UNIVERSITY OF CALIFORNIA, SAN DIEGO

B and C class MADS-box genes and the developmental genetics of maize flower development

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requirements for the degree Doctor of Philosophy

in

Biology

by

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The dissertation of Clinton J. Whipple is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2006

DEDICATION

To my son Jacob and my daughter Claire

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It is reprinted with permission from all co-authors and the publisher. I was the primary author and researcher, and supervised the research which forms the basis of this chapter.

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POSTER ABSTRACTS

Whipple, C., Ambrose, B., and Schmidt R. (2004). *Maize Genetics Conference Abstracts*. 46:P88. Assessing the Functional Redundancy in the Maize C-Class Control of Carpel and Stamen Identity.

Whipple, C., Ambrose, B., Mena, M., and Schmidt R. (2003). *Maize Genetics Conference Abstracts* 45:P110. Transgenic analyses of a duplicate pair of maize MADS-box genes, Zag1 and Zmm2, suggest protein subfunctionalization of the C class in the traditional ABC model of flower development.

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ABSTRACT OF THE DISSERTATION

B and C class MADS-box genes and the developmental genetics of maize flower development

by

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Professor Robert J. Schmidt, Chair

The ABC model of flower development describes how a flower is patterned and the genes necessary for floral organ identity. However, it is not clear that the ABC model can be generally applied to the flowering plants, as it was based solely on genetic studies from the core eudicot species *Arabidopsis* and *Antirrhinum*. This dissertation describes an examination of maize orthologs of B and C class genes, and compares their function with B and C class genes of *Arabidopsis* to understand the degree to which the ABC model is conserved.

B class genes from maize were found to rescue *Arabidopsis* B class mutants, and the maize B class proteins were shown to bind DNA as an obligate heterodimer as has been demonstrated in *Arabidopsis*. These findings indicate conservation in biochemical function of the maize and *Arabidopsis* B class proteins. Furthermore, these findings support the conclusion that the lodicule, a grass specific organ of

uncertain homology, represents a modified petal. A comparative expression approach was used to further verify the relationship of lodicules to the organs of non-grass flowers. B class genes were shown to be expressed in a whorl of foliar organs outside the stamens in *Streptochaeta*, a basal grass that diverged before the evolution of lodicules, and in the petals of the outgroup species *Joinvillea* and *Chondropetalum* strongly supporting the interpretation that lodicules are modified petals, and further supporting conservation of B class function between *Arabidopsis* and maize.

Zag1 and *Zmm2* are duplicate pair of C class genes from maize that are hypothesized to have partitioned the C class function of establishing stamen and carpel identity. Rescue of the *Arabidopsis* C class mutant *ag* with the two maize genes confirms that their protein products have subfunctionalized, with ZAG1 better able to promote carpel identity, and ZMM2 better able to promote stamen identity. A more recent duplicate of *Zmm2* was isolated, *Zmm23*, as were mutant alleles of *zmm2* and *zmm23*. While the *zmm2 zmm23* double mutant had no phenotype, the *zag1 zmm2 zmm23* showed a considerable enhancement of the previously described *zag1* phenotype substantiating a C class function for *Zmm2* and *Zmm23*.

CHAPTER I

Introduction: Genetics of grass flower development

Abstract

The developmental genetic analyses of floral organ specification that led to the well-known ABC model of flower development were primarily performed in eudicot model species. To better understand how pathways controlling flower development have either been conserved or modified more broadly in the angiosperms, it is necessary to examine the genetic basis of flowering in plant groups more distantly related to *Arabidopsis* and *Antirrhinum*. Maize and rice are grass species with genomics and genetic resources that make them amenable to both forward and reverse genetics. A combination of these two strategies is beginning to elucidate how the ABC model is conserved, as well as ways in which grass flower development differs from eudicots. The ability to investigate the degree of conservation in developmental pathways, the evolution of derived morphologies, and the consequences of gene duplication events make the grass family an excellent model for studies on the evolution of flower development.

Introduction

The most striking characteristic feature of angiosperms was the evolution of the flower, which, although it has been extremely modified in multiple angiosperm lineages, is basically composed of four organ types: 1. sepals, bract-like protective leaves 2. petals, modified leaves serving as pollinator attractors 3. the stamen, or male reproductive organ, and 4. the carpel, or female reproductive organ. These organs are found almost always (with the only known exception being *Lacandonia schismatica*

(Ambrose et al., 2006)) in a stereotypical order of concentric whorls starting from the outside and moving inwards: sepal, petal, stamen, carpels. There are, of course, many species with missing whorls, or highly modified organs. Nevertheless, conservation of these organ types and this basic body plan, allows us to recognize these shared features in even very modified and bizarre flowers.

Early developmental genetic research in model eudicot species *Arabidopsis thaliana* and *Antirrhinum majus* led to the establishment of a simple and powerful model for the genetic establishment of floral organ identity (Coen and Meyerowitz, 1991)(Fig. 1.1). The original ABC model held that three classes of genes worked alone or in combination with another class to specify the identity of each of the four organ whorls. A class alone provides sepal identity. A and B classes combine to establish petal identity. B and C classes together promote stamen identity, and C class alone is responsible for carpel identity. As the genes underlying these activities were cloned, nearly all turned out to belong to a large family encoding transcription factors containing the conserved MADS domain, a protein domain that binds to the consensus CArG-box sequence in promoters of prospective target genes (Huang *et al.*, 1993; Mueller and Nordheim, 1991; Riechmann *et al.*, 1996). The only exception is *APETELA2*, a classically defined A class homeotic gene, that belongs to a different family of putative transcription factors. As more was learned about the genetic control of flowering in *Arabidopsis*, it was clear that the ABC model would need to be modified to include the activity of another set of MADS-box genes with redundant roles in regulating the activity of B and C class MADS proteins (Pelaz et al 2001).

Other studies revealed further roles of additional MADS-box genes in specifying the identity of ovules, which together led to the proposal for the expansion of the ABC model to include D and E classes, with D activity specifying ovule identity and E activity being required for the activity of B and C classes (Theissen 2001). E class activity was later shown to be necessary for A class gene function in addition to B and C (Ditta et al., 2004). Although it is not clear that ovule development should be included in a model that was designed to describe floral organ specification, it serves as a useful categorization of MADS-box gene members for the purpose of this review. These studies in model eudicot species have established the prominent role of so many different MADS-box genes in eudicot flower and ovule development, resulting in a large amount of interest and research on this family of genes. This is especially true for researchers interested in the evolution of developmental differences (evo-devo) in the origin and diversification of flowers.

It was recognized early that modifications in the expression of the ABC genes could be very useful in describing some of the floral variation seen in other angiosperms groups, such as the petaloid outer tepals seen in some monocots (van Tunen *et al.*, 1993). More recently, modifications of the ABC model have been invoked to describe morphological diversity in some basal eudicot flowers (Kramer *et al.*, 2003). However, there is still a paucity of genetic or functional evidence to understand the degree to which the ABC model is conserved outside the core eudicots.

There are at least two questions of broad general interest for understanding the evolution of flowers. First, what is conserved? That we can recognize a diversity of morphologies as flowers seems to indicate that there is a conserved genetic mechanism establishing the floral ground plan and organ identities. At first glance, it would seem that the ABC model genes make good candidates for such a mechanism. The second question is: What is different? In other words, what kinds of changes are responsible for the often striking differences in floral morphologies that occur in the angiosperms? Do modifications in ABC genes or their expression domains correlate with changes in morphology, or is the ABC model a basic ground plan, which most angiosperms maintain, and the morphological diversity of flowers is controlled by other pathways? In order to make progress on these two questions it is important to have a model group or species that is outside the core eudicots, one which has available forward and reverse genetic resources to rigorously test gene function. Additionally, to address the second question a group is needed that has some derived floral features. We would argue that the grasses, and maize (*Zea mays*) and rice (*Oryza sativa*) in particular, make an excellent choice for just such an inquiry. As monocots, grasses are distantly related to the species serving as plant models in the core eudicots. They have a derived floral morphology, especially in the two sterile outer whorls. Furthermore, an increasing array of genetic and genomic resources is being developed for these species. It is thus possible to begin examining grass ABC model genes for conservation of function. It is also possible, through forward screens, to identify grass-specific factors involved in the derived morphology of this group.

In this review we will discuss the genetics of grass flowering. Other reviewers have dealt broadly with advances in understanding grass inflorescence development (Bommert et al., 2005; McSteen et al., 2000, Malcomber et al., this issue). For this reason, we will focus specifically on the genetics of flower development. As these studies lead naturally to a discussion of comparative floral development, we will finish by presenting the case for using grasses, with their emerging genomic resources, as a model group for evo-devo studies.

Grass Floral Morphology

Mature floral morphology in the grasses differs significantly from the typical monocot flower, so much so that traditional assignments of homology have been problematic (Clifford, 1987) (see Fig. 1.2). It is clear however, from recent phylogenetic analyses of the Poales (GPWG, 2001; Michelangeli et al., 2003) that the closest extant outgroups to the grasses, *Joinvillea* and *Ecdeiocolea*, have a typical monocot floral plan with two outer whorls of tepals (more or less differentiated between the inner and outer tepal whorl), a whorl of stamens, and a central whorl of carpels. As typical in the monocots, the floral organs in each whorl occur in multiples of three. In the grasses, the basic unit of the inflorescence is the spikelet, or a small spike composed of one to many florets and subtended by two sterile bracts called glumes. The florets themselves consist of: 1. a **lemma**, often considered to be a bract in the axil of which the flower arises, 2. a **palea**, a generally two-veined bract-like organ, occasionally

interpreted as the prophyll of the floral branch subtended by the lemma, 3. **lodicules**, small scale-like or fleshy organs that swell at anthesis to open the floret and exsert the anthers. In some grasses (mostly bamboos), there are three lodicules that alternate with the anthers. However, in most grasses the medial (adaxial) lodicule aborts leaving only the two abaxial lodicules adjacent to the lemma. 4. **Stamens**, the male reproductive organs, generally occur in one or two whorls of three. 5. **Carpels**, the female reproductive unit is composed of three fused carpels, generally with two stigmas and a single ovary.

The homology of the reproductive organs in grass flowers with those of eudicot flowers is clear and not in question. However, the outer sterile organs are derived in the grasses leading to their grass specific nomenclature of lemma, palea, and lodicule. By making assumptions about the homology of the sterile floret organs, it is possible to adapt the ABC model to the grass floret and propose a hypothesis of how ABC genes may act in patterning a grass flower Fig. 1.1 (Ambrose *et al.*, 2000). We will come back to a possible scenario for the evolution of the grass floret, and ways to test it.

Genetics of Grass Flower Development

Both forward and reverse genetic approaches have been taken to understand the genetic control of flowering in grasses. Forward genetics starts with a mutant known to cause a defect in a particular trait, but without any knowledge of the gene that has been mutated. This powerful approach was used in eudicot species to establish the

ABC model. While forward genetics is theoretically possible in any plant, practical difficulties in cloning genes underlying grass mutations initially limited its utility. However, candidate genes revealed from *Arabidopsis* and *Antirrhinum* could be examined in grasses (see below). As these genes were cloned, their expression patterns were observed by a combination of *in situ* hybridization and northern blot analyses, with the thought that conserved patterns of expression would indicate conserved function. Eventually more rigorous tests of function for some genes involved isolating transposon insertion mutants and specific knockdown of transcription by transgenes. Fortunately, increased genomic resources have recently made forward genetics more practical in grasses. This combination of forward and reverse genetics is beginning to unravel the developmental mechanisms of flowering in this interesting and economically important plant family.

MADS-box genes and grass flower development: rounding up the usual suspects

With studies in maize and rice, grasses emerged early as a model group to test the function of homeotic MADS-box genes outside the eudicots. While members from all classes (ABCD and E) have been identified, little is known about the function of these genes beyond the B and C classes. We will briefly review what is known about these groups in the grasses classifying the genes according to published phylogenetic work and focusing on studies that elucidate individual gene function. Although the ABCDE model in Fig. 1.1 suggests that genes in these five classes have clear roles in organ identity, this is not always the case as will be discussed. However, genes in each class

tend to be related phylogenetically, and thus the division into classes A-E is a convenient classification.

A Class

According to the ABC model, A class genes in *Arabidopsis* are necessary for sepal and petal identity. *Arabidopsis* A class includes the MADS box gene *APETELA1* (*AP1*) and the non MADS-box gene *APETELA2*. While mutations in *AP1* cause difficulties in sepal and petal development, it is not clear that *AP1* contributes directly to a discreet A function for organ identity. These phenotypes may in fact be the result of incomplete loss of floral meristem identity as indicated by the phenotype of the triple mutant combination of *ap1* with two closely related genes in *Arabidopsis, cauliflower* (*cal*) and *fruitful* (*ful*) (reviewed in Litt and Irish (2003)). Also, a true A class was never defined in *Antirrhinum*, even though the *squamosa* mutant was shown to encode the *AP1* ortholog (Huijser *et al*., 1992). Furthermore, *LIP1* and *LIP2*, the redundant *Antirrhinum* orthologs of the non MADS-box A class gene *AP2* do not have a completely conserved function with the *Arabidopsis* gene (Keck *et al*., 2003), nor apparently does the *Petunia hybrida AP2* homolog *PhAP2A* (Maes et al., 2001). Thus, while the literature commonly refers to A class specification of floral organ identity, the situation may be more subtle and complicated.

Whatever the contribution of *AP1* to A class gene function, there are clearly grass genes that belong to the *AP1*/*FUL* group, and these appear to fall into three distinct lineages (see Fig. 1.3A) (Münster *et al*., 2002; Schmitz *et al*., 2000). One lineage includes *Zap1a* from maize and its duplicate *ZmMADS3 (Zap1b)*, *BM8* from

barley (*Hordeum vulgare*), and *OsMADS15* from rice (Heuer *et al*., 2001; Mena *et al*., 1995; Moon *et al*., 1999; Schmitz *et al*., 2000). A second lineage includes *Zmm4* and *15* from maize, *OsMADS14* from rice, and *BM5* from barley (Fischer *et al*., 1995; Moon *et al*., 1999; Münster *et al*., 2002; Schmitz *et al*., 2000). A third lineage includes *Zmm28* from maize, *OsMADS18* from rice and *BM3* from barley (Moon *et al*., 1999; Münster *et al*., 2002; Schmitz *et al*., 2000).

Based on RNA blotting, expression of the first member of this group to be cloned, *Zea AP1a* (*Zap1a*), suggested a conserved pattern of expression with that of *AP1*, namely that transcripts were restricted to the non-reproductive organs of the maize floret (Mena *et al*., 1995). This indicated a conserved role for *Zap1a* in specifying the outer whorls of the grass floret but not the inner whorls. More revealing expression studies based on *in situ* hybridizations have been documented for genes from these lineages in rice, *Lolium temulentum* and barley (*Hordeum vulgare*). In agreement with the observations in maize, expression of the rice *RAP1* (*OsMADS14*) was not detected in developing stamen and carpel primordia, but was detected in developing palea, lemma and lodicule (Kyozuka et al., 2000). The situation in barley appears more complex, where all three genes (*BM8*, *BM5*, and *BM3*) appeared to be expressed in all developing floral organ primordia (Schmitz *et al*., 2000). Unfortunately, only late stage inflorescences were examined, so the earliest onset of expression was not determined. Expression of the *Lolium* genes *LtMADS1* and *LtMADS2* (orthologs of *OsMADS14* and *OsMADS15,* respectively) was seen in the apical inflorescence meristem as well as the spikelet and floret meristems (Gocal *et*

al., 2001). Because the *in situ* hybridization studies in these different grass species did not always include equivalent stages of inflorescence development, it is difficult to extrapolate a consensus pattern of expression, if indeed one exists. How any of these genes function in specifying grass floral organ or meristem identity is unknown, although the expression patterns are consistent with a role in meristem identity as seen for eudicot *AP1* genes, and for at least some of the species analyzed, the expression is consistent with a possible role in specifying or promoting the sterile outer whorls of the grass flower.

A possible role in promoting the transition to flowering for the *OsMADS14/BM5/Zmm4,15* clade is suggested by a study in which the diploid wheat (*Triticum monococum*) ortholog of this lineage mapped directly to the *Vrn1* locus which controls flowering time in response to cold treatment (Yan *et al*., 2003). Spring wheat varieties that require no vernalization contain the dominant allele *Vrn1*, while winter wheat varieties that do require vernalization have the recessive allele *vrn1*. Interestingly, three different spring wheat *Vrn1* alleles carry different size deletions that disrupt a putative CArG box in the 5' proximal promoter of this *Triticum AP1* ortholog. Expression of this wheat *AP1* gene was shown to be induced by vernalization only in winter wheat varieties. These expression studies were confirmed by a study of the same gene in triploid bread wheat *Triticum aestivum*, in which co-suppression of this gene leads to delayed flowering (Murai *et al*., 2003).

Function for the *Zmm28*/*OsMADS18* clade has been investigated in only a single study. Overexpression of *OsMADS18* in rice leads to early flowering, however a transgeneic knockdown by RNAi had no obvious phenotype (Fornara *et al.*, 2004). As noted by the authors in this study, early flowering caused by overexpression is consistent with other studies in which *AP1*-like genes were overexpressed in rice and *Arabidopsis*, and thus may indicate a role in promoting flowering for this clade as well.

Although there is still limited understanding of the roles that A class genes may play in grass flower development, it is interesting to note that the genes are commonly expressed in meristems other than the floral meristem, most notably in meristems that precede the floral meristem such as the spikelet meristem. In *Arabidopsis*, *AP1* and another non MADS-box gene *LEAFY* (*LFY*) are both necessary and sufficient for establishing a floral meristem identity (Mandel *et al.*, 1992; Mandel and Yanofsky, 1995; Weigel *et al.*, 1992; Weigel and Nilsson, 1995). Grasses are different from *Arabidopsis* in that the first meristem that branches from the inflorescence meristem has a distinct identity that is not a floral meristem (McSteen *et al.*, 2000), namely the spikelet or spikelet pair meristem (Fig. 1.4). However, at least in some of the grasses, the *AP1* orthologs and the *LFY* orthologs are expressed in these early meristems without conferring a floral meristem identity (Gocal et al. 2001, Bomblies et al. 2003). This suggests that either they have been recruited to new roles in these earlier grass meristems, or that their role in promoting a floral meristem identity is somehow repressed until the later arising floral meristem is produced. The

loss of function phenotype for the redundant maize *LFY* orthologs *Zfla* and *Zflb* supports the latter hypothesis. While the *zfla zflb* double mutant has floral defects similar to the *Arabidopsis lfy* mutant, indicating a conservation between monocots and eudicots in this pathway of promoting a floral identity, there are no apparent defects in the spikelet or other meristem identities (Bomblies *et al*., 2003). It remains to be seen if grass A class mutants will also have defects outside of the floral meristem, or if, like *Zfla* and *Zflb*, they are expressed in these earlier meristems without effecting their identities. If it is true that their floral-promoting function is repressed during the early phases of grass inflorescence development, then there must be an unknown factor or factors responsible for this repression that are yet to be discovered in the grasses.

