). (B) Developing spikelet surrounded by inner (Gi) and outer (Go) glumes. 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).

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 a 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 heterodimeriztion 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

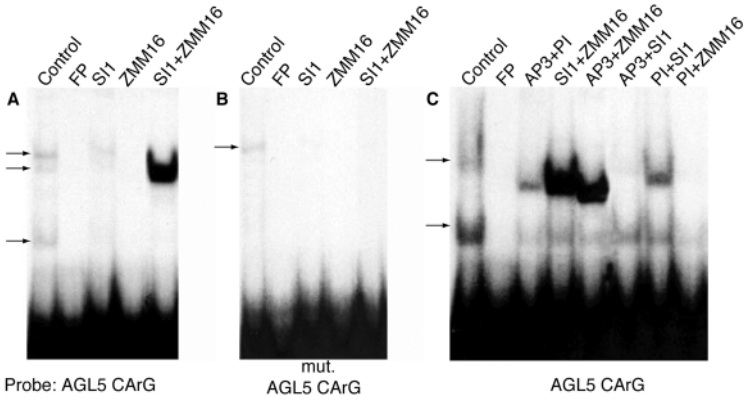

Fig. 3. In vitro DNA-binding assay of maize and *Arabidopsis* B-class proteins. (A) Autoradiogram of in vitro transcribed and translated SI1 and ZMM16 proteins incubated with labeled *AGL5* CArG-box probe. Neither SI1 nor ZMM16 alone are capable of binding the *AGL5* CArG-box, but together (SI1+ZMM16 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 ^{35}S-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 functionally replacing their *Arabidopsis* orthologs, we created rescue constructs using the *Arabidopsis AP3* promoter (*AP3*pro) to drive expression of the maize *Si1* and *Zmm16* cDNAs in whorls two and three of developing *Arabidopsis* flowers. As a control, we also fused the *AP3* promoter to the *AP3* cDNA to ensure that our *AP3*pro fragment contained sufficient regulatory information to rescue an *ap3* mutant. The *AP3*pro:*AP3* and *AP3*pro:*Si1* transgenes were transformed into *Arabidopsis* plants heterozygous for *ap3-3*, a null allele caused by a stop codon in the MADS-box (Jack et al., 1992). The *AP3*pro:*Zmm16* construct was transformed into plants heterozygous for *pi-1*, a null mutation caused by stop codon in the I-domain (Goto and Meyerowitz, 1994). Transformants both containing the appropriate transgenes and homozygous for either *ap3-3* or *pi-1*, respectively, were identified by PCR and Southern blot (see Materials and methods). For all three constructs, independent transformants were identified showing a range of phenotypes (Table 1). Most of the *AP3*pro:*AP3* lines showed complete (4/9) or strong (2/9) rescue. Twelve independent *AP3*pro:*Si1* transformants were identified that were homozygous for *ap3-3*, together with 12 independent *AP3*pro:*Zmm16* transformants homozygous for *pi-1*. In neither case did rescue result in a phenotype indistinguishable from wild type, as was observed in some of the transformants carrying the *AP3*:pro*AP3* construct. Four of the *AP3*pro:*Si1* lines showed relatively strong rescue (see below), and six *AP3*pro:*Zmm16* lines showed a similar level of rescue. Medium to weak complementation was also seen for both lines, in which neither stamens nor petals were completely rescued. In none of the lines was there a rescue of stamen identity without a similar level of petal rescue. One of each strongly rescued line was selected for more detailed phenotypic analyses.

Table 1. Transgenic rescue of *Arabidopsis* B-class mutants

	Full*	Strong†	Medium‡	Weak§
*AP3*pro:*AP3* in *ap3-3*	4 (44%)	2 (22%)	1 (11%)	2 (22%)
*AP3*pro:*Si1* in *ap3-3*	0 (0%)	4 (33%)	4 (33%)	4 (33%)
*AP3*pro:*Zmm16* in *pi-1*	0 (0%)	6 (50%)	2 (17%)	4 (33%)

*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.