B Class

Angiosperm B class genes belong to one of two groups that are the result of a duplication early in the evolution of angiosperms: *APETELA3*(*AP3*)/*DEFICIENS*(*DEF*) and *PISTILATA*(*PI*)/*GLOBOSA*(*GLO*) (Stellari *et al*., 2004). In the core eudicots, it is clear that these genes play a conserved role in conferring stamen and petal identity. As originally demonstrated in *Arabidopsis* (Goto and Meyerowitz, 1994; Jack *et al*., 1992) and *Antirrhinum* (Sommer *et al*., 1990; Trobner *et al*., 1992) and more recently in *Petunia* (Vandenbussche *et al*., 2004), loss of B class function leads to homeotic transformation of stamens into carpels and petals into sepals. Furthermore, AP3/DEF proteins and PI/GLO proteins are known to interact as obligate heterodimers to bind CArG-box elements in the promoters of

target genes (Goto and Meyerowitz, 1994; Jack *et al*., 1994; Riechmann *et al*., 1996; Schwarz-Sommer *et al*., 1992).

A duplication in the *AP3*/*DEF* lineage that occurred at the base of the core eudicots resulted in the paleo*AP3* and eu*AP3* lineages. These two lineages are distinguished by a frame shift in the C terminus that gave rise to the conserved euAP3 motif, distinct from the paleoAP3 motif found in basal eudicot, monocot, basal angiosperm and core eudicot paleo*AP3* genes (Kramer *et al*., 1998; Vandenbussche *et al*., 2003). The expression of paleo*AP3* genes in the basal eudicots is consistently strong in stamens but often weak or patchy in petals (Kramer and Irish, 1999). This expression in petals is distinct from the strong stamen *and* petal expression seen for eu*AP3* genes in core eudicots. These observations have lead to the proposal that B-class genes evolved a novel role in specifying petal identity in core eudicots coincident with the duplication creating the eu*AP3* lineage (Kramer and Irish, 1999; Kramer and Irish, 2000). This hypothesis also appears consistent with classical morphological investigations of petal evolution, where petals are thought to have evolved independently multiple times in the angiosperms from either stamens or subtending sepals/bracts (Takhtajan, 1991). In this view, core eudicot petals are all interpreted as homologous organs derived a single time from stamens. A key prediction of the hypothesis proposed by Irish and Kramer is that paleo*AP3* genes would play no major role in specifying petal identity.

So far, the only data for paleo*AP3* function comes from the grasses where knockouts have been characterized in both maize and rice. In both cases there is a

single *AP3/DEF* ortholog *Silky1* (*Si1*) in maize and *SUPERWOMAN1* (*SPW1*) in rice. In both *si1* and *spw1*, the same mutant phenotypes are seen, with homeotic conversion of stamens into carpels and lodicules into bract-like lemma/palea-type organs (Ambrose *et al*., 2000; Nagasawa *et al*., 2003). This has led to an interpretation of lodicules as second whorl organs homologous to petals, and more loosely of palea/lemma-like bracts as first whorl organs equivalent to sepals. The latter was further supported by the spikelet phenotype of the *silky1 zag1-mum* double mutant where these bract-like organs with palea/lemma characteristics proliferated within an otherwise normal pair of glumes (Ambrose *et al*., 2000). This double mutant phenotype was reminiscent of the mutant floral phenotypes observed in *ap3 ag* double mutants of *Arabidopsis* where only sepals are produced (Bowman et al., 1989; Bowman et al., 1991). Together, these observations led to the conclusion that B class function is largely conserved across monocots and eudicots. However, another interpretation holds that lodicules may not be homologous to petals, and that B class function was independently recruited to specify a lodicule identity in grasses (Irish, 2000). Whether B class function in grasses should be interpreted as conserved or derived critically depends on the relationship of lodicules to second whorl organs (petals or tepals) in other monocots, a subject addressed below.

Unlike the single grass *AP3/DEF* lineage, there are two paralogous *PI/GLO* gene lineages in the grasses: *OsMADS2/Zmm16* and *OsMADS4/Zmm18/29* (see Fig. 1.3B) (Chung *et al*., 1995; Münster *et al*., 2001). Expression of these *PI/GLO*-like genes in maize and rice is consistent with a role in B class function, as their

transcripts are present in developing stamen and lodicules (Chung et al., 1995; Kyozuka et al., 2000; Münster et al., 2001; Whipple et al., 2004). The only functional studies of these genes come from rice where an antisense suppression of *OsMADS4* resulted in homeotic conversion of lodicules and stamens similar to those observed in *spw1* and*si1* mutants (Kang *et al*., 1998). However, the reported phenotype may not reflect a reduction in *OsMADS4* alone, as the authors did not rule out the possibility that the antisense construct used to silence *OsMADS4* may well have silenced *OsMADS2* as well. A more focused study by (Prasad and Vijayraghavan, 2003) used RNAi to specifically reduce *OsMADS2* levels, resulting in a loss of lodicule identity but no effect on stamens. This second study raises the interesting possibility that *OsMADS2* and *OsMADS4* have diverged in function, with *OsMADS2* playing a more key role in lodicule specification than stamen. The possibility of subfunctionalization is supported by the observation of Kyozuka et al. (2000) that *OsMADS2* expression is maintained in the lodicule, but quickly down regulated in the developing stamen primordia of the rice floret. A further understanding of the grass *PI*/*GLO*-like gene functions will require a more careful analysis of *OsMADS4* function as well as of the role of the corresponding maize genes.

The homeotic phenotype of the *si1* and *spw1* mutants discussed above make a strong case for conservation of B class gene function in specifying organ identity. If it is accepted that lodicules are modified petals, then it appears that B class function was conserved in the common ancestor of monocots and eudicots. A study of the biochemical function of maize B class genes is consistent with this view (Whipple *et al*., 2004). In this study it was shown that the maize B class genes *Si1* and *Zmm16* were capable of rescuing both petal and stamen identity in *Arabidopsis*. Furthermore,

it was shown that the maize SILKY1 and ZMM16 proteins interact as an obligate heterodimer pair to bind DNA, as is the case with core eudicot AP3/DEF and PI/GLO proteins. Together these findings appear consistent with a conserved B class biochemical function between monocots and eudicots. However, the observation of obligate heterodimerization in maize is not so unambiguously interpreted as a conserved biochemical interaction as will be discussed later.

C Class

C class genes in the eudicots control stamen and carpel organ identities, as well as conferring determinacy upon the floral meristem (Bowman *et al*., 1989). In *Arabidopsis*, C class is controlled by the action of a single gene *AGAMOUS* (Yanofsky *et al*., 1990), and loss of function mutants result in stamens converted to petals and a new flower arising in the position of the carpel reiterating the pattern: sepal, petal, petal, new flower…. In maize, two C class genes were initially identified: *Zag1*, and *Zmm2* (Mena *et al*., 1996; Schmidt *et al*., 1993; Theissen *et al*., 1995). A knockout in *Zag1* shows that it plays a role in floral meristem determinacy (Mena *et al*., 1996). However, the *zag1* mutant has little if any effect on carpel identity, and no discernable defect in stamen identity. This does not necessarily mean that maize C class genes have no role in organ identity since *Zmm2* may be acting redundantly with *Zag1* to specify stamens and carpels. Interestingly, expression of *Zag1* and *Zmm2* is overlapping but not identical. Both genes are expressed in stamens and carpels as would be expected for C class genes, but *Zag1* is expressed more strongly in carpels and *Zmm2* is expressed more strongly in stamens (Mena *et al*. 1966). This has led to

the speculation that C class function has been partitioned in maize, as would be expected from subfunctionalization following a gene duplication event (Force *et al.*, 1999). Overexpression and complementation studies in *Arabidopsis* indicate that such subfunctionalization is present at the level of protein function in addition to expression since *Zag1* is more capable of promoting carpel identity and *Zmm2* is better able to promote stamen identity (C. Whipple, B. Ambrose, M. Mena, and R. Schmidt, unpublished observation). More recently, *Zmm23*, a duplicate of *Zmm2* was also isolated from maize (Münster *et al.*, 2002). Further analysis of the maize C class will require isolation of loss of function mutations for *Zmm2* and *Zmm23* to understand how they may contribute to organ identity, and if the apparent subfunctionalization at the level of gene expression is consistent with their mutant phenotypes, alone and in combination with *zag1*.

Studies of C class function in rice focused initially on the *Zmm2* ortholog *OsMADS3* (Kang et al., 1995; Kyozuka et al., 2000). Overexpression of *OsMADS3* in tobacco leads to organ transformations consistent with C class function (Kang *et al.*, 1995). A more recent study in which *OsMADS3* was constitutively expressed in rice showed that it could convert lodicules into stamens, as would be predicted by the ABC model, but had no effect on lemma or palea identity (Kyozuka and Shimamoto, 2002). Kang et al. (1998) expressed a transgenic *OsMADS3* anti-sense construct in an attempt to reduce *OsMADS3* expression in rice, with results largely consistent with a C class function: stamens partially converted to lodicules, and carpels replaced by multiple flowers of undifferentiated carpels and stamens.

More recently, expression and a functional characterization of both *OsMADS3* (the rice *Zmm2* ortholog) and *OsMADS58* (the rice *Zag1* ortholog), was reported (Yamaguchi et al., 2006). Both genes are expressed in the floral meristem before the initiation of floral organs. Later on *OsMADS58* is maintained in both the developing stamen and carpel primordia, while *OsMADS3* expression is rapidly restricted to the ovule primordia. Two insertion alleles of *OsMADS3* had a phenotype, the strong T-DNA insertion line *osmads3-3* showed a near complete conversion of stamens into lodicules and a partial loss of meristem determinacy, while the weaker *Tos17* insertion line *osmads3-2* had a partial transformation of stamens and only an occasional loss of determinacy. The *OsMADS58* RNAi line *osmads58-s1* showed a complete loss of floral meristem determinacy. Additionally carpel development was significantly affected, but only an occasional loss of stamen identity was reported. The phenotypes of *osmads58-s1* plants are similar to *zag1* mutants, although *OsMADS58* seems to have a more prominent role in carpel development.

The mutant phenotypes of the *OsMADS3* and *OsMADS58* suggest that the rice C class has been partitioned such that *OsMADS3* plays a more crucial role in the third whorl, and *OsMADS58* plays a more crucial role in the fourth whorl. While these results can largely be viewed as consistent with subfunctionalization of the two rice C class genes as has been hypothesized for the maize C class genes, some questions remain. For instance, it is still not clear what role C class genes have in establishing carpel identity. The authors do not interpret the carpel defects seen in *osmads58* RNAi lines as loss of carpel identity, but rather as an indication that *OsMADS58* plays

a later role in carpel development (it should be noted, however, that the carpels in *osmads58-s1* plants produce trichomes which are not formed on carpels of *wt* plants and may indicate a partial loss of carpel identity). It is possible that *OsMADS3* is redundant with *OsMADS58* in establishing carpel identity. The authors report that silencing *OsMADS58* in the hypomorphic *osmads3-2* does not enhance the carpel defects seen in *osmads58-s1* plants. Since the *osmads3-2* allele retains some partial function, one can not yet rule out a redundant role in establishing carpel identity. Another possibility, in light of an apparent role for *DL* in establishing carpel identity, is that the rice C class genes are redundant with *DL* (see discussion below).

Another interesting observation of Yamaguchi et al. (2006) was that *osmads3* mutants appear to develop extra lodicules in the second whorl, in the position where the medial lodicule aborts in wild type. Additionally, *OsMADS3* expression is detected in the position where the medial lodicule should develop. This may suggest that the rice C class genes play a role in the abortion of the medial lodicule (abortion of the medial lodicule is common in many grasses although some bamboos have three). A role for C class genes in establishing the lodicule asymmetry of grass flowers would represent an as yet undescribed function for this class of genes. There are some problems with this interpretation, however. If true, one would expect ectopic expression of *OsMADS3* in the second whorl to cause abortion of lodicules. However, Kyozuka and Shimamoto (2002) report that constitutive *OsMADS3* expression leads to conversion of lodicules to stamens. Furthermore, more than three lodicules develop in the second whorl of *osmad3-3* plants indicating that *OsMADS3* does not simply

repress the medial lodicule. Indeed, the indeterminacy of *osmads3-3* mutant flowers may obscure the whorl boundaries, such that these ectopic lodicules are produced by a larger, more indeterminate third whorl.

D Class

D class genes were proposed as ovule identity genes when the *Petunia* MADS box genes *FBP7* and *FBP11* were shown to be necessary for ovule specification (Angenent *et al*., 1995; Colombo *et al*., 1995). *FBP11* was also capable of forming ectopic ovules when constitutively expressed (Colombo *et al*., 1995). More recently *SEEDSTICK,* the *Arabidopsis* homolog of this conserved clade (closely related to C class genes), was shown to play a role in ovule development (Pinyopich *et al*., 2003). A phylogenetic analysis of C and D class genes indicates that there are two clades of grass D class genes (see Figure 1.3C) (Kramer *et al*., 2004). The first clade includes *Zag2* and *Zmm1* from maize and *OsMADS13* from rice (Lopez-Dee *et al*., 1999; Schmidt *et al*., 1993; Theissen *et al*., 1995). The second clade includes *Zmm25* from maize and the rice predicted gene P0408G07.14 (Kramer *et al*., 2004; Münster *et al*., 2002). There are, as yet, no informative functional studies for the grass D class genes, but the maize *Zag2* and the rice *OsMADS13* expression patterns are consistent with other known D class gene patterns, with expression early in the carpel primordia and subsequent restriction to the ovule (Lopez-Dee *et al*., 1999; Schmidt *et al*., 1993). Functional studies of these genes in grasses would provide insight into any conserved role they may play in ovule identity.

E Class

The *Arabidopsis SEPALLATA1, 2, 3,* and *4* (*SEP1-4)* genes have been shown in a series of elegant genetic studies to be required for the activity of A B and C class genes in specifying all the floral organs (Ditta *et al*., 2004; Pelaz *et al*., 2000). Without *SEP* function, the flower is replaced by an indeterminate shoot of lateral organs with a leaf-like identity (Ditta *et al*., 2004). It is thought that the SEP proteins function as transcriptional activators in complexes of MADS-box proteins to mediate the activity of the ABC genes (Honma and Goto, 2001; Pelaz *et al*., 2001; Theissen and Saedler, 2001). The *SEP* clade is large and the result of both ancient and recent duplication events in the angiosperms (Zahn *et al*., 2005). These complex duplication patterns are also evident in the grass representatives of the *SEP* clade (Fig. 1.3D) (Malcomber and Kellogg, 2004; Zahn *et al*., 2005).

According to a recent phylogenetic analysis of *SEP* genes including basal angiosperms (Zahn *et al*., 2005), there appear to be two large clades in the angiosperms: the *SEP3*(*AGL9*) clade and the *SEP1, 2, 4*(*AGL2, 4, 3*) clade. In the *SEP3* clade, a duplication has lead to two grass lineages. The first clade, *OsMADS7*, includes maize *Zmm6* and rice *OsMADS7*, while the second, *OsMADS8*, clade includes maize *Zmm27* and rice *OsMADS8* (Fischer *et al*., 1995; Kang *et al*., 1997). In the *SEP1, 2, 4* clade there are three grass lineages: 1. The *OsMADS34* clade, including maize *Zmm24* and *Zmm31* and rice *OsMADS34* (also *OsMADS19*) (Münster *et al*., 2002) (Pelucchi et al., 2002; Shinozuka et al., 1999), 2. The *LHS1* clade, with maize *ZmLHS1a* and *ZmLHS1b* (aka *Zmm8* and *Zmm14*) and rice *LEAFY HULL*

STERILE1 (*LHS1* aka *OsMADS1*) (Cacharrón *et al*., 1999) (Chung *et al*., 1994; Jeon *et al*., 2000; Malcomber and Kellogg, 2004), 3. The *OsMADS5* clade includes the maize *Zmm3* and rice *OsMADS5* (Fischer *et al*., 1995; Kang and An, 1997).

It is clear that duplications have lead to complexity in grass *SEP* lineages. Studies of grass *SEP* gene expression in both maize and rice, indicate that their expression patterns are as complex as their lineages (for a review see Malcomber and Kellogg (2004)). The rice *leafy hull sterile1* is the only described mutant in a grass *SEP*, and this lineage has been the most studied. The *lhs1* mutant results in flowers with lemma, palea, and lodicule transformed into leaf-like organs (Jeon *et al*., 2000). However, *lhs1* is a semidominant mutation. For this reason, it is not clear what the true *lhs1* loss of function phenotype is. Nevertheless, expression in floral organs, and the semidominant mutant phenotype both indicate that this gene has a role in floral organ identity. Reduction of *OsMADS1* by RNAi led to a phenotype similar to *lhs1*, however some lines also had a conversion of stamens and carpels to leaf/glume-like organs indicating that *OsMADS1* is involved in establishing organ identity in all four whorls (Prasad et al., 2005). A careful study of *LHS1* orthologs in multiple grass species has shown that expression patterns of this *SEP* lineage are highly variable (Malcomber and Kellogg, 2004). Such complexity in expression and lineage duplications, in combination with the relative lack of functional studies, make it difficult to understand how these genes may function in grasses. However, such complexity and lability in expression patterns also make this lineage an exciting group for continued study.

Analyzing the grass ABCDE MADS-box genes summarized in Table I, it becomes apparent that there is a lot of work yet to be accomplished to understand the function of these genes. True loss-of-function mutants are only available for two maize genes (*si1* and *zag1)* and two rice genes (*spw1,* and *osmads3*). While patterns of gene expression and the few available mutant phenotypes appear consistent with what is predicted about their functions based on studies in eudicots, this appears mostly true for those genes controlling B and C organ identify function. Perhaps this is not surprising, since the reproductive organs are thought to be homologous among all angiosperms it would seem logical that a single ancestral genetic mechanism for their specification would be conserved in divergent angiosperm groups. Little is known about how the non-reproductive organs of the grass flower are specified, and what role if any these MADS-box genes play. The major exception is the role of B class genes in lodicule identity, although the interpretation of this remains controversial. Consequently, it will be interesting to see how genes predicted to have a role in first and second whorl organ identity (specifically A and E class genes) actually affect grass flower development.

Thinking outside of the MADS box: how forward screens reveal non-MADS-box genes important for grass floral patterning

It is clear from the work accomplished so far in grasses that MADS-box genes are playing a role in grass floral organ identity and patterning. It is not clear, however, that all aspects of the model are rigidly conserved. Testing the conservation of the

ABC model in grasses relies on the assumption that genes important in eudicots are good candidates for reverse genetic studies in maize and rice. While this strategy has been fruitful with B and C class genes, there is no reason *a priori* to believe that only MADS-box genes will be the major homeotic genes in grasses. In fact, recent work has shown that non MADS-box genes may be playing crucial roles in grass floral patterning. These mutants demonstrate the importance of blind forward genetic strategies to understand the genes controlling grass floral development.

In rice, the *DROOPING LEAF* (*DL*) mutation causes a homeotic conversion of carpels into stamens (Nagasawa *et al*., 2003). In addition, *dl* mutants have a loss of floral meristem determinacy such that up to seven ectopic stamens are produced in severe alleles. *DL* was cloned and shown to be the rice ortholog of *CRABS CLAW* (*CRC*), a gene of the *YABBY* family that is important for proper development of the *Arabidopsis* carpel (Yamaguchi *et al*., 2004). Unlike *CRC,* which is expressed primarily in abaxial tissues of the developing carpel, *DL* is expressed from the earliest stages of carpel initiation and maintained throughout all tissues of the carpel except the developing ovary, consistent with a direct role in carpel identity. Thus it appears that a non MADS-box gene plays a crucial role in carpel specification in the grasses and that, unlike *Arabidopsis*, the *AG* pathway may not be the primary regulator of carpel identity. It is interesting that *Arabidopsis* also has an *AG*-independent pathway to specify carpels, as can be seen by the ectopic carpel growth in an *ag ap2* double mutant (Bowman *et al*., 1991). This ectopic carpel identity is lost by removing *CRC* function, indicating that *CRC* and *AG* act in parallel pathways to specify *Arabidopsis*

carpels, but that the *AG* pathway is more important (Alvarez and Smyth, 1999). In grasses, *DL* may have a more important role in establishing carpel identity than does its ortholog in *Arabidopsis*. It is also noteworthy that expression of a *CRC* ortholog in the basal angiosperm *Amborella trichopoda* is more similar to the abaxial pattern of expression observed in Arabidopsis, indicating that the carpel specification role of *DL* may be a derived function in the lineage leading to the grasses (Fourquin *et al*., 2005).

While the data appear consistent with an important role for *DL* in establishing grass carpel identity, a more trivial explanation may be that *DL* is necessary for proper carpel *development* (not identity) as in *Arabidopsis*, and the homeotic transformation is an indirect result of *DL*'s role in keeping B class expression out of the fourth whorl. A key prediction of this hypothesis is that removing B class function from the *dl* mutant would result in a flower with malformed carpels in the central whorl. This experiment was done by (Nagasawa *et al*., 2003), and they reported the growth of floral organs of "unknown identity" in the fourth whorl of a *spw1 dl* double mutant. It is possible that these organs are, in fact, the malformed carpels that would be predicted if *DL* function in rice was conserved with *CRC* function in *Arabidopsis*. Regardless of which explanation is more accurate, it is clear from the *dl* phenotype that the rice *CRC* ortholog has functions not seen in *Arabidopsis* that include control of the B class expression domain, conferring determinacy upon the floral meristem, as well as a role in midrib development. These clear differences in *CRC* function between *Arabidopsis* and rice are interesting and demonstrate how the function of important developmental genes can change in the course of evolution.

Recently the *palealess* (*pal*) mutant has been described in rice, which has defects in palea identity, but the other floral organs (lemma, lodicule, stamen, and carpel) are unaffected (Luo *et al*., 2005). Most grasses have a palea with two prominent vascular bundles, but in the *pal* mutant two distinct leaf-like organs develop in the place of the palea. The authors suggest that the gene responsible for the *pal* phenotype could be a palea identity gene and thus may represent a grass A class member. Interestingly, the *pal* mutation was mapped to a single BAC that has been sequenced, but does not contain a MADS-box gene nor an *AP2*-like gene, although there is another predicted transcription factor that may be *PAL*. Given that the mutant phenotype is only present in the palea without affecting the lodicules, and that it is not a MADS-box gene, the interpretation of *PAL* as an A class gene seems premature. It will be interesting to learn the identity of *PAL*, and if further characterization is consistent with it being a palea identity gene. If true, this would suggest that grasses either have evolved a distinct mechanism to specify an organ unique to grasses, or that the grass outer 'sepal' whorl is specified differently than what has been described in eudicots. Investigation of this gene in other grasses and outgroups may shed light on the evolution of the grass floret.

These two examples of non-MADS-box gene mutants affecting grass floral organ identity illustrate the importance of taking a forward, in addition to reverse, genetic approach to understand grass flowering. Unfortunately, grass floral organs are tightly enclosed in the developing spikelet, making large-scale mutant screens difficult. It is likely that many interesting grass mutants with defects in florets have

been missed in the genetic screens performed so far. Our lab has recently undertaken a careful screen of spikelets and florets from ~1000 segregating EMS-mutagenized M2 maize families (for a searchable database of whole-plant phenotypes see http://www.maizegdb.org/ems-phenotype.php). Tassel branches were collected, dried and stored until they could be carefully screened with the aid of a dissecting microscope for spikelet and floral phenotypes. Our initial results indicate that even in maize, which contains a significant number of duplicate genes, novel mutant phenotypes are to be found. Of the families screened, 12 mutants affecting spikelet and/or floret development were identified by this approach that were missed by field-based screens of the same families for inflorescence defects. Of these, at least two have floret phenotypes that haven't been described previously. Such careful screens in a species with fewer gene duplications, such as rice, are likely to be even more productive.

Physical interactions among MADS-box proteins: function and evolution.

MADS-box proteins are known to interact to form dimers and higher order complexes. The quartet model has been proposed to describe how tetramers ofMADS-box proteins could interact to form a transcriptional activation complex sufficient to establish the identity of each of the four floral whorls (Theissen and Saedler, 2001). Support for the quartet model is drawn in part from simultaneous overexpression of *Arabidopsis* MADS-box proteins known to interact *in vitro*, such as AP1/AP3/PI/SEP (a combination of A, B, and E class proteins respectively), which results in leaves transformed into a petal identity (Honma and Goto, 2001; Pelaz *et al*., 2001). These

studies make it clear that the apparent protein-protein interactions of MADS-box gene products are critical to their function.

Protein-protein interaction among MADS-box gene products have been described in rice. Favaro et al. (2002) showed that the rice D class protein OsMADS13 interacts with the E class SEP homologs OsMADS8 and OsMADS7 in a yeast two-hybrid assay. An interaction of the rice A class protein OsMADS18 with these same two SEPs, was shown by yeast two-hybrid (Fornara *et al*., 2004), and further verified by co-immunoprecipitation. Yeast two-hybrid interaction for the B class protein SPW1 with OsMADS8 has also been reported (Lee *et al*., 2003). Interestingly, these studies all indicate an interaction between the diverse A, B, and D class rice proteins and rice SEP orthologs. Such an interaction would be predicted from a conserved quartet model, where SEP proteins are necessary for the function of florally expressed MADS-box proteins. It is also interesting to note that each of these studies further indicated an interaction with the rice protein OsMADS6, an ortholog of the *Arabidopsis* AGL6. Although this gene has not been functionally characterized in *Arabidopsis*, it is closely related to the *SEP* subfamily (Zahn *et al*., 2005), suggesting the *OsMADS6* lineage may also have an important role in grass floral development.

Unfortunately, while the above studies suggest conservation of protein-protein interactions of grass SEP proteins with other ABCD proteins, little is known about the *in vivo* relevance of such interactions in the grasses. Among MADS-box proteins analyzed, most form homodimers capable of binding DNA in Electrophoretic Mobiltiy Shift Assays (EMSAs) (Riechmann *et al*., 1996). One exception to this ability to

dimerize is the eudicot B class proteins AP3 and PI which are known to interact as obligate heterodimers. This obligate heterodimerization appears to have direct functional consequences. Unlike other duplicate MADS-box genes such as *APETALA1*, *CAULIFLOWER*, and *FRUITFUL* or *SEPALLATA1*, *2*, *3*, and *4*, which are known to act redundantly in *Arabidopsis*, the duplicate B class genes show no apparent functional redundancy, and mutations in either gene give nearly identical phenotypes, with no more severe phenotype in the double mutant. This is likely a result of obligate heterodimerization since any protein complex containing a single B class group member would be incapable of binding DNA and thus incapable of promoting any aspect of B class function. Recently there has been interest in how B class proteins interact, and how obligate heterodimerization evolved.

Winter et al. (2002) used a phylogenetic approach to examine the evolution of B class protein interactions. A combination of EMSA and yeast two-hybrid assays indicated that a *Gnetum gnemon* (a gymnosperm) B class protein and a *Lilium regale* (a monocot) PI/GLO ortholog were both able to form homodimers capable of binding DNA. Interestingly the *Lilium* AP3/DEF ortholog required its PI/GLO partner to bind DNA. The most parsimonious interpretation of these findings is that the ancestral state for B class proteins is to form homodimers, and that this was successively lost, first in the AP3/DEF lineage, and eventually in the PI/GLO lineage resulting in the obligate heterodimerization system described in eudicots. This study, while useful as an initial broad comparison, was unfortunately limited by a small sampling of angiosperm B class proteins, and a robust parsimony reconstruction of this ancestral

state would require more sampling. Another potential drawback is that it did not include any basal angiosperm members of the AP3 and PI lineages. The *Gnetum* B class protein descended from a lineage that diverged prior to the duplication event that created the AP3 and PI lineages. Consequently, the ancestral states in the angiosperm AP3 and PI lineages are still ambiguous. It is entirely possible that obligate heterodimerization evolved early in the history of the angiosperms, and was subsequently lost in the *Lilium* PI/GLO lineage. Another possibility is that dimerization specificity is labile in non-eudicot B class proteins, resulting in multiple gains and losses, which would further complicate a limited survey of angiosperm B class proteins. A larger analysis of basal monocot and basal angiosperm B class proteins will help to clarify these issues.

That there could be lability in the dimerization properties of B class genes is suggested by a study of the maize B class proteins SILKY1 and ZMM16. Whipple *et al*. (2004) show that these proteins form an obligate heterodimer pair similar to *Arabidopsis* B class proteins. Furthermore, no maize B class homodimer is likely to be functional in the traditional B class roles of specifying second and third whorl organ identities because the *silky1* mutant shows complete loss of organ identity and the expected homoeotic conversions in these whorls, indicating no functional redundancy and consistent with obligate heterodimerization. That two monocot PI/GLO proteins have different dimerization properties further complicates the parsimony reconstruction of Winter *et al*. (2002). Did obligate heterodimerization evolve independently in the grasses, or was it simply lost in the *Lilium* PI/GLO

lineage? In order to address these questions our lab has begun examining the DNA binding properties of B class proteins in *Joinvillea ascendens*, a close outgroup to the grasses. Our results indicate that the *Joinvillea* PI protein (JaPI), unlike ZMM16, is capable of binding DNA as a homodimer, while the *Joinvillea* AP3 protein (JaAP3) is not (Fig. 1.5).

The ability of JaPI to bind DNA as a homodimer is similar to the other monocot PI proteins that have been analyzed. Together these results suggest that the ancestral state in the monocots is for PI proteins to homodimerize, and that the maize PI protein ZMM16 (and by extension other grass proteins of this lineage) lost this capacity. It is still unclear whether the other maize PI proteins, ZMM18 and 29, function as homodimers or as obligate heterodimers like ZMM16.

That JaPI can homodimerize, but the closely related maize ZMM16 cannot, suggests that this protein-protein interaction can be quickly lost. Such plasticity makes it problematic to assign an ancestral state to the monocot PI lineage. The possibility that this protein-protein interaction is labile can only be discarded after a broad and thorough analysis of monocot PI proteins. If it is true that homodimerization is easily gained and/or lost, perhaps it is a selectively neutral character and of little functional importance. If, however, it is found that the grasses have independently evolved another case of obligate heterodimerization, then it seems more likely that this change seen in the grass PI-like protein (compared to JaPI) is not neutral and has a functional relevance. What the function of a PI homodimer could be in the monocots is unclear. It is possible that the grass ZMM18, 29/OsMADS4 PI-like

clade maintains the ability to homodimerize, in which case it would be possible to begin assessing any differential function by knocking out the genes in both *PI*-like clades in rice or maize.

Another avenue of research that could prove enlightening is to examine the domains and amino acids involved in this evolutionary change from JaPI to ZMM16. Domain swaps could be done between JaPI and ZMM16 to identify the region of the protein that is responsible. B class MADS-box proteins have the stereotypical MIKC structure (MADS DNA binding domain, Intervening region, Keratin-like coiled-coil domain, and C-terminal domain) (Riechmann and Meyerowitz, 1997). The K domain is thought to be important for heterodimerization (Yang et al., 2003; Yang and Jack, 2004), although other domains may also play a role in protein-protein interactions. It will be interesting to see if the K domain is responsible for the difference between JaPI and ZMM16. After determining the domain, the high amino-acid conservation between these closely related proteins could even allow identification of the critical aimino acid(s), which could be tested by site-directed mutagenesis providing valuable structure-function insight into this evolutionary change of protein interactions.

The Grass Family as a Model System for Evo-devo

So far this review has discussed primarily what is known about MADS-box gene function in the grasses, with emphasis on genetic studies from rice and maize. Unfortunately there is as yet no other angiosperm genetic model species outside of the eudicots. Most floral evo-devo work has relied on extrapolations from gene

expression patterns, inferring gene function based on what is known about the orthologous gene activity in *Arabidopsis* or other model species, and ectopic overexpression studies in *Arabidopsis* to indicate gene activity from gain of function phenotypes -- all approaches that are prone to artifacts and ambiguous interpretations. Without some rigorous test of gene function, evo-devo hypotheses will continue to be hopeful 'just-so stories' at best. The ability in maize and rice to obtain and analyze loss of function phenotypes has made the grasses an important group for understanding the conservation of genetic mechanisms elucidated primarily in eudicot model species. Maize and rice have established communities with a number of genetic resources that make them excellent for use in evo-devo studies, including approaches for performing forward and reverse genetics, and robust transformation technologies. We have discussed some ways these genetic strategies have already proven useful in understanding flower development in an angiosperm group distantly related to the core eudicots. Now we would like to consider some characteristics of the grass family itself that make it a model group for evo-devo, including some characteristics that at first glance might be considered drawbacks.

Gene duplication and subfunctionalization

Phylogenetic studies of diverse MADS-box gene families in the angiosperms give evidence of gene duplication events occurring at multiple taxonomic levels (Kramer *et al*., 1998; Kramer *et al*., 2004; Litt and Irish, 2003; Stellari *et al*., 2004; Zahn *et al*., 2005). It is intriguing to speculate as to why so many duplicate copies have been

maintained resulting in a radiation in MADS-box genes. An initial examination of MADS-box gene phylogenies shows that the gene radiations often correlate with radiations of successful plant groups, including the duplications at the base of the angiosperms and core eudicots. It is possible that duplications increase the molecular complexity necessary for morphological evolution, and thus mediate adaptive radiations. In other words, groups with more regulators of flower development can more easily evolve new morphologies and thus adapt more easily to diverse niches leading to increased speciation. Testing such speculative hypotheses will first require a careful examination of the roles of duplicate genes.

Ohno (1970) followed by Force *et al.* (1999), provided the theoretical background to the possible consequences of a gene duplication event. They define three processes that should occur after duplication. The first possibility is **nonfunctionalization**, in which one of the duplicate copies begins to accumulate deleterious mutations until it becomes a nonfunctional psuedogene, and eventually disappears completely. Nonfunctionalization is though to be common since duplications create completely redundant copies and there is no reason for selection to maintain both genes. Genome sequences show that such pseudogenes exist. Furthermore, that paralogs are often lost entirely is suggested by the existence of single copy genes even though multiple other gene lineages are duplicated following a genome wide duplication event as happened in an ancestor of maize. The second process, **neofunctionalization** results in one of the gene copies gaining a new function that is selectively advantageous, and explains why some paralogs could be maintained.

A neofunctionalization event is likely to be rare since most mutational events are deleterious. In the final process, **subfunctionalization**, both genes are maintained because they accumulate complementary mutations that make it necessary to have both paralogs present to maintain the function of the pre-duplication gene. As most genes are expressed in multiple tissues, mutations could occur in enhancer elements of the duplicates with the result that each gene is now expressed in different, but complimentary, subdomains of the ancestral gene. Thus only by keeping both duplicates is the entire ancestral expression pattern maintained. Finally, mutations could also occur in the coding sequence of duplicate genes resulting in subfunctionalization at the level of protein function.

An interesting case of subfunctionalization appears to have occurred in the eudicot C class lineage. In *Arabidopsis*, the C class functions of organ identity and floral meristem determinacy are performed by *AG*, while in *Antirrhinum* the same activities are preformed by *PLENA* (*PLE*) (Coen and Meyerowitz, 1991), which was the purported ortholog based on its close sequence identity and function. However, recent evidence from more roboust gene sequence comparisons (Kramer *et al*., 2004) and a direct comparison of syntenic regions from *Arabidopsis* and *Antirrhinum* (Causier et al., 2005) have demonstrated that *AG* and *PLE* are not orthologs, as previously believed, but paralogs. In *Arabidopsis*, the *AG* duplicate *SHATTERPROOF* (actually encoded by two more recent, redundant duplicates *SHP1* and *2*) has an essential role in establishing valve margin identity in the *Arabidopsis* fruit (Ferrandiz *et al*., 2000; Liljegren *et al*., 2000). In *Antirrhinum* the *AG* ortholog is

FARINELLI (*FAR*), while the *SHP* ortholog is actually *PLE*. Interestingly, the organ identity and meristem determinacy functions in *Antirrhinum* are not controlled by the *AG* ortholog *FAR*, but by the *SHP* ortholog *PLE*. *far* mutants only have defects in some aspects of stamen development (Davies *et al*., 1999), and so the function of *FAR*, like *SHP*, is more limited in scope. It is interesting that orthologous genes do not have the same function in *Arabidopsis* and *Antirrhinum*, but closely related paralogs do. This indicates that following the duplication event that created *AG*/*FAR* and *SHP*/*PLE* lineages, the paralogs subfunctionalized differently in each lineage. It remains an open question if such differential subfunctionalization is common, or if this represents and unusual occurrence. Either way it is clear that analyzing duplicate genes in divergent species that contain both genes can yield surprising insights into the evolution of developmental mechanisms. It also raises a cautionary note concerning assignment of functional orthology between closely related genes from different species even when evidence of similar function can be obtained.

As can be seen from Table I, multiple MADS-box lineages have been duplicated in the grass family. Duplications present in both rice and maize appear to be the result of a genome wide polyploidization event that occurred before the radiation of the grasses (Paterson *et al*., 2004). A more recent allotetraploidy event in a common ancestor of *Tripsacum* and maize (Bomblies and Doebley, 2005; Gaut and Doebley, 1997) has led to additional duplications in maize gene lineages. Such duplications can confound initial reverse genetic attempts to assign function to the grass genes due to redundancy in function among closely related paralogs. However,

such duplications are also an excellent chance to study the fate of duplicate genes involved in flower development, and test the proposals of Force and Lynch about subfunctionalization and neofunctionalization that should be responsible for maintaining these genes. If there is an obvious subfunctionalization of expression patterns for a duplicate pair in either maize or rice, other grasses can be examined to see if the pattern of subfunctionalization is conserved for orthologs, or if there is variability in the manner in which gene duplicates are expressed during the course of evolution. With a nearly complete rice genome and the maize genome project just beginning, it may be possible in the near future to use bioinformatics methods to quickly identify potential enhancers that are responsible for the expression differences using methods such as those described to identify conserved noncoding sequences (CNSs) (Guo and Moose, 2003; Inada et al., 2003; Kaplinsky et al., 2002). A complementary method to investigate subfunctionalization would be to compare the phenotypes from loss of function mutations for both duplicates in both maize and rice, which phylogenetically span the majority of grass diversity. Reverse genetic resources including transposon insertions (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/, http://mtm.cshl.edu/) and TILLING (Till *et al*., 2004) have been established, allowing such investigations of grass duplicate genes. Such reverse genetic methods have confirmed subfunctionalization of the duplicate rice C class genes, although a full comparison with the maize C class must await characterization of *Zmm2* and *Zmm23*.

These reverse genetic studies will take time, especially for maize where many of the genes have undergone an additional duplication that has occurred more recently. However, two established genetic model species make the grasses ideal to study family level duplications, and will likely lead to insights into what happens when transcription factors controlling important developmental processes radiate.

The evolution of derived morphologies

As discussed above, grasses have highly modified flowers relative to other monocots. This is especially true for the non-reproductive organs where homology assignments are difficult. This can make interpreting grass floral mutants and expression patterns in relation to other monocots or eudicots difficult. However, these difficulties also present an opportunity to explore how derived morphologies evolve.