In comparison to wild type, early flowers of a strong *AP3*pro:*Si1* rescue plant had short stamens that did not shed

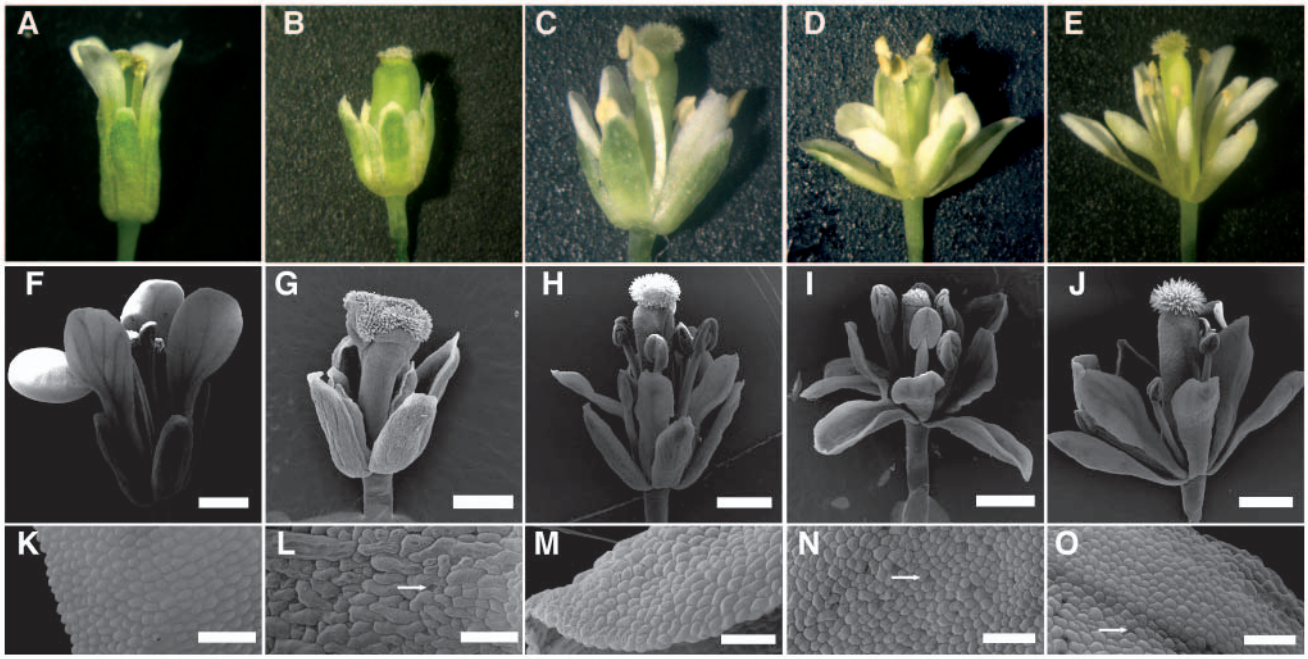

Fig. 4. Complementation of *Arabidopsis* B-class mutants *ap3* and *pi* by their maize orthologs. (A-E) *Arabidopsis* flowers of (A) wild type, (B) *ap3-3* mutant, (C) *ap3-3* with *AP3*pro:*Si1* transgene, (D) *pi-1* mutant with *AP3*pro:*Zmm16* transgene (note white sepal margins), and (E) *ap3-3 pi-1* double mutant with both *AP3*pro:*Si1* and *AP3*pro:*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 epidermis (K) are rounded and no guard cells are present. Cells of second whorl 'sepal' epidermis of *ap3-3* mutant (L) are elongated and irregular, with many guard cells. Rescued mutants (M-O) have epidermal cells that are intermediate in shape between those seen in K and L, with occasional guard cells. Note the lack of elongated epidermal cells characteristic of sepals. Arrows in L, N and O indicate guard cells. Scale bars: 0.5 mm in F-J; 50 μm in K-O.