A prerequisite for any such study is a robust phylogeny, which has been worked out for the grasses by the Grass Phylogeny Working Group (GPWG, 2001) (Fig. 1.6). This phylogeny indicates that there are two major clades, which contain the majority of grass species. Members of these two clades have the standard grass spikelet as described above. There are also some basal clades of herbaceous bamboos represented by *Pharus*, which also have a spikelet. However, the most basal extant grasses do not have a traditional spikelet and include the genera *Streptochaeata* and *Anomochloa* (Judziewicz and Soderstrom, 1989). *Anomochloa*, has one species and is only known from a single population in Brazil. The *Anomochloa* spikelet equivalent consists of two successive bracts that initiate opposite each other, with the second

bract enclosing the flower. There appear to be no sterile outer whorls as in other grasses, but a ciliated fringe does surround the stamen whorl. Unfortunately, *Anomochloa* is endangered and is not readily available for comparative studies. *Streptochaeta*, on the other hand, is more common and grows readily in greenhouse conditions, making it possible to examine expression of candidate genes. The *Streptochaeta* spikelet equivalent is composed of twelve bracts (generally designated I-XII). The first five basal bracts (I-V) are spirally arranged, and can occasionally contain axilary meristems that grow into spikelets. Bract VI is large and encloses the rest of the floral organs, and appears to be modified for animal dispersal of the mature seed. Generally two shorter, pointed bracts (VII-VIII) develop opposite the large bract VI, although occasionally the remnant of a third bract (IX) can be detected in this whorl, indicating that it usually aborts. There is a whorl of three overlapping bracts (X-XII) that surround the stamens and enclose them until they emerge for pollination. Two whorls of three stamens each, and a gynoecium with three stigmas develop inside bracts X-XII. The closest outgroups to the grasses include the families *Joinvilleaceae*, *Ecdeiocoleaceae*, *Restionaceae*, and *Flagellariaceae*. These outgroups have a standard monocot floral plan, with two alternating whorls of outer tepals.

Knowing the phylogenetic position of *Streptochaeta* helps to form a hypothesis about how the derived grass floret evolved. The *Streptochaeta* spikelet equivalent is more likely to represent an ancestral grass floral morphology since it diverged before the evolution of true spikelets and the stereotypical grass floral organs. Even a cursory look at *Streptochaeta* in relation to true spikelets and outgroup flowers suggests that

Streptochaeta can be interpreted as intermediate in morphology. Although it has no lodicules, it does have three laminar bracts with a distinct morphology in the position of lodicules, which also correspond to the innter tepal whorl of out groups. Bracts VII and VIII are then in the position of an outer tepal whorl, with abortion of the medial bract IX. By fusion of bracts VII and VIII you could produce a palea of a true grass floret. This would suggest that the grass palea represents a modified outer tepal whorl. Consistent with this view is the observation (discussed above) that the *spw1* mutant of rice and the *si1* mutant of maize transforms lodicules into organs with a palea-like identity. It is also interesting that the rice *pal* mutant results in two unfused bracts growing in the place of a palea, indicating that the palea is in fact a fusion of two distinct primordia. This would then suggest that the large bract VI of *Streptocheata* is an intermediate between the lemma and subtending floral bract of outgroups. Similar interpretations of *Streptochaeta* have been suggested previously, although there is little consensus in the literature about the proper interpretation of the *Streptochaeta* spikelet equivalent (for reviews, see (Judziewicz and Soderstrom, 1989; Page, 1951; Soderstrom, 1981)).

This hypothesis of floret evolution can be tested using grass genes that are consistent markers of organ identity. For example, B class genes are known to mark stamen and lodicule identity in grasses with a true spikelet. If bracts X-XII of *Streptocheata* are, in fact, intermediates between the inner tepals of outgroups and the lodicules of other grasses, then one would expect to see B class gene expression in stamens and bracts X-XII of *Streptochaeta*. Additionally there should be expression

in stamens and the inner tepal whorl of monocot outgroups. Our lab has isolated B class genes from *Streptochaeta* as well as outgroups, and *in situ* expression patterns of these genes are entirely consistent with this hypothesis (Whipple, C.J., Zanis, M., Kellogg, E.A., and Schmidt, R. J., unpublished data). Unfortunately there are no robust markers of palea identity yet. However, as we learn more about genes controlling grass flowering other aspects of this hypothesis can be tested.

Synteny and the grass family as an integrated genetic system

Comparative mapping studies in diverse grasses have indicated that grass genomes have a high degree of colinearity, or synteny, meaning broad regions of chromosomes in different species share the same genes in roughly the same order (Moore *et al.*, 1995). This colinearity among grass genomes has important implications for comparative studies of evolution and development in the grass family as has been noted by others (Freeling, 2001). With a complete draft sequence of the rice genome, and beginning sequencing of the maize and sorghum genomes, it is possible to use these species as 'reference' genomes for mapping studies in non-model grasses. If it is possible to form fertile F1 hybrids from closely related species or subspecies that differ in morphological traits of interest, then it is possible to use the powerful methods of Quantitative Trait Loci (QTL) mapping to define the approximate number and location of genes affecting the trait. An excellent example of this work is that of Doebley and colleagues in identifying the QTL that differentiate maize from its wild ancestor teosinte (Doebley and Stec, 1991). In theory it should be possible to map the loci differentiating any two grasses that form a fertile F1. With more sophisticated

mapping techniques it is becoming reasonable to clone the genes underlying individual QTL (Salvi and Tuberosa, 2005).

Just such a QTL study with the non-model domesticated grass *Setaria italica* and its wild progenitor *Setaria viridis* was recently begun (Doust *et al*., 2004; Doust *et al*., 2005). These two species differ significantly in vegetative and inflorescence branching habits. Maize RFLP probes were used to create an initial map of the *Setaria* genome, and a QTL analysis was performed using the same markers. Interestingly, candidate genes such as *Tb1* from maize, a major determinant of vegetative branching differences between maize and teosinte, did not explain as much of the branching differences as did two other QTLs to which no obvious candidate genes in maize mapped. This suggests that a QTL approach has identified genes for branching in *Setaria* that have yet to be identified in maize (perhaps due to maize gene redundancy, as discussed above), or that these mapping studies have identified grass genes that evolved more significant roles in this developmental pathway in the *Setaria* lineage than they did in maize. Markers flanking QTLs of interest were located to syntenous regions on the rice genome and used to identify potential candidate genes that underlie important QTL. As these candidates are evaluated, new genes important for morphological changes selected during domestication should come to light.

The domestication studies in maize and *Setaria* demonstrate the power of QTL analysis to reveal the genetic basis of morphological variation among closely related species. That these techniques were applied successfully to a non-model grass is hopeful. The majority of QTL studies performed to date have been on agricultural

species, and thus are biased towards traits selected by humans. The critical next step is to understand the genetic basis of morphological differences in wild, undomesticated species. There is impressive morphological variation among grass species, especially in inflorescence architecture. All that is needed is a fertile F1 from two wild species that differ for traits of interest. The work of Doust *et al*. (2004; 2005), demonstrates that it is not unreasonable to use grass synteny to move from an initial QTL study to a list of candidate genes to evaluate, even with little sequence information available for the grass species being studied.

Conclusions

Because of its history as a great genetic system and due to its development as one of the first plant systems for reverse genetics, maize emerged early as a model for testing the applicability of the ABC model of flower development. Rice, with its comparative ease of transformation, provided another grass species that was amenable to dissecting gene function through antisense and later, RNAi approaches. These two grasses afforded functional insights beyond gene sequence comparisons and comparative expression analyses of floral organ identify genes in other grass species. From these studies it appears that B class gene activities are largely conserved, with these genes in the grasses specifying lodicule and stamen development, as compared to petal and stamen development in the core eudicots. C class gene activity appears also conserved in terms of pattern of gene expression, having a role in stamen and possibly carpel identity and floral meristem determinacy, although as discussed, there may be

elaborations on this program of development that are unique to the grasses. Gene redundancy in maize has complicated a thorough functional analysis to date. This is especially true regarding the role of genes in the A and E class gene lineages, where the lineage of *AP1*/*FUL*-like and *SEP*-like genes in the grasses appear to have radiated early in their evolutionary history. However, a combination of reverse genetics to understand the role of grass ABCDE class genes, with forward genetics to uncover unknown genes important to grasses, promises to further advance our understanding of the genetics of grass flower development. Lastly, the combined use of rice and maize genomic resources has facilitated map-based cloning of maize genes (Bortiri et al., 2006; Wang et al., 2005) portending a time when walking to a gene in these species will be as commonplace as in *Arabidopsis*.

Upon the completion of the closely related sorghum and maize genome sequences, the combined genomic resources for maize, sorghum and rice will provide a strong platform from which to explore the evolution floral development not only in these species, but across all the grasses. With rice and maize representing the majority of species diversity in the grass family, the analysis of the gene function and corresponding gene sequence changes that have occurred during their 50-60 million years of evolution will provide important insights into developmental genetics and comparative evolution of all the grasses. Approaches including comparative functional analyses of duplicated genes important in development, as well QTL studies in morphologically distinct species will provide a wealth of information on the processes by which evolution modifies morphology and developmental pathways. As derived

members of the monocot lineage, the analysis of not only floral development, but many aspects of angiosperm development in these groups will continue to provide important comparisons with the model eudicots.

Acknowledgements

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Whipple, C. J., and Schmidt R. J. (2006 in press). Developmental Genetics of Grass Flower development. *Advances in Botanical Research: Developmental Genetics of the Flower* volume 44, ed. Soltis, Leebens-Mack, and Soltis.

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Figures and Figure Legends

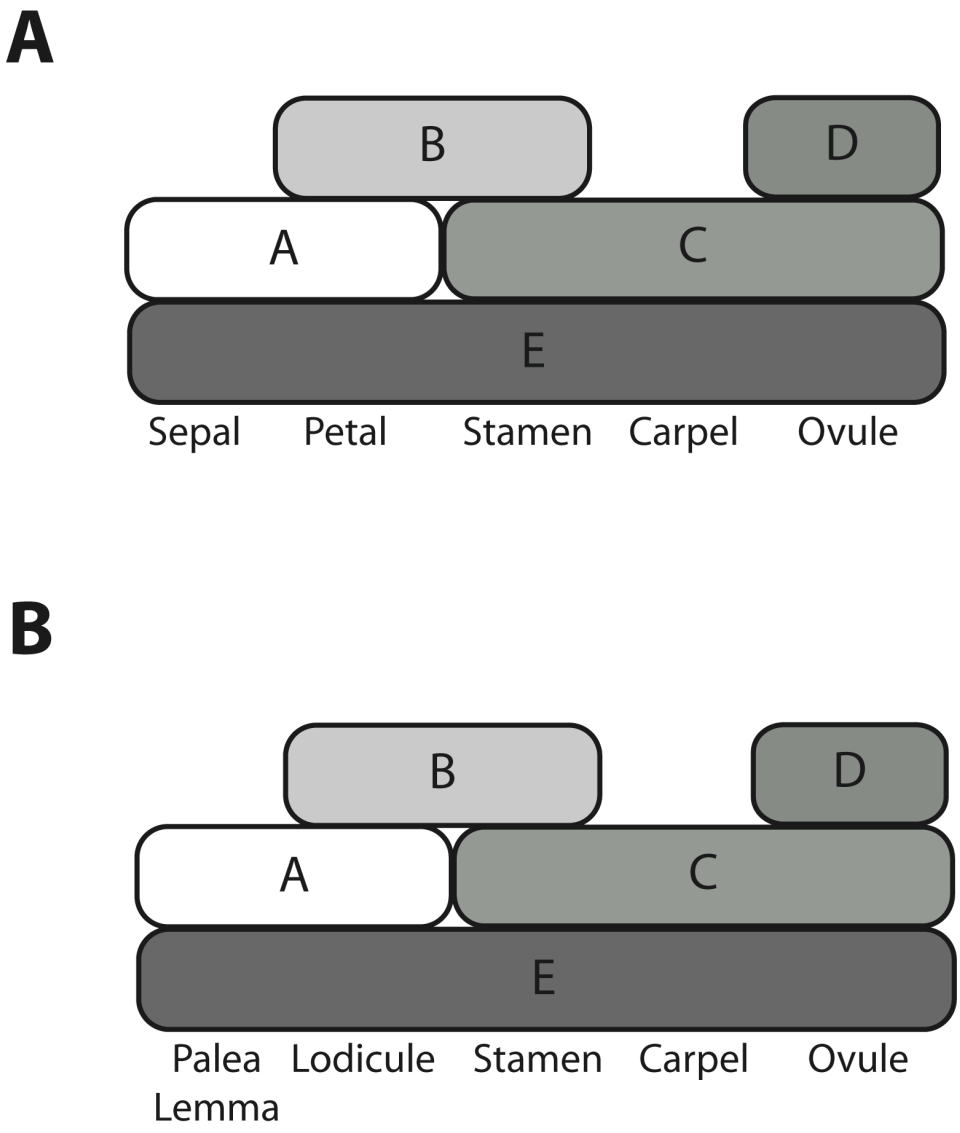

Figure 1.1
The ABCDE model illustrating the genetic interactions necessary for floral organ specification.

A. The ABCDE model as it applies to eudicots, from Theissen (2001) with changes incorporating recent data from Ditta et al. (2004).
B. A modification of the ABCDE model to explain the patterning of a grass floret, modified from Ambrose et al. (2000).

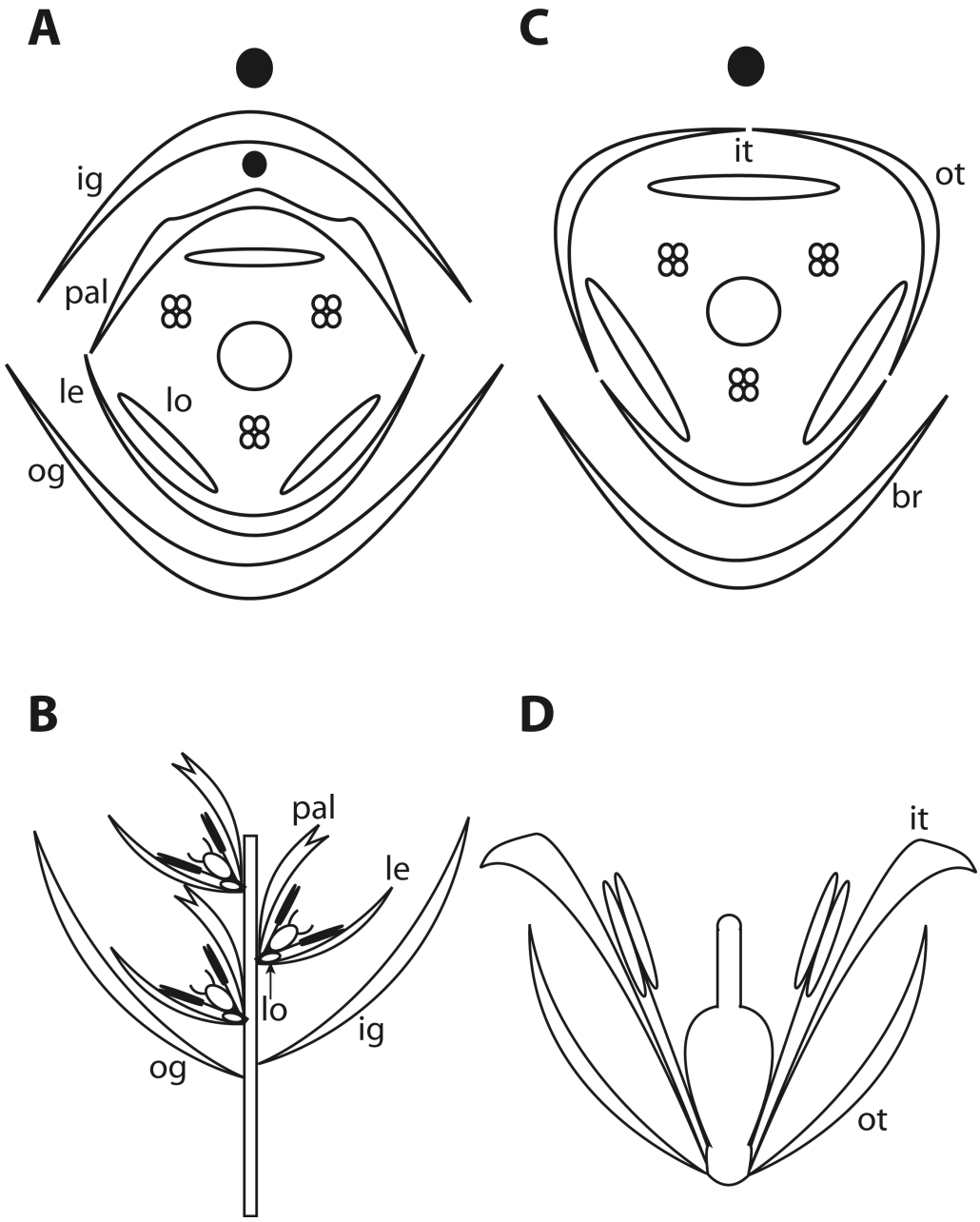

Figure 1.2
Schematics for the grass spikelet and typical monocot flower

A-**B**. Grass spikelet diagram
C-**D**. Monocot flower diagram

ig = inner glume, **og** = outer glume, **pal** = palea, **le** = lemma, **lo** = lodicule, **br** = bract, **ot** = outer tepal, **it** = inner tepal , black dots represent the relative position of the stem

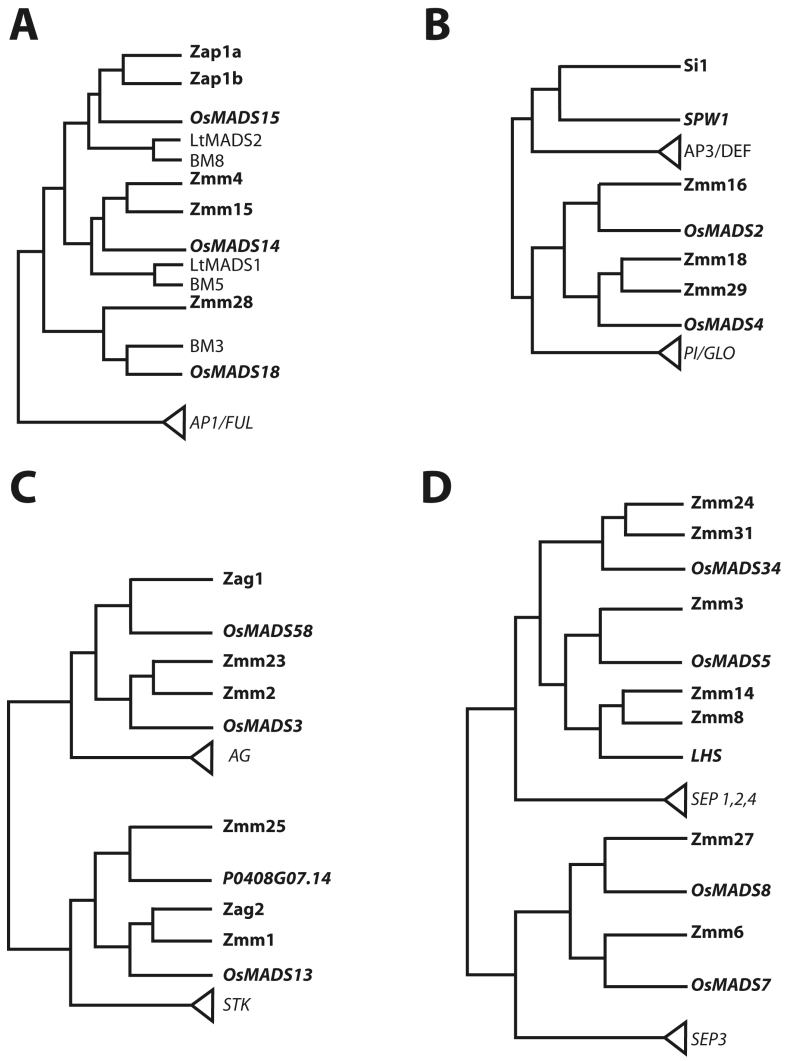

Figure 1.3
Summary of phylogenetic relationships among grass ABCD and E classes of MADS-box genes

A. A class genes
B. B class genes
C. C and D class genes
D. E class genes

Maize genes in **bold**, rice genes in ***bold italics***, barley and *Lolium* genes in normal typeface, and closest eudicot lineage (represented by *Arabidopsis*) in *italics*. Relationships in (**A**) and (**B**) are adapted from Münster et al. (2002), in (**C**) adapted from Kramer et al. (2004), and in (**D**) adapted from Zahn et al. (2005).

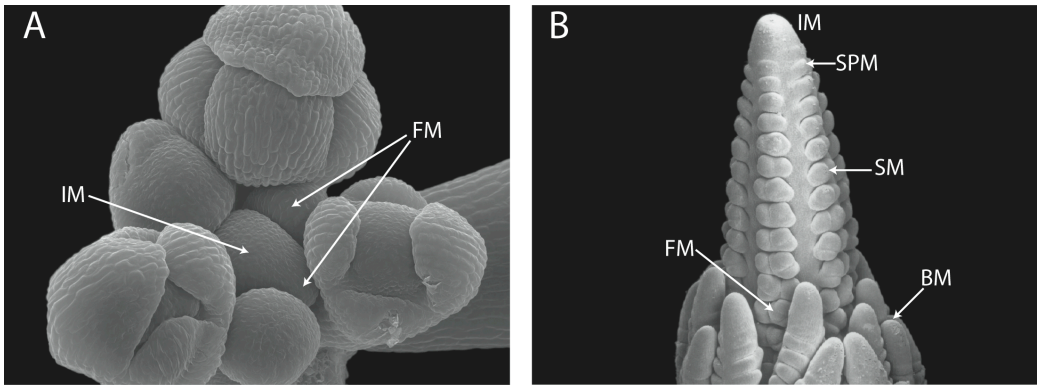

Figure 1.4
Reproductive meristems in *Arabidopsis* versus the grasses

A. The first meristem to form on the flanks of the *Arabidopsis* inflorescence meristem (**IM**) is the floral meristem (**FM**), which expresses *LFY* and *AP1*, thus conferring a floral meristem identity.
B. With the exception of branch meristems (**BM**), the first meristems to form from the grass inflorescence meristem are either a spikelet pair meristem (**SPM**) or a spikelet meristem (SM). Shown here is maize, which produces a spikelet pair meristem that divides, forming two spikelet meristems. The spikelet meristems initiate two glumes and finally produce the floral meristems. Grass *AP1*-like genes and *Zfl* are both expressed in the spikelet meristem and spikelet pair meristem without conferring a floral identity.

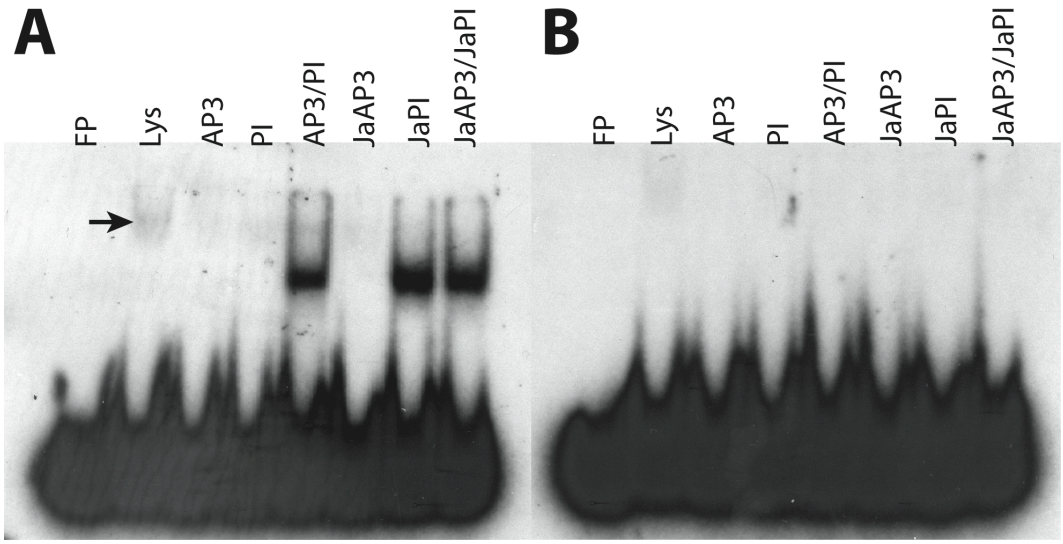

Figure 1.5
Electromobility Shift Assay (EMSA) of *Arabidopsis* (AP3 and PI) and *Joinvillea ascendens* (JaAP3 and JaPI) B class proteins.

EMSA was performed as previously described (Whipple *et al*., 2004). Binding specificity was tested using radiolabled probe containing a CArG box (5'-TTAGGCAATACTTTCCATTTTTGGTAACTC-3', CArG box underlined) derived from the *Arabidopsis AP3* promoter (**A**), or a mutated version **(B)**.
A. Neither *Arabidopsis* protein binds DNA alone, but do together (AP3/PI lane) as indicated by upward shift in labeled probe. JaAP3 does not bind DNA alone, but JaPI does. Shift in JaAP3/JaPI lane could be due to JaPI homodimer or JaAP3-JaPI heterodimer, or a combination of the two.
B. A mutant *AP3* CArG-box (5'-TTAGGCAATACTTT**GG**ATTTTT**CC**TAACTC-3', mutations in bold) abolishes all binding by both *Arabidopsis* and *Joinvillea* B class proteins, indicating that the binding seen in **A** is specific.
Joinvillea B class genes (*JaAP3*, and *JaPI*) were amplified by PCR on cDNA from immature inflorescence tissue using a degenerate B-class specific MADS box primer and a poly-T primer. Phylogenetic analysis indicated that the B class genes were sister to the grass genes (unpublished results C. Whipple, M. Zanis, E. Kellogg, and R. Schmidt). All B class cDNAs were subcloned into the pSPUTK vector (Stratagene), then transcribed and translated using the TNT Quick Coupled transcription translation system (Promega).
Arrow in **A** indicates a shift due to background proteins present in the lysate. **FP** = free probe, **Lys** = lysate control without plasmid added.

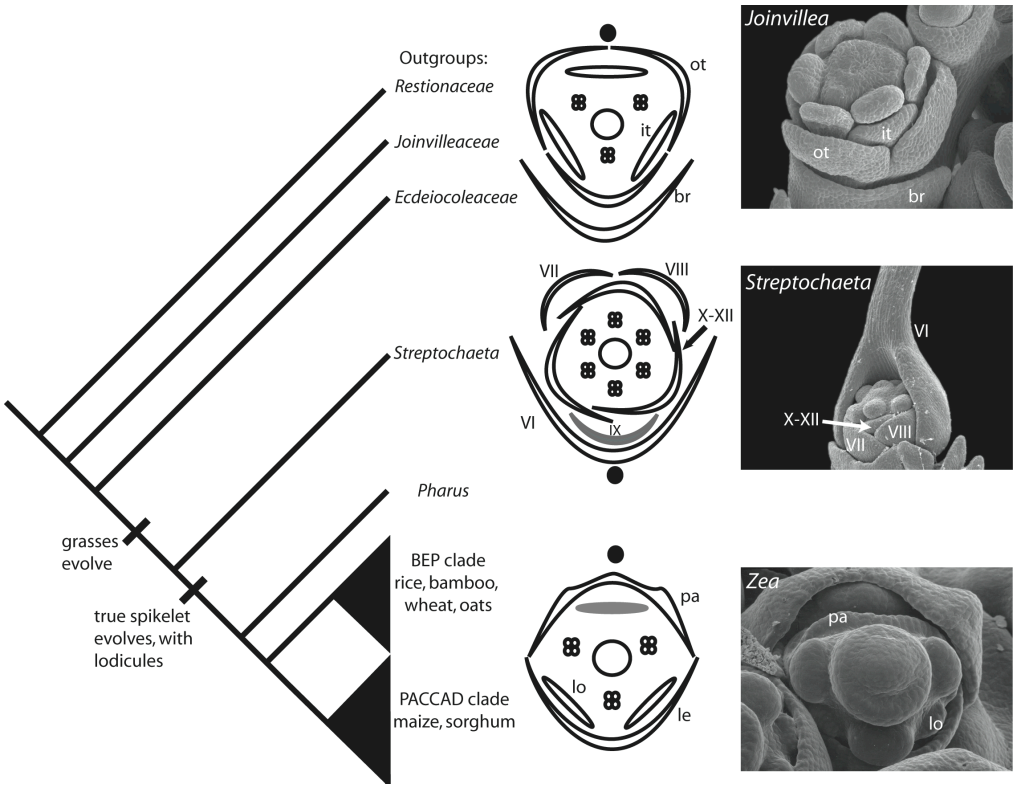

Figure 1.6
Evolution of the grass spikelet morphology

Phylogeny of grasses and outgroups, with generalized floral diagrams for a grass spikelet, an outgroup monocot flower, and the intermediate morphology of the *Streptochaeta* spikelet equivalent. Bracts **X**-**XII** are in the relative position where lodicules (**lo**) are normally found in the standard grass floret, and also in the position where inner tepals (**it**) of outgroup monocot flowers are typically located. Bracts **VII**-**IX** of *Streptochaeta* are in the relative position of outer tepals (**ot**) of a standard monocot flower. Fusion of bracts **VII** and **VIII**, and abortion of bract **IX** would lead to the palea (**pa**) of the grass floret. The large bract **VI** could then correspond to a floral bract (**br**) in outgroups and the lemma (**le**) of the grass spikelet.
Gray shaded organs in *Streptochaeta* and the grass floret indicate common abortion of bract IX and the medial lodicule. Black dots represent the relative position of the stem. BEP clade includes the grass subfamilies Bambusoideae, Ehrartoideae, and Pooideae, while the PACCAD clade includes Panicoideae, Arundinoideae, Centothecoideae, Chloridoideae, Aristidoideae, and Danthonioideae.

Table 1.1 Summary of grass ABCD and E class MADS-box genes classified according to their phylogenetic relationships.

The first column indicates the closest eudicot lineage to each of the grass genes in that row. Bold genes have characterized mutants, and genes with a * indicate co-suppression or other studies of gene function.

	Eudicot lineaege	Maize	Rice	Other
A class	*AP1/CAL/FUL*	*Zap1a, Zap1b* (*ZmMADS3*)*	*OsMDAS15*	*BM8, LtMADS2*
	"	*Zmm4, Zmm15*	*OsMADS14*	*BM5, LtMADS1, Vrn1**
	"	*Zmm28*	*OsMADS18*	*BM3*
B Class	*AP3/DEF*	***Si1***	***SPW***	
	PI/GLO	*Zmm16*	*OsMADS2**	
	"	*Zmm18, Zmm29*	*OsMADS4**	
C Class	*AG*	***Zag1***	*OsMADS58**	
	"	*Zmm2, Zmm23*	***OsMADS3****	
D Class	*FBP11/STK*	*Zag2, Zmm1*	*OsMADS13*	
	"	*Zmm25*	P0408G07.14	
E Class	*SEP3*	*Zmm6*	*OsMADS7*	
	"	*Zmm27*	*OsMADS8*	
	SEP1, 2, 4	*Zmm24, Zmm31*	*OsMADS34*	
	"	*ZmLHS1a, ZmLHS1b*	***LHS1****	
	"	*Zmm3*	*OsMADS5*	

References

Alvarez, J. and Smyth, D.R. (1999). CRABS CLAW and SPATULA, two Arabidopsis genes that control carpel development in parallel with AGAMOUS. *Development*. **126**, 2377-2386.

Ambrose, B.A., Espinosa-Matias, S., Vazquez-Santana, S., Vergara-Silva, F., Martinez, E., Marquez-Guzman, J. and Alvarez-Buylla, E.R. (2006). Comparative developmental series of the Mexican triurids support a euanthial interpretation for the unusual reproductive axes of Lacandonia schismatica (Triuridaceae)
Am. J. Bot. **93**, 15-35.

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F. and Schmidt, R.J. (2000). Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. *Molecular Cell*. **5**, 569-579.

Angenent, G.C., Franken, J., Busscher, M., van Dijken, A., van Went, J.L., Dons, H.J. and van Tunen, A.J. (1995). A novel class of MADS box genes is involved in ovule development in petunia. *Plant Cell*. **7**, 1569-1582.

Bomblies, K. and Doebley, J.F. (2005). Molecular evolution of FLORICAULA/LEAFY orthologs in the Andropogoneae (Poaceae). *Molecular Biology and Evolution*. **22**, 1082-1094.

Bomblies, K., Wang, R.L., Ambrose, B.A., Schmidt, R.J., Meeley, R.B. and Doebley, J. (2003). Duplicate FLORICAULA/LEAFY homologs zfl1 and zfl2 control inflorescence architecture and flower patterning in maize. *Development*. **130**, 2385-2395.

Bommert, P., Satoh-Nagasawa, N., Jackson, D. and Hirano, H.Y. (2005). Genetics and evolution of inflorescence and flower development in grasses. *Plant and Cell Physiology*. **46**, 69-78.

Bortiri, E., Chuck, G., Vollbrecht, E., Rocheford, T., Martienssen, R. and Hake, S. (2006). ramosa2 Encodes a LATERAL ORGAN BOUNDARY Domain Protein That Determines the Fate of Stem Cells in Branch Meristems of Maize. *Plant Cell*.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989). Genes directing flower development in Arabidopsis. *Plant Cell*. **1**, 37-52.

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

Cacharrón, J., Saedler, H. and Theissen, G. (1999). Expression of MADS box genes ZMM8 and ZMM14 during inflorescence development of Zea mays discriminates between the upper and the lower floret of each spikelet. *Development Genes and Evolution*. **209**, 411-420.

Causier, B., Castillo, R., Zhou, J., Ingram, R., Xue, Y., Schwarz-Sommer, Z. and Davies, B. (2005). Evolution in action: following function in duplicated floral homeotic genes. *Curr Biol*. **15**, 1508-1512.

Chung, Y.-Y., Kim, S.-R., Kang, H.-G., Noh, Y.-S., Chul, P.M., Finkela, D. and An, G. (1995). Characterization of two rice MADS box genes homologous to GLOBOSA. *Plant Science*. **109**, 45-56.

Chung, Y.Y., Kim, S.R., Finkel, D., Yanofsky, M.F. and An, G. (1994). Early flowering and reduced apical dominance result from ectopic expression of a rice MADS box gene. *Plant Molecular Biology*. **26**, 657-665.

Clifford, H.T. (1987). Spikelet and Floral Morphology. *In* "Grass Systematics and Evolution" (T. R. Soderstrom, K. W. Hilu, C. S. Campbell and M. E. Barkworth, eds.), pp. 21-30. Smithsonian Institution Press. Washington D. C.

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

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C. and van Tunen, A.J. (1995). The petunia MADS box gene FBP11 determines ovule identity. *Plant Cell*. **7**, 1859-1868.

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1999). PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. *Embo Journal*. **18**, 4023-4034.

Ditta, G., Pinyopich, A., Robles, P., Pelaz, S. and Yanofsky, M.F. (2004). The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. *Current Biology*. **14**, 1935-1940.

Doebley, J. and Stec, A. (1991). Genetic analysis of the morphological differences between maize and teosinte. *Genetics*. **129**, 285-295.

Doust, A.N., Devos, K.M., Gadberry, M.D., Gale, M.D. and Kellogg, E.A. (2004). Genetic control of branching in foxtail millet. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **101**, 9045-9050.

Doust, A.N., Devos, K.M., Gadberry, M.D., Gale, M.D. and Kellogg, E.A. (2005). The genetic basis for inflorescence variation between foxtail and green millet (poaceae). *Genetics*. **169**, 1659-1672.

Favaro, R., Immink, R.G., Ferioli, V., Bernasconi, B., Byzova, M., Angenent, G.C., Kater, M. and Colombo, L. (2002). Ovule-specific MADS-box proteins have conserved protein-protein interactions in monocot and dicot plants. *Molecular Genetics and Genomics*. **268**, 152-159.

Ferrandiz, C., Liljegren, S.J. and Yanofsky, M.F. (2000). Negative regulation of the SHATTERPROOF genes by FRUITFULL during Arabidopsis fruit development. *Science*. **289**, 436-438.

Fischer, A., Baum, N., Saedler, H. and Theissen, G. (1995). Chromosomal mapping of the MADS-box multigene family in Zea mays reveals dispersed distribution of allelic genes as well as transposed copies. *Nucleic Acids Research*. **23**, 1901-1911.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L. and Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*. **151**, 1531-1545.

Fornara, F., Parenicova, L., Falasca, G., Pelucchi, N., Masiero, S., Ciannamea, S., Lopez-Dee, Z., Altamura, M.M., Colombo, L. and Kater, M.M. (2004). Functional characterization of OsMADS18, a member of the AP1/SQUA subfamily of MADS box genes. *Plant Physiology*. **135**, 2207-2219.

Fourquin, C., Vinauger-Douard, M., Fogliani, B., Dumas, C. and Scutt, C.P. (2005). Evidence that CRABS CLAW and TOUSLED have conserved their roles in carpel development since the ancestor of the extant angiosperms. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **102**, 4649-4654.

Freeling, M. (2001). Grasses as a single genetic system: reassessment 2001. *Plant Physiology*. **125**, 1191-1197.

Gaut, B.S. and Doebley, J.F. (1997). DNA sequence evidence for the segmental allotetraploid origin of maize. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **94**, 6809-6814.

Gocal, G.F., King, R.W., Blundell, C.A., Schwartz, O.M., Andersen, C.H. and Weigel, D. (2001). Evolution of floral meristem identity genes. Analysis of Lolium temulentum genes related to APETALA1 and LEAFY of Arabidopsis. *Plant Physiology*. **125**, 1788-1801.

Goto, K. and Meyerowitz, E.M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes and Development*. **8**, 1548-1560.

Grass Phylogeny Working Group (2001). Phylogeny and subfamilial classification of the grasses (Poaceae). *Annals of the Missouri Botanical Garden*. **88**, 373-457.

Guo, H. and Moose, S.P. (2003). Conserved noncoding sequences among cultivated cereal genomes identify candidate regulatory sequence elements and patterns of promoter evolution. *Plant Cell*. **15**, 1143-1158.

Heuer, S., Hansen, S., Bantin, J., Brettschneider, R., Kranz, E., Lorz, H. and Dresselhaus, T. (2001). The maize MADS box gene ZmMADS3 affects node number and spikelet development and is co-expressed with ZmMADS1 during flower development, in egg cells, and early embryogenesis. *Plant Physiology*. **127**, 33-45.

Honma, T. and Goto, K. (2001). Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. *Nature*. **409**, 525-529.