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* 'sepals' (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* sepals 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 *AP3*pro:*Si1* plants, early flowers of the *AP3*pro:*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 *AP3*pro:*Si1* flowers, with small round cells and occasional stomata. Interestingly, sepals of the *AP3*pro:*Zmm16* plants had a partial transformation to petal identity (Fig. 4N). Generally the sepal margins of *AP3*pro:*Zmm16* plants were white, and occasionally there would be patches of white within the sepal. Similar sepal transformation phenotypes are seen in *Arabidopsis* plants ectopically expressing *PI* under the control of the constitutive 35S viral promoter (Krizek and Meyerowitz, 1996; McGonigle et al., 1996). One explanation for these results is that the *AP3* promoter drives some weak expression in sepals. Consequently, we interpret these mosaic sepals as the result of weak expression of *Zmm16* in the sepals, where the resulting ZMM16 protein is able to interact with native AP3 to promote petal identity. The very last flowers to be produced by the inflorescence meristem of the *AP3*pro:*Zmm16* plants often showed weaker rescue, with sepaloid petals and carpeloid stamens that had ectopic ovules (not shown). Like the *AP3*pro:*Si1* plant, the *AP3*pro:*Zmm16* plant was self fertile, and subsequent generations had a similar level of rescue.

These results suggest that both SI1 and ZMM16 proteins are capable of interacting with their *Arabidopsis* partners in vivo to rescue B-class mutants. However, it is not clear from these experiments whether the maize genes are sufficient, in the absence of an *Arabidopsis* partner, to rescue *Arabidopsis* B-class mutants. In the rescued plants, it is possible that the native *Arabidopsis* AP3 or PI proteins only needed to dimerize with their maize partner to enter the nucleus and bind DNA, but were otherwise sufficient by themselves to activate the appropriate downstream genes. Consequently we created an *ap3 pi* double mutant containing both *AP3*pro:*Si1* and *AP3*pro:*Zmm16* transgenes by crossing strongly rescued *AP3*pro:*Si1* and *AP3*pro:*Zmm16* plants. The rescued double mutant showed a combination of traits of the individually rescued plants. Early flowers showed a weak rescue with very short stamens and greenish sepaloid petals (not shown). Later flowers had elongated stamens and white petals with the involuted margins of the *AP3*pro:*Zmm16* plants (Fig. 4E,J). The stamens of later flowers produced fertile pollen and the plants were self fertile. The sepals of these plants showed even more dramatic petaloid characteristics than the *AP3*pro:*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).

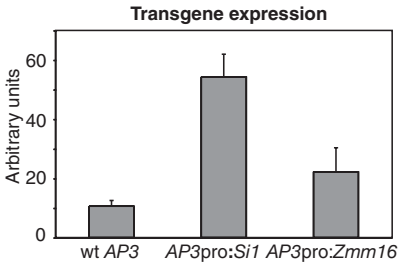

Fig. 5. Maize transgene expression levels compared with wild-type *AP3* levels. Total RNA collected from inflorescences of strongly rescued *AP3*:*Si1* and *AP3*:*Zmm16* plants, was spotted onto nylon filters and hybridized with transgene specific probes or an *AP3* gene-specific probe having identical specific activities. Expression levels are arbitrarily designated by the ImageQuant software after exposure to a phosphoimager screen.

F2 progeny resulting from the above cross were also identified in which the *AP3*pro:*Si1* transgene was present in a *pi-1* homozygous background, and *AP3*pro:*Zmm16* was present in the *ap3-3* homozygous background. In both cases, the transgene failed to rescue the mutant, as the plants were indistinguishable from the *ap3* and *pi* mutants (data not shown).

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 *AP3*pro:*Si1* line contained 7-11 copies of the transgene, whereas the *AP3*pro:*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 *AP3*pro:*Si1* transgene was expressed at approximately five times the level of the wild-type *AP3*, whereas *AP3*pro:*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 *Si1*, 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 *Si1*, and suggests that the maize B-class genes are tightly co-regulated.

That *Si1* and *Zmm16* expression domains overlap in the stamen and the lodicule primordia suggests that SI1 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 *Si1* 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 *si1* 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 gnemon* (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 *Si1* 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 *Si1* 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, *Si1* is a member of the paleo*AP3* lineage, whereas *AP3* is a member of the higher eudicot eu*AP3* 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 eu*AP3* 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 *Si1* and *Zmm16*, we feel that there is a strong case for functional equivalence when one considers the similarity in mutant phenotypes of *Si1* 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 (Takthajan, 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 *Si1* 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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