Huang, H., Mizukami, Y., Hu, Y. and Ma, H. (1993). Isolation and characterization of the binding sequences for the product of the Arabidopsis floral homeotic gene AGAMOUS. *Nucleic Acids Research*. **21**, 4769-4776.

Huijser, P., Klein, J., Lonnig, W.E., Meijer, H., Saedler, H. and Sommer, H. (1992). Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in Antirrhinum majus. *Embo Journal*. **11**, 1239-1249.

Inada, D.C., Bashir, A., Lee, C., Thomas, B.C., Ko, C., Goff, S.A. and Freeling, M. (2003). Conserved noncoding sequences in the grasses. *Genome Res*. **13**, 2030-2041.

Irish, V.F. (2000). Variations on a theme: flower development and evolution. *Genome Biology*. **1**, 1015.1011-1015.1014.

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

Jack, T., Fox, G.L. and Meyerowitz, E.M. (1994). Arabidopsis homeotic gene APETALA3 ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell*. **76**, 703-716.

Jeon, J.S., Jang, S., Lee, S., Nam, J., Kim, C., Lee, S.H., Chung, Y.Y., Kim, S.R., Lee, Y.H., Cho, Y.G. and An, G. (2000). Leafy hull sterile1 is a homeotic mutation in a rice MADS box gene affecting rice flower development. *Plant Cell*. **12**, 871-884.

Judziewicz, E.J. and Soderstrom, T.R. (1989). Morphological, anatomical, and taxonomic studies in *Anomochloa* and *Streptochaeta* (Poaceae: Bambusoideae). *Smithsonian Contributions to Botany*. **68**, 1-52.

Kang, H.G. and An, G. (1997). Isolation and characterization of a rice MADS box gene belonging to the AGL2 gene family. *Molecular Cells*. **7**, 45-51.

Kang, H.G., Jang, S., Chung, J.E., Cho, Y.G. and An, G. (1997). Characterization of two rice MADS box genes that control flowering time. *Molecular Cells*. **7**, 559-566.

Kang, H.G., Jeon, J.S., Lee, S. and An, G. (1998). Identification of class B and class C floral organ identity genes from rice plants. *Plant Molecular Biology*. **38**, 1021-1029.

Kang, H.G., Noh, Y.S., Chung, Y.Y., Costa, M.A., An, K. and An, G. (1995). Phenotypic alterations of petal and sepal by ectopic expression of a rice MADS box gene in tobacco. *Plant Molecular Biology*. **29**, 1-10.

Kaplinsky, N.J., Braun, D.M., Penterman, J., Goff, S.A. and Freeling, M. (2002). Utility and distribution of conserved noncoding sequences in the grasses. *Proc Natl Acad Sci U S A*. **99**, 6147-6151.

Keck, E., McSteen, P., Carpenter, R. and Coen, E. (2003). Separation of genetic functions controlling organ identity in flowers. *Embo Journal*. **22**, 1058-1066.

Kramer, E.M., Di Stilio, V.S. and Schlüter, P.M. (2003). Complex patterns of gene duplication in the *APETALA3* and *PISTILLATA* lineages of the ranunculaceae. *International Journal of Plant Sciences*. **164**, 1-11.

Kramer, E.M., Dorit, R.L. and Irish, V.F. (1998). Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. *Genetics*. **149**, 765-783.

Kramer, E.M. and Irish, V.F. (1999). Evolution of genetic mechanisms controlling petal development. *Nature*. **399**, 144-148.

Kramer, E.M. and Irish, V.F. (2000). Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *International Journal of Plant Sciences*. **161**, S29-S40.

Kramer, E.M., Jaramillo, M.A. and Di Stilio, V.S. (2004). Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. *Genetics*. **166**, 1011-1023.

Kyozuka, J., Kobayashi, T., Morita, M. and Shimamoto, K. (2000). Spatially and temporally regulated expression of rice MADS box genes with similarity to Arabidopsis class A, B and C genes. *Plant Cell Physiol*. **41**, 710-718.

Kyozuka, J. and Shimamoto, K. (2002). Ectopic expression of OsMADS3, a rice ortholog of AGAMOUS, caused a homeotic transformation of lodicules to stamens in transgenic rice plants. *Plant and Cell Physiology*. **43**, 130-135.

Lee, S., Jeon, J.S., An, K., Moon, Y.H., Chung, Y.Y. and An, G. (2003). Alteration of floral organ identity in rice through ectopic expression of OsMADS16. *Planta*. **217**, 904-911.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L. and Yanofsky, M.F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature*. **404**, 766-770.

Litt, A. and Irish, V.F. (2003). Duplication and diversification in the APETALA1/FRUITFULL floral homeotic gene lineage: implications for the evolution of floral development. *Genetics*. **165**, 821-833.

Lopez-Dee, Z.P., Wittich, P., Enrico Pe, M., Rigola, D., Del Buono, I., Gorla, M.S., Kater, M.M. and Colombo, L. (1999). OsMADS13, a novel rice MADS-box gene expressed during ovule development. *Developmental Genetics*. **25**, 237-244.

Luo, Q., Zhou, K., Zhao, X., Zeng, Q., Xia, H., Zhai, W., Xu, J., Wu, X., Yang, H. and Zhu, L. (2005). Identification and fine mapping of a mutant gene for palealess spikelet in rice. *Planta*. **221**, 222-230.

Maes, T., Van de Steene, N., Zethof, J., Karimi, M., D'Hauw, M., Mares, G., Van Montagu, M. and Gerats, T. (2001). Petunia Ap2-like genes and their role in flower and seed development. *Plant Cell*. **13**, 229-244.

Malcomber, S.T. and Kellogg, E.A. (2004). Heterogeneous expression patterns and separate roles of the SEPALLATA gene LEAFY HULL STERILE1 in grasses. *Plant Cell*. **16**, 1692-1706.

Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. (1992). Molecular characterization of the Arabidopsis floral homeotic gene APETALA1. *Nature*. **360**, 273-277.

Mandel, M.A. and Yanofsky, M.F. (1995). A gene triggering flower formation in Arabidopsis. *Nature*. **377**, 522-524.

McSteen, P., Laudencia-Chingcuanco, D. and Colasanti, J. (2000). A floret by any other name: control of meristem identity in maize. *Trends in Plant Science*. **5**, 61-66.

Mena, M., Ambrose, B.A., Meeley, R.B., Briggs, S.P., Yanofsky, M.F. and Schmidt, R.J. (1996). Diversification of C-function activity in maize flower development. *Science*. **274**, 1537-1540.

Mena, M., Mandel, M.A., Lerner, D.R., Yanofsky, M.F. and Schmidt, R.J. (1995). A characterization of the MADS-box gene family in maize. *Plant Journal*. **8**, 845-854.

Michelangeli, F.A., Davis, J.I. and Stevenson, D.W. (2003). Phylogenetic relationships among Poaceae and related families as inferred from morphology, inversions in the plastid genome, and sequence data from the mitochondrial and plastid genomes. *American Journal of Botany*. **90**, 93-106.

Moon, Y.H., Kang, H.G., Jung, J.Y., Jeon, J.S., Sung, S.K. and An, G. (1999). Determination of the motif responsible for interaction between the rice APETALA1/AGAMOUS-LIKE9 family proteins using a yeast two-hybrid system. *Plant Physiology*. **120**, 1193-1204.

Moore, G., Devos, K.M., Wang, Z. and Gale, M.D. (1995). Cereal genome evolution. Grasses, line up and form a circle. *Current Biology*. **5**, 737-739.

Mueller, C.G. and Nordheim, A. (1991). A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. *Embo Journal*. **10**, 4219-4229.

Münster, T., Deleu, W., Wingen, L.U., Ouzunova, M., Cacharrón, J., Faigl, W., Werth, S., Kim, J.T.T., Saedler, H. and Theissen, G. (2002). Maize MADS-box genes galore. *Maydica*. **47**, 287-301.

Münster, T., Wingen, L.U., Faigl, W., Werth, S., Saedler, H. and Theissen, G. (2001). Characterization of three GLOBOSA-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. *Gene*. **262**, 1-13.

Murai, K., Miyamae, M., Kato, H., Takumi, S. and Ogihara, Y. (2003). WAP1, a wheat APETALA1 homolog, plays a central role in the phase transition from vegetative to reproductive growth. *Plant and Cell Physiology*. **44**, 1255-1265.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H. and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. *Development*. **130**, 705-718.

Ohno, S. (1970). "Evolution by Gene Duplication". Springer-VerlagNew York

Page, V.M. (1951). Morphology of the Spikelet of Streptochaeta. *Bulletin of the Torrey Botanical Club*. **78**, 22-37.

Paterson, A.H., Bowers, J.E. and Chapman, B.A. (2004). Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **101**, 9903-9908.

Pelaz, S., Ditta, G.S., Baumann, E., Wisman, E. and Yanofsky, M.F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature*. **405**, 200-203.

Pelaz, S., Tapia-Lopez, R., Alvarez-Buylla, E.R. and Yanofsky, M.F. (2001). Conversion of leaves into petals in Arabidopsis. *Current Biology*. **11**, 182-184.

Pelucchi, N., Fornara, F., Favalli, C., Masiero, S., Lago, C., Pè, M.E., Colombo, L. and Kater, M. (2002). Comparative analysis of rice MADS-box genes expressed during flower development. *Sexual Plant Reproduction*. **15**, 113-122.

Pinyopich, A., Ditta, G.S., Savidge, B., Liljegren, S.J., Baumann, E., Wisman, E. and Yanofsky, M.F. (2003). Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature*. **424**, 85-88.

Prasad, K., Parameswaran, S. and Vijayraghavan, U. (2005). OsMADS1, a rice MADS-box factor, controls differentiation of specific cell types in the lemma and palea and is an early-acting regulator of inner floral organs. *Plant J*. **43**, 915-928.

Prasad, K. and Vijayraghavan, U. (2003). Double-stranded RNA interference of a rice PI/GLO paralog, OsMADS2, uncovers its second-whorl-specific function in floral organ patterning. *Genetics*. **165**, 2301-2305.

Riechmann, J.L., Krizek, B.A. and Meyerowitz, E.M. (1996). Dimerization specificity of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **93**, 4793-4798.

Riechmann, J.L. and Meyerowitz, E.M. (1997). MADS domain proteins in plant development. *Biological Chemistry*. **378**, 1079-1101.

Salvi, S. and Tuberosa, R. (2005). To clone or not to clone plant QTLs: present and future challenges. *Trends in Plant Science*. **10**, 297-304.

Schmidt, R.J., Veit, B., Mandel, M.A., Mena, M., Hake, S. and Yanofsky, M.F. (1993). Identification and molecular characterization of ZAG1, the maize homolog of the Arabidopsis floral homeotic gene AGAMOUS. *Plant Cell*. **5**, 729-737.

Schmitz, J., Franzen, R., Ngyuen, T.H., Garcia-Maroto, F., Pozzi, C., Salamini, F. and Rohde, W. (2000). Cloning, mapping and expression analysis of barley MADS-box genes. *Plant Molecular Biology*. **42**, 899-913.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.E., Saedler, H. and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *Embo Journal*. **11**, 251-263.

Shinozuka, Y., Kojima, S., Shomura, A., Ichimura, H., Yano, M., Yamamoto, K. and Sasaki, T. (1999). Isolation and characterization of rice MADS box gene homologues and their RFLP mapping. *DNA Research*. **6**, 123-129.

Soderstrom, T.R. (1981). Some evolutionary trends in the Bambusoideae (Poaceae). *Annals of the Missouri Botanical Garden*. **68**, 15-47.

Sommer, H., Beltran, J.P., Huijser, P., Pape, H., Lonnig, W.E., Saedler, H. and Schwarz-Sommer, Z. (1990). Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. *Embo Journal*. **9**, 605-613.

Stellari, G.M., Jaramillo, M.A. and Kramer, E.M. (2004). Evolution of the APETALA3 and PISTILLATA lineages of MADS-box-containing genes in the basal angiosperms. *Molecular Biology and Evolution*. **21**, 506-519.

Takhtajan, A. (1991). "Evolutionary trends in flowering plants". Columbia University PressNew York

Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. *Current Opinion in Plant Biology*. **4**, 75-85.

Theissen, G. and Saedler, H. (2001). Plant biology. Floral quartets. *Nature*. **409**, 469-471.

Theissen, G., Strater, T., Fischer, A. and Saedler, H. (1995). Structural characterization, chromosomal localization and phylogenetic evaluation of two pairs of AGAMOUS-like MADS-box genes from maize. *Gene*. **156**, 155-166.

Till, B.J., Reynolds, S.H., Weil, C., Springer, N., Burtner, C., Young, K., Bowers, E., Codomo, C.A., Enns, L.C., Odden, A.R., Greene, E.A., Comai, L. and Henikoff, S. (2004). Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biology*. **4**, 12.

Trobner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lonnig, W.E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1992). GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. *Embo Journal*. **11**, 4693-4704.

van Tunen, A.J., Eikelboom, W. and Angenent, G.C. (1993). Floral organogenesis in *Tulipa*. *Flowering Newsletter*. **16**, 33-37.

Vandenbussche, M., Theissen, G., Van de Peer, Y. and Gerats, T. (2003). Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. *Nucleic Acids Research*. **31**, 4401-4409.

Vandenbussche, M., Zethof, J., Royaert, S., Weterings, K. and Gerats, T. (2004). The duplicated B-class heterodimer model: whorl-specific effects and complex genetic interactions in Petunia hybrida flower development. *Plant Cell*. **16**, 741-754.

Wang, H., Nussbaum-Wagler, T., Li, B., Zhao, Q., Vigouroux, Y., Faller, M., Bomblies, K., Lukens, L. and Doebley, J.F. (2005). The origin of the naked grains of maize. *Nature*. **436**, 714-719.

Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F. and Meyerowitz, E.M. (1992). LEAFY controls floral meristem identity in Arabidopsis. *Cell*. **69**, 843-859.

Weigel, D. and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. *Nature*. **377**, 495-500.

Whipple, C.J., Ciceri, P., Padilla, C.M., Ambrose, B.A., Bandong, S.L. and Schmidt, R.J. (2004). Conservation of B-class floral homeotic gene function between maize and Arabidopsis. *Development*. **131**, 6083-6091.

Winter, K.U., Weiser, C., Kaufmann, K., Bohne, A., Kirchner, C., Kanno, A., Saedler, H. and Theissen, G. (2002). Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. *Molecular Biology and Evolution*. **19**, 587-596.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G. and Hirano, H.Y. (2006). Functional Diversification of the Two C-Class MADS Box Genes OSMADS3 and OSMADS58 in Oryza sativa. *Plant Cell*. **18**, 15-28.

Yamaguchi, T., Nagasawa, N., Kawasaki, S., Matsuoka, M., Nagato, Y. and Hirano, H.Y. (2004). The YABBY gene DROOPING LEAF regulates carpel specification and midrib development in Oryza sativa. *Plant Cell*. **16**, 500-509.

Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T. and Dubcovsky, J. (2003). Positional cloning of the wheat vernalization gene VRN1. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **100**, 6263-6268.

Yang, Y., Fanning, L. and Jack, T. (2003). The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. *Plant J*. **33**, 47-59.

Yang, Y. and Jack, T. (2004). Defining subdomains of the K domain important for protein-protein interactions of plant MADS proteins. *Plant Mol Biol*. **55**, 45-59.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. *Nature*. **346**, 35-39.

Zahn, L.M., Kong, H., Leebens-Mack, J.H., Kim, S., Soltis, P.S., Landherr, L.L., Soltis, D.E., Depamphilis, C.W. and Ma, H. (2005). The evolution of the SEPALLATA subfamily of MADS-box genes: a preangiosperm origin with

multiple duplications throughout angiosperm history. *Genetics*. **169**, 2209-2223.

CHAPTER II

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

Research article

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.

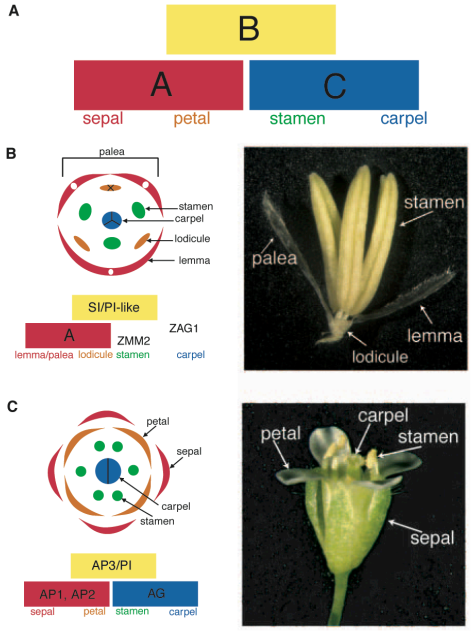

Fig. 1. ABC Model for patterning maize florets and *Arabidopsis* flowers. (A) Diagram of the traditional ABC model. (B) ABC model adapted for the maize floret (Ambrose et al., 2000). Maize genes are indicated in their appropriate domains (an aborted lodicule is indicated by X). A photo of a maize floret, 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 ∝l. The average amount of TNT reaction (or 'protein input') used in one DNA-binding assay was about 5 ∝l. 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).

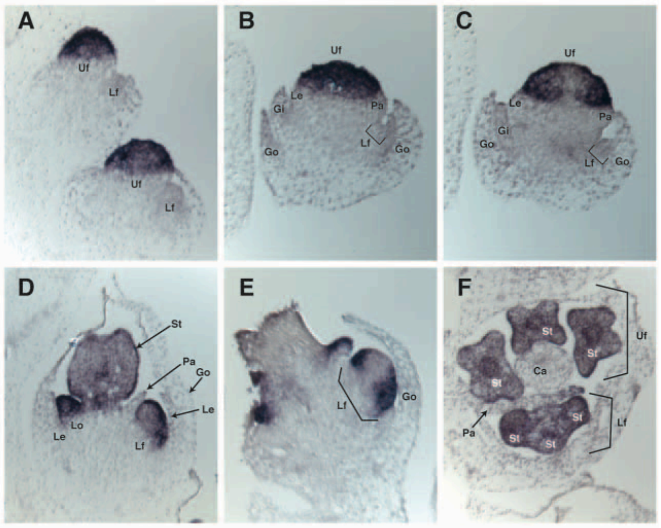

Fig. 2. *Zmm16* RNA in situ hybridization of maize developing male spikelets. (A) Two young male spikelets with *Zmm16* expression throughout the upper floral meristem (Uf) and absent from the emerging lower floral meristem (Lf). (B) Developing spikelet surrounded by inner (Gi) and outer (Go) glumes.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

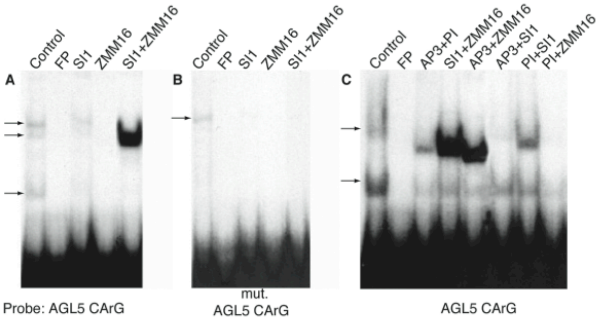

Fig. 3. In vitro DNA-binding assay of maize and *Arabidopsis* B-class proteins. (A) Autoradiogram of in vitro transcribed and translated SI1 and ZMM16 proteins incubated with labeled *AGL5* CArG-box probe. Neither SI1 nor ZMM16 alone are capable of binding the *AGL5* CArG-box, but together (SI1+ZMM16 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

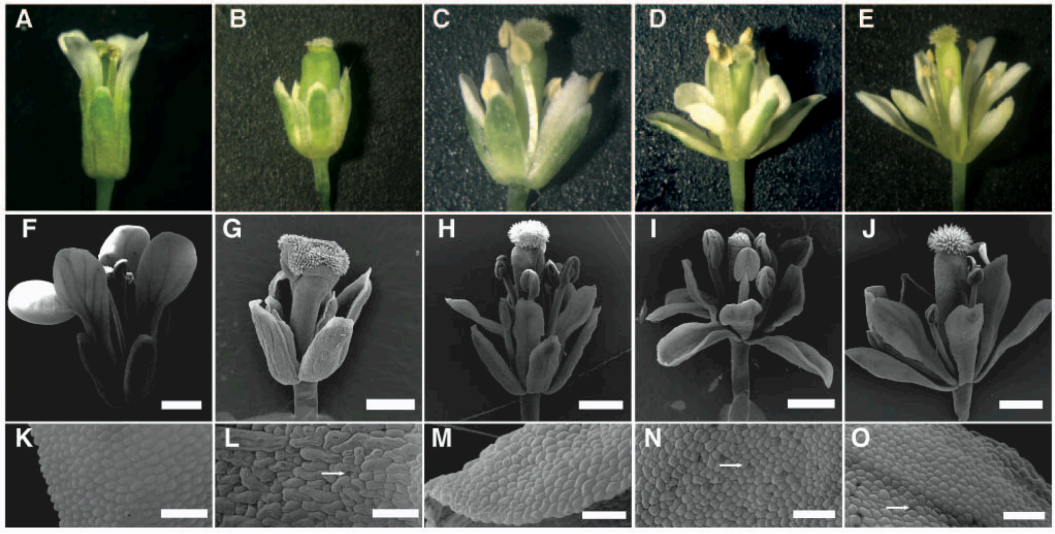

Fig. 4. Complementation of *Arabidopsis* B-class mutants *ap3* and *pi* by their maize orthologs. (A-E) *Arabidopsis* flowers of (A) wild type, (B) *ap3-3* mutant, (C) *ap3-3* with *AP3*pro:*Si1* transgene, (D) *pi-1* mutant with *AP3*pro:*Zmm16* transgene (note white sepal margins), and (E) *ap3-3 pi-1* 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 ∝m 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).

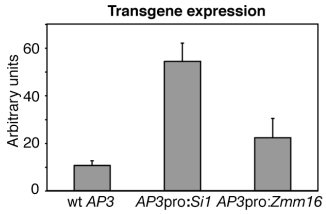

Fig. 5. Maize transgene expression levels compared with wild-type *AP3* levels. Total RNA collected from inflorescences of strongly rescued *AP3*:*Si1* and *AP3*:*Zmm16* plants, was spotted onto nylon filters and hybridized with transgene specific probes or an *AP3* gene-specific probe having identical 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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References

Ambrose, B. A., Lerner, D. R., Ciceri, P., Padilla, C. M., Yanofsky, M. F. and Schmidt, R. J. (2000). Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. *Mol. Cell* **5**, 569-579.

Angenent, G. C., Franken, J., Busscher, M., van Dijken, A., van Went, J. L., Dons, H. J. and van Tunen, A. J. (1995). A novel class of MADS box genes is involved in ovule development in petunia. *Plant Cell* **7**, 1569-1582.

Burr, B., Burr, F. A., Thompson, K. H., Albertson, M. C. and Stuber, C. W. (1988). Gene mapping with recombinant inbreds in maize. *Genetics* **118**, 519-526.

Chung, Y.-Y., Kim, S.-R., Kang, H.-G., Noh, Y.-S., Chul, P. M., Finkela, D. and An, G. (1995). Characterization of two rice MADS box genes homologous to GLOBOSA. *Plant Sci.* **109**, 45-56.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **16**, 735-743.

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

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H. J., Angenent, G. C. and van Tunen, A. J. (1995). The petunia MADS box gene FBP11 determines ovule identity. *Plant Cell* **7**, 1859-1868.

Fukui, M., Futamura, N., Mukai, Y., Wang, Y., Nagao, A. and Shinohara, K. (2001). Ancestral MADS box genes in Sugi, Cryptomeria japonica D. Don (Taxodiaceae), homologous to the B function genes in angiosperms. *Plant Cell Physiol.* **42**, 566-575.

Goto, K. and Meyerowitz, E. M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes Dev.* **8**, 1548-1560.

Irish, V. F. and Yamamoto, Y. T. (1995). Conservation of floral homeotic gene function between Arabidopsis and antirrhinum. *Plant Cell* **7**, 1635-1644.

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

Jack, T., Fox, G. L. and Meyerowitz, E. M. (1994). Arabidopsis homeotic gene APETALA3 ectopic expression: transcriptional and posttranscriptional regulation determine floral organ identity. *Cell* **76**, 703-716.

Kang, H. G., Jeon, J. S., Lee, S. and An, G. (1998). Identification of class B and class C floral organ identity genes from rice plants. *Plant Mol. Biol.* **38**, 1021-1029.

Kanno, A., Saeki, H., Kameya, T., Saedler, H. and Theissen, G. (2003). Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (Tulipa gesneriana). *Plant Mol. Biol.* **52**, 831-841.

Kellogg, E. A. (2000). The grasses: a case study in macroevolution. *Annu. Rev. Ecol. Syst.* **31**, 217-238.

Kramer, E. M. and Irish, V. F. (1999). Evolution of genetic mechanisms controlling petal development. *Nature* **399**, 144-148.

Kramer, E. M. and Irish, V. F. (2000). Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *Int. J. Plant Sci.* **161**, S29-S40.

Kramer, E. M., Dorit, R. L. and Irish, V. F. (1998). Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. *Genetics* **149**, 765-783.

Krizek, B. A. and Meyerowitz, E. M. (1996). The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. *Development* **122**, 11-22.

Kyozuka, J. and Shimamoto, K. (2002). Ectopic expression of OsMADS3, a rice ortholog of AGAMOUS, caused a homeotic transformation of lodicules to stamens in transgenic rice plants. *Plant Cell Physiol.* **43**, 130-135.

Lamb, R. S. and Irish, V. F. (2003). Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. *Proc. Natl. Acad. Sci. USA* **100**, 6558-6563.

Lloyd, A. M., Walbot, V. and Davis, R. W. (1992). Arabidopsis and Nicotiana anthocyanin production activated by maize regulators R and C1. *Science* **258**, 1773-1775.

Mathews, S., Tsai, R. C. and Kellogg, E. A. (2000). Phylogenetic structure in the grass family (Poaceae): evidence from the nuclear gene phytochrome B. *Am. J. Bot.* **87**, 96-107.

McGonigle, B., Bouhidel, K. and Irish, V. F. (1996). Nuclear localization of the Arabidopsis APETALA3 and PISTILLATA homeotic gene products depends on their simultaneous expression. *Genes Dev.* **10**, 1812-1821.

Mena, M., Mandel, M. A., Lerner, D. R., Yanofsky, M. F. and Schmidt, R. J. (1995). A characterization of the MADS-box gene family in maize. *Plant J.* **8**, 845-854.

Mena, M., Ambrose, B. A., Meeley, R. B., Briggs, S. P., Yanofsky, M. F. and Schmidt, R. J. (1996). Diversification of C-function activity in maize flower development. *Science* **274**, 1537-1540.

Moon, Y. H., Jung, J. Y., Kang, H. G. and An, G. (1999). Identification of a rice APETALA3 homologue by yeast two-hybrid screening. *Plant Mol. Biol.* **40**, 167-177.

Mouradov, A., Hamdorf, B., Teasdale, R. D., Kim, J. T., Winter, K. U. and Theissen, G. (1999). A DEF/GLO-like MADS-box gene from a gymnosperm: Pinus radiata contains an ortholog of angiosperm B class floral homeotic genes. *Dev. Genet.* **25**, 245-252.

Münster, T., Wingen, L. U., Faigl, W., Werth, S., Saedler, H. and Theissen, G. (2001). Characterization of three GLOBOSA-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. *Gene* **262**, 1-13.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H. and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. *Development* **130**, 705-718.

Neff, M. M., Neff, J. D., Chory, J. and Pepper, A. E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in Arabidopsis thaliana genetics. *Plant J.* **14**, 387-392.

Page, V. M. (1951). Morphology of the Spikelet of Streptochaeta. *Bull. Torrey Club* **78**, 22-37.

Pelaz, S., Ditta, G. S., Baumann, E., Wisman, E. and Yanofsky, M. F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* **405**, 200-203.

Prasad, K. and Vijayraghavan, U. (2003). Double-stranded RNA interference of a rice PI/GLO paralog, OsMADS2, uncovers its second-whorl-specific function in floral organ patterning. *Genetics* **165**, 2301-2305.

Riechmann, J. L. and Meyerowitz, E. M. (1997). MADS domain proteins in plant development. *Biol. Chem.* **378**, 1079-1101.

Riechmann, J. L., Krizek, B. A. and Meyerowitz, E. M. (1996). Dimerization specificity of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA, and AGAMOUS. *Proc. Natl. Acad. Sci. USA* **93**, 4793-4798.

Savidge, B., Rounsley, S. D. and Yanofsky, M. F. (1995). Temporal relationship between the transcription of two Arabidopsis MADS box genes and the floral organ identity genes. *Plant Cell* **7**, 721-733.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P. J., Hansen, R., Tetens, F., Lonnig, W. E., Saedler, H. and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J.* **11**, 251-263.

Sommer, H., Beltran, J. P., Huijser, P., Pape, H., Lonnig, W. E., Saedler, H. and Schwarz-Sommer, Z. (1990). Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. *EMBO J.* **9**, 605-613.

Sundstrom, J. and Engstrom, P. (2002). Conifer reproductive development involves B-type MADS-box genes with distinct and different activities in male organ primordia. *Plant J.* **31**, 161-169.

Sundstrom, J., Carlsbecker, A., Svensson, M. E., Svenson, M., Johanson, U., Theissen, G. and Engstrom, P. (1999). MADS-box genes active in developing pollen cones of Norway spruce (Picea abies) are homologous to the B-class floral homeotic genes in angiosperms. *Dev. Genet.* **25**, 253-266.

Takhtajan, A. (1991). *Evolutionary trends in flowering plants*. New York, NY: Columbia University Press.

Trobner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lonnig, W. E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1992). GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. *EMBO J.* **11**, 4693-4704.

van Tunen, A. J., Eikelboom, W. and Angenent, G. C. (1993). Floral organogenesis in *Tulipa*. *Flowering Newslett.* **16**, 33-37.

Vandenbussche, M., Theissen, G., van de Peer, Y. and Gerats, T. (2003). Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. *Nucleic Acids Res.* **31**, 4401-4409.

Walker, A. R., Davison, P. A., Bolognesi-Winfield, A. C., James, C. M., Srinivasan, N., Blundell, T. L., Esch, J. J., Marks, M. D. and Gray, J. C. (1999). The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *Plant Cell* **11**, 1337-1350.

Weigel, D. and Meyerowitz, E. M. (1994). The ABCs of floral homeotic genes. *Cell* **78**, 203-209.

Wikstrom, N., Savolainen, V. and Chase, M. W. (2001). Evolution of the angiosperms: calibrating the family tree. *Proc. R. Soc. Lond., B, Biol. Sci.* **268**, 2211-2220.

Winter, K. U., Becker, A., Münster, T., Kim, J. T., Saedler, H. and Theissen, G. (1999). MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. *Proc. Natl. Acad. Sci. USA* **96**, 7342-7347.

Winter, K. U., Saedler, H. and Theissen, G. (2002a). On the origin of class B floral homeotic genes: functional substitution and dominant inhibition in Arabidopsis by expression of an orthologue from the gymnosperm Gnetum. *Plant J.* **31**, 457-475.

Winter, K. U., Weiser, C., Kaufmann, K., Bohne, A., Kirchner, C., Kanno, A., Saedler, H. and Theissen, G. (2002b). Evolution of class B floral homeotic proteins: obligate heterodimerization originated from homodimerization. *Mol. Biol. Evol.* **19**, 587-596.

Zik, M. and Irish, V. F. (2003). Global identification of target genes regulated by APETALA3 and PISTILLATA floral homeotic gene action. *Plant Cell* **15**, 207-222.

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CHAPTER III

Conservation of B class MADS-box gene expression in the second whorl of a basal grass and outgroups links the origin of lodicules and petals

Abstract

Molecular and genetic studies of flower development in core eudicot species have established a central role for B class genes in specifying petal and stamen (second and third whorl) identities. In grass species, mutations in B class orthologs have shown that B class genes control stamen and lodicule identity, suggesting conservation of B class gene activity across angiosperms providing one equates the grass lodicule with the petal. However, as lodicules are grass-specific organs with a morphology distinct from petals, their true homology to eudicot and non-grass monocot floral organs has been a topic of debate. In addition, expression studies in basal eudicots suggest that B class genes may not be playing a conserved role in petal identity outside the core eudicots, casting some doubt on the degree of B class functional conservation. If lodicules represent modified second whorl organs (i.e. petals), then it would appear that B class control of second and third (stamen) whorl organ identities was present in the common ancestor of monocots and eudicots, and that B class function is largely conserved. To understand the relationship of lodicules to the sterile floral organs of non-grass monocots we have isolated and observed the expression of B class genes from a basal grass *Streptochaeta* that diverged before the evolution of lodicules, as well as the outgroup species *Joinvillea* and *Chondropetalum* which have a typical monocot floral plan. Our results support the interpretation of lodicules as modified second whorl organs. The results further suggest that B class genes control second whorl organ identity in a broader sense than simply "petal" identity, as the second

whorl organs in the grasses and outgroups have a distinct morphology, but do not have the showy characteristics of petals.

Introduction

The ABC model of floral patterning, developed from studies of the model species *Arabidopsis* and *Antirrhinum*, proposes that three classes of genes act alone or in combination to establish the identities of the four concentric whorls of floral organs (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). A class genes alone establish sepal identity, A class genes combine with B class genes to establish petal identity, B and C class genes combine to establish stamen identity, and C class genes act alone to confer carpel identity and floral meristem determinacy. As these genes were cloned they were found to belong, with the exception of *APETALA2*, to the conserved MADS-box family of transcription factors. Since establishment of this simple model, there has been interest in determining the degree to which it applies to other more distantly related angiosperms (Soltis et al., 2002). However, there remains little functional evidence that ABC MADS-box genes are playing conserved roles in flower development outside of the core eudicots. Recent work in grasses has shown that mutations in B and C class genes result in similar phenotypes as observed in B and C class mutants found in the higher eudicot species *Arabidopsis,* and *Antirrhinum*. Thus, these genetic analyses suggest that the B and C class functions of the ABC model may have been established early in the history of the angiosperms. The lack of

genetic knockout or knockdown data in many non-model species of angiosperms makes the testing of this model difficult

The origin and the number of times the petal has evolved has long been of interest to botanist (Albert et al., 1998; Takhtajan, 1991; Zanis et al., 2003). In addition to the origin of the petal, the origin of novel structures such as the palea, lemma, and lodicules of grasses have also been of interest (Clifford, 1987; Page, 1951; Piper, 1906). Current research has focused on the potential use of B class gene expression as a marker for petal identity and on the role of B class genes specifying petal identity outside the core eudicots. Formulation of an unambiguous and general definition of petal identity has been difficult, but generally it is considered to include a combination of morphological characteristics: 1) position- in a whorl just outside the stamens but internal to sepals, 2) appearance – compared to the sepals, generally larger, colored or otherwise non-green and more delicate, 3) epidermal cell morphology- characteristic conical cells. The original B class mutants described in *Arabidopsis* and *Antirrhinum* have homeotic transformations in the second whorl (petal) and third whorl (stamen) organs such that petals are transformed into sepals and stamens transformed into carpels (Goto and Meyerowitz, 1994; Jack et al., 1992). There are two B class mutants in both *Antirrhinum* and *Arabidopsis*, and when cloned these were shown to be a pair of closely related MADS box genes: *APETALA3* (*AP3*) (Jack et al., 1992) and *PISTILLATA* (*PI*) (Goto and Meyerowitz, 1994) in *Arabidopsis*, and their *Antirrhinum* orthologs *DEFECIENS* (*DEF*) (Sommer et al., 1990) and *GLOBOSA* (*GLO*) (Trobner et al., 1992) respectively. The paralogous *AP3*/*DEF* and

PI/*GLO* lineages are the result of a duplication that occurred near the base of the angiosperms (Stellari et al., 2004). Furthermore, a duplication in the *AP3*/*DEF* lineage at the base of the core eudicots,gave rise to the eu*AP3* and paleo*AP3* lineages which have distinct motifs in their protein C-terminal regions (Kramer et al., 1998). Core eudicot species have both the paleo*AP3* and eu*AP3* genes (although the paleo*AP3* ortholog was lost in *Arabidopsis*), while basal eudicots, monocots and basal angiosperms have *AP3* genes containing the paleo*AP3* motif. Core eudicot paleo*AP3* genes have not been functionally characterized, but rescue of the *Arabidopsis ap3* mutant with a chimeric AP3 protein containing a paleoAP3 C-terminus from a basal eudicot paleoAP3 resulted in stamen rescue, but no rescue of petal identity (Lamb and Irish, 2003). These results suggest that the paleo*AP3* functions primarily in stamen identity, and perhaps the eu*AP3* evolved a new role for specifying petal identity in the core eudicots.

Further evidence that the eu*AP3* lineage evolved to specify core eudicot petal identity comes from an examination of B class gene expression in non-core eudicots. This study showed that while B class genes are strongly expressed throughout stamen development of basal eudicot species, expression is often weak or patchy in petals (Kramer and Irish, 1999). This contrasts with core eudicots, where expression is strong throughout petal development as well. In light of morphological and anatomical evidence that petals evolved multiple times independently during the evolution of angiosperms (Albert et al., 1998; Takhtajan, 1991; Zanis et al., 2003), it was proposed that B class genes were recruited to a central role in petal identity in a

common ancestor of the core eudicots, but that in other angiosperm lineages B class genes are not necessarily specifying petaloidy (Kramer and Irish, 1999; Kramer and Irish, 2000).

A critical test of this hypothesis would be to disrupt B class gene function in a non-core eudicot species. To date such a disruption of B class gene function has only been described for the paleo*AP3* genes of the grass species maize and rice which both have highly derived floral organs (Ambrose et al., 2000; Nagasawa et al., 2003). These mutants show transformation of stamens to carpels as seen in higher eudicots, in addition to transformation of the grass specific organ lodicule into a lemma/palea –like organ. This phenotype is consistent with an interpretation of lodicules as modified grass petals, and palea and/or lemma as grass sepals. This, if a correct interpretation, would suggest that B class gene function is conserved in the common ancestor of monocots and eudicots and is consistent with a subsequent analysis indicating conservation of the biochemical function of the maize and *Arabidopsis* B class proteins (Whipple et al., 2004). Other than position, however, little in the mature morphology of lodicules indicates homology with petals, raising the possibility that B class genes were independently recruited to specify lodicule fate in the grasses (Irish, 2000; Irish, 2003). To help distinguish between these two opposing interpretations of grass B class gene function we have isolated and observed the expression pattern of B class genes from *Streptochaeta angustifolia*, a basal grass species that diverged before the evolution of lodicules, as well as from non-grass outgroups *Joinvillea ascendens* and *Chondropetalum elephantinum* that have a typical monocot floral plan. Our

results indicate that lodicules are indeed modified second whorl organs, and that B class genes appear to mark the fate of the second and third floral whorls. Furthermore, these expression patterns suggest that B class gene activity specifies a second whorl identity independent of the showy characteristics commonly interpreted as petaloid. These results provide further evidence that B class control of second and third whorl organ identities is conserved between monocots and dicots.

Materials and Methods

Plant material

Inflorescence and young floral primordia were collected from *Streptochaeta angustifolia*; plants were grown from seed in a growth room at 22˚C under constant light conditions. *Joinvillea ascendens* tissue was collected from plants growing in the National Tropical Botanical Garden in Kalaheo, HI. *Chondropetalum elephantinum* tissue was collected from plants growing in the private collection of Monique and Lambert Devoe of San Diego, CA. cDNA of *Pharus virescens* was collected from plants growing in a greenhouse of the Missouri Botanical Gardens (St. Louis, MO).

Isolation of B class genes

cDNA was synthesized from RNA isolated from young flowers using the SuperScript First-Strand cDNA Synthesis kit (Invitrogen). A polyT primer with a 5' adapter sequence was used in the cDNA synthesis step (5'-CCGGATCCTCTAGAGCGGCCGCTTTTTTTTTTTTTTTT-3'). PCR of B class

genes from grass species and outgroups was performed with a degenerate MADS-box sequence forward primer (5'-ATGGGBMGNGGVARKATHGAGA-3') and the polyT adapter primer. These PCR products were subcloned into pGEM-Teasy (Promega) or TOPO-TA (Invitrogen) and sequenced. Isolation of AP3 and PI orthologs from some species required a second round of PCR using internal primers: Grass PIrev (5'-YTSCTGBARRTTGGGRTG-3'), and Grass AP3rev (5'-YYARCCSAGGCGSAGGTCGTG-3'). Following isolation of a partial sequence, complete cDNA coding sequence was obtained using 5' and/or 3' RACE. DNA sequences were submitted to Genbank with the following accession numbers: *PvPI1* XXXXXXXX, *PvPI2* XXXXXXXX, *SaPI1* XXXXXXXX, *SaPI2* XXXXXXXX, *JaPI* XXXXXXXX, *CePI* XXXXXXXX, *SaAP3* XXXXXXXX, *JaAP3* XXXXXXXX, *CeAP3a* XXXXXXXX, *CeAP3b* XXXXXXXX.

Phylogenetic analysis

Initial DNA sequence alignment was performed with ClustalX, followed by manual adjustments using MacClade4. ModelTest was then used to evaluate these alignments for the optimal model of evolution to be used. Based on the results from Akaike Information Criterion (AIC) the GTR + G model was selected and Bayesian phylogenetic analysis was performed using MrBayes v3.1 with 2 million generations, a sample frequency of 100, and a burnin value of 5,000 (25%) for both the *AP3* and *PI* data sets. Maximum Likelihood bootstrap values were determined from a total of 100 replicates, also with the GTR + G model. Published sequences used in the analysis

had the following accession numbers: *OsMADS2* L37526, *ZMM16* AJ292959, *HvPI1* BU996044, *WPI2* AB107992, *OsMADS4* L37527, *ZMM18* AJ292960, *ZMM29* AJ292961, *HvPI2* AY541066, *WPI1* AB107991, *AhPI* AY621156, *SPW1* AF454259, *Si1* AF181479, *TaAP3* AB107993, *HvAP3* AY541065, *AhAP3* AY621154.

Scanning electron microscopy

Developing inflorescences were dissected and fixed in freshly prepared FAA (3.7% formaldehyde, 50% ethanol, 5% Acetic Acid) containing 0.1% Triton-X. Samples were dehydrated through an ethanol series, and dried with a critical point drier. Dried samples were mounted and dissected when necessary to reveal internal floral organs, then sputter coated with gold-palladium and viewed with a Quanta 600 environmental scanning electron microscope (ESEM).

RNA *in situ* hybridization

Freshly collected samples (except for *Joinvillea* which was collected 24-48 hrs prior to fixation) were fixed overnight at 4˚C in FAA, dehydrated through an ethanol series, cleared with histoclear, and embedded in paraplast. 8μm sections were cut with a microtome and mounted on Probe-on-Plus slides (Fisher). Slides with sections were prepared, hybridized, washed and exposed as described (Long et al., 1996) http://www.its.caltech.edu/~plantlab/protocols/insitu.htm. Probes were created by PCR amplification of the IKC domains and 3'UTRs of cDNAs and subcloning this

fragment upstream of the T7 promoter of pGEM-Teasy (Promega) or pBluescript (Stratagene). Antisense, digoxygenen-labelled UTP probe was synthesized using T7 polymerase with either a PCR amplified DNA template, or linearized plasmid.

Results

Isolation of B class genes from basal grass species and outgroups

Grass B class genes were originally isolated from maize (*Zea mays*) and rice (*Oryza sativa*) (Ambrose et al., 2000; Chung et al., 1995; Moon et al., 1999; Münster et al., 2001). In both species there appears to be a single *AP3* ortholog, *Silky1* (*Si1*) in maize and *SUPERWOMAN1* (*SPW1*) in rice, mutations of which result in a strong B class homeotic phenotype (Ambrose et al., 2000; Münster et al., 2001; Nagasawa et al., 2003). However, a previous analysis of the grass *PI*-like genes indicates that a duplication event in a common ancestor of maize and rice lead to two paralogous lineages, one containing the rice *OsMADS2* and maize *Zmm16* and the other containing rice *OsMADS4* and the maize genes *Zmm18* and *Zmm29* (the latter two appear to be a result of a more recent tandem duplication) (Münster et al., 2001). To examine expression of *AP3* and *PI* orthologs, as well as to more confidently place the duplication event in the grass *PI* genes, we isolated *AP3* and *PI* orthologs from the basal grass species *Pharus* and *Streptocheata*, as well as from two closely related outgroup species *Joinvillea* and *Chondropetalum*. Bayesian phylogenetic estimate of the grass *AP3* genes closely matches the consensus topology published by the Grass Phylogeny Working Group (GPWG) (Group, 2001), and is in agreement with a single

lineage for *AP3*-like genes in the grasses (Fig. 3.1B). This is consistent with a single *AP3* ortholog in the complete rice genome sequence, and a non-redundant (i.e. strong) B-class phenotype when this gene is disrupted in rice s*pw1* and maize *si1* mutants. However, two *AP3*-like genes were isolated from *Chondropetalum,* which appear to be the result of a duplication event sometime in the evolution of the Restionaceae.

In the *PI* phylogeny, two well-supported clades of grass *PI*s, named here *PI1* and *PI2*, are apparent with the *JoinvilleaPI* as sister to both clades (Fig. 3.1A). In both clades there is a *PI* ortholog from each grass species, and the topology matches the GPWG topology with only slight, unsupported, variations. These results are consistent with a *PI* duplication event occurring at or near the base of the grass family. This position coincides with a putative genome-wide duplication event early in the evolution of the grass family (Paterson et al., 2004). The placement of the *PI* duplication is also consistent with that of duplications in other MADS-box genes e.g. AP1/FUL (E. A. Kellogg, unpublished results). The results of a Maximum Likelihood analysis gave *AP3* and *PI* trees with the same topology as the Bayesian analysis, but with weaker support for some of the clades, particularly in the *PI* tree (Fig. 3.1A).

Morphology of second whorl organs in *Streptochaeta*, *Joinvillea*, and *Chondropetalum* is distinct from that of other organs, but not petaloid

The grass flower, relative to other monocots, is a derived structure, in which the sterile organs are of uncertain homology and have a grass specific nomenclature (Clifford, 1987). The lodicule is such an organ unique to the grasses that occurs in a whorl just

outside the stamens. Lodicules can be fleshy or scale-like, and generally swell at anthesis to allow the stamens to extend and the lemma and palea to separate. The two most basal genera of grasses *Anomochloa* and *Streptochaeta*, do not have lodicules (Arber, 1929; Judziewicz and Soderstrom, 1989). In *Streptochaeta*, three leaf or bract-like organs surround the stamens, while in *Anomochloa* a hairy "perigonate anulus" surrounds the stamens, indicating that lodicules evolved after the lineages for *Streptochaeta* and *Anomochloa* diverged (Kellogg, 2001). Outgroups to the grasses including *Joinvillea* and Restionaceae have a typical monocot floral plan in which the sterile organs occur in two separate whorls, the inner and outer tepals. Considering the hypothesis that lodicules are modified petals (inner tepals), we wished to observe the floral ontogeny of *Streptochaeta*, *Joinvillea* and *Chondropetalum* (Restionaceae) to better understand the morphology of their second whorl organs.

The *Streptocheata* spikelet equivalent has been described as a complex arrangement of twelve bracts (I-XII) that initiate before the reproductive organs (Judziewicz and Soderstrom, 1989). Bracts I-V initiate in a spiral, are small and can occasionally develop axillary spikelet equivalents of their own. Bract VI is large with a long curled awn that can entangle passing animals for seed dispersal. After bract VI, an apparent whorl of smaller bracts develops, with the two bracts (VII and VIII) opposite VI developing into shorter pointed structures, and the third member of this whorl (IX) adjacent to VI either reduced or absent. Bracts X-XII are similar and develop into an overlapping whorl that elongates and hardens at maturity to enclose the ovary and developing seed. Early developmental stages show that bracts VII and

VIII initiate in an apparent whorl, and quickly grow to cover the inner organs (Fig. 3.2A-C). Bracts X-XII initiate as a whorl outside the stamens and inside the VII-VIII-(IX) whorl, and because of their position are often interpreted as lodicules (Page, 1951) although they have none of the morphological characteristics of lodicules. Dissecting the large bract VI from the flower and imaging from behind shows that the X-XI-XII whorl overlaps and surrounds the developing stamens and is distinct in shape from the VII-VIII-(IX) whorl (Fig. 3.2 C, D). If the VII-VIII-(IX) bracts are interpreted as the first or outer whorl and X-XI-XII as the second or inner whorl of a *Streptochaeta* flower that is subtended by the large bract VI, then there is a clear differentiation in morphology between the first and second floral whorls in this basal grass.

The typical monocot floral plan has whorls of organs occurring in multiples of three with the first two whorls generally composed of three members each (Rudall and Bateman, 2004). These first two whorls can be either distinct or similar in adult morphology. If they are distinct, and the second whorl is clearly modified to attract pollinators they are called sepals and petals respectively, similar to flowers of eudicots. When they are similar, they are referred to as tepals, which can be either large and showy, "petaloid", or non-showy, "sepaloid". The closest extant relatives to the grasses include *Joinvillea* and the Restionaceae, which have two similar whorls of non-showy tepals. However, close examination of the development of these flowers shows that, while similar, the first and second whorls do have distinct morphologies (Fig. 3.2E-L). In *Chondropetalum*, the outer tepals initiate sequentially rather than in

a whorl and are hooded (Fig. 3.2F, G), while the inner tepals are more laminar and develop as an overlapping whorl that surrounds the stamens (Fig. 3.2H). As the flower matures the inner tepals elongate and continue to enclose the reproductive organs (Fig. 3.2E). In *Joinvillea*, the outer tepal whorl is more hooded and elongated with a smooth margin, while the inner tepal whorl is more laminar and triangular with a papillate margin (Fig. 3.2K, L).

Thus, in both *Joinvillea* and *Chondropetalum* the inner and outer tepals are distinct in morphology, although it is not clearly a sepal/petal distinction as the second whorl does not have the characteristic showy features of petals. A distinct morphology for the first and second whorl has also been described for other taxa in the same family as *Chondropetalum* (Decraene et al., 2002). Additionally, the *Streptochaeta* inner whorl (interpreting bracts VII-VIII-IX as the outer whorl) has a distinct morphology. Similarly lodicules have a distinct morphology in the grasses. Although in grasses it is not clear what organs should be interpreted as the first whorl (Clifford, 1987), some evidence suggests the palea and/or lemma could be (Ambrose et al., 2000; Nagasawa et al., 2003). The position and distinct morphology of the second whorl in *Streptochaeta*, *Joinvillea* and *Chondropetalum* along with the grass lodicules naturally suggests the hypothesis that lodicules are modified second whorl organs or inner tepals, and that the apparent second whorl of *Streptochaeta* could be an intermediate step in the evolution of lodicules.

B-class MADS box genes mark the second and third whorls of *Streptochaeta*, *Chondropetalum*, and *Joinvillea*

Considering our observations about the distinct morphology of the second whorl organs of *Streptochaeta* and non-grass outgroups, in addition to genetic data from maize and rice that B class genes control the identity of the second whorl lodicules, we think one possibility is that B class genes control second whorl organ identity in a broader sense than just petal identity as in eudicots or lodicule identity as in the grasses. If true, one would expect to see B class gene expression in the young second whorl organ primordia of *Streptochaeta* and grass outgroups. Such expression would also further support interpretation of lodicules as modified second whorl organs. Consequently, we performed RNA *in situ* hybridization on developing flowers of these species using probes derived from the B class genes we had isolated.

In *Streptochaeta* we examined expression of the *AP3* ortholog *SaAP3* as well as the *PI* ortholog *SaPI2*. For *SaAP3* there was strong expression in the stamens and second whorl primordia. Additionally, weaker expression was observed in the developing carpel and ovules (Fig. 3.3A). Such expression in the fourth whorl is not uncommon, and has been reported for *PI* and *DEF* at early stages of floral development (Goto and Meyerowitz, 1994; Schwarz-Sommer et al., 1992) as well as for the maize *PI* orthologs at later stages (Münster et al., 2001). For *SaPI2*, expression was only observed in the stamen whorl and the second whorl (Fig. 3.3B). In *Chondropetalum*, we performed an *in situ* with both of the *AP3* orthologs *CeAP3a* and *CeAP3b*. For *CeAP3a* we observed strong expression in the developing stamen and

second whorl primordia (Fig. 3.3C). A similar result was seen with *CeAP3b* (Fig. 3.3D), although expression was possibly weaker overall compared to *CeAP3a* (not shown). We were able to obtain tissue of *Joinvillea*, although the floral stages were not as young as for *Streptochaeta* and *Chondropetalum*, and fresh tissue was not available for fixing. For these reasons *in situ* hybridization of the *Joinvillea AP3* (*JaAP3*) and *PI* (*JaPI*) was not as robust. Nevertheless, for both *JaAP3* and *JaPI*, expression was observed in the stamen whorl as well as the second whorl, but apparently absent from the first whorl (Fig. 3.3E, F).

Discussion

B class gene expression supports a second whorl origin for the grass lodicule

B class genes in angiosperms are consistently expressed in stamens (third whorl), and it is thought that specification of the male reproductive organs in flowers is derived from a role in specification of male cone identity in Gymnosperms (Fukui et al., 2001; Mouradov et al., 1999; Sundstrom et al., 1999; Sundstrom and Engstrom, 2002; Winter et al., 1999). In the core eudicots, B class genes also have a role in specifying the sterile organs of the second floral whorl. In grasses there is no petal whorl, but a derived organ, the lodicule, is present in the same location. In order to understand the relationship of lodicules to the sterile organs of other monocot flowers, we have isolated B class genes from a basal grass without lodicules and from outgroups to the grasses, and see that B class genes are consistently expressed in stamens and the organ whorl just outside the stamens in these species. In most grasses this whorl outside the

stamens is composed of lodicules, while in the basal grass *Streptochaeta* this whorl is composed of bracts X-XII and in outgroups to the grasses like *Joinvillea* and *Chondropetalum* this whorl is the inner tepals. In *Asparagus officinalis*, another non-grass monocot with non-showy tepals, the *AP3* and *PI* orthologs are similarly expressed in stamens and inner tepals (Park et al., 2004; Park et al., 2003). Taken together, these data strongly suggest that bracts X-XII of *Streptochaeta* and lodicules of other grasses evolved by modification the inner tepal or second whorl of a typical monocot flower (Fig. 3.4).

It is important to interpret our expression results in light of the loss of function phenotype for *AP3* orthologs in the grasses. Expression of B class genes in monocot second whorl organs is not of itself sufficient evidence that these genes control their identity. However, the *si1* and *spw1* mutants clearly show that the *AP3* ortholog in grasses is necessary for lodicule identity. If B class genes were independently recruited to specify lodicule identity in the grasses, then we would not expect B class expression in the second whorl of *Streptochaeta* and the grass outgroups. Alternatively, we would have to hypothesize that B class expressing organs in *Streptochaeta* and the outgroups were lost over evolutionary time, and were replaced with lodicules in the same position and expressing the same regulatory genes. We feel this is unlikely. The much simpler hypothesis, that B class genes specify a second whorl identity in monocots and that lodicules are modified second whorl organs, is entirely consistent with the data. Further confirmation must await a B class loss of function mutant for a non-grass monocot.

B class genes and second whorl identity as opposed to petal identity

Our results suggest that B class genes may have two separable roles. The first is a role in establishing a differentiated second whorl organ identity, while the second role is to promote petaloid cell identities. B class genes are expressed in the second whorl of grasses and outgroups even though this whorl has few of the characteristics generally considered petaloid. Likewise, *Asparagus* has two whorls of sepaloid tepals, but B class genes are still expressed in the second whorl. Monocots with two whorls of sepaloid tepals could have lost this second aspect of B class function through changes in the B class genes themselves or changes in their downstream targets.

Many monocots, including lilies and tulips, have two whorls of petaloid tepals. A modified ABC model was proposed to explain the presence petaloid characteristic of the outer tepal whorl in these species (van Tunen et al., 1993). In this modified model, B class gene expression expands to include the outer tepal whorl, in addition to the inner tepal whorl and the stamens, resulting in the petal-like outer tepal whorl characteristic of liliod monocots. Northern analysis of B class gene expression in tulip agrees with the modified ABC model (Kanno et al., 2003), although it has also been reported that the protein can not be detected in the outer tepal whorl of lily even though the RNA is present (Tzeng and Yang, 2001). Unfortunately, *in situ* hybridizations on early stage floral primordia of these species have not been reported. Such data are necessary to ensure that B class genes are expressed from the inception of the outer tepals in these species. It is entirely possible that B class genes are only

expressed at later stages of outer tepal development, but this is sufficient to give petaloid characteristics. A careful examination of B class gene expression by *in situ* hybridization with diverse monocot species having two whorls of petaloid tepals (lily or tulip) would help clarify this question.

Petal evolution and B class genes

We find compelling evidence that B class genes are important for establishing second whorl organ identity in the monocots, suggesting that eudicot petals and inner tepals in the monocots inherited a common mechanism for their specification involving B class MADS-box genes. The independent evolution of petals as described by morphologists could simply be the result of this B class petal program shifting to new organ whorls (Baum and Whitlock, 1999; Kramer and Jaramillo, 2005). The data presented here demonstrate how expression of B class genes can be used in combination with morphological, ontological and genetic data to establish the identity of organs of uncertain homology. Expression data, functional genetic data (Ambrose et al., 2000; Nagasawa et al., 2003; Whipple et al., 2004), and morphological data taken together suggest that lodicules represent modified second whorl organs, likely petals or second whorl tepals. Although we present evidence here that B class genes have a conserved role in establishing second whorl identity in both grass and non-grass monocots, the fascinating question remains of how lodicules evolved their distinct morphology. It is likely that unique genetic pathways underlie the novel morphology of lodicules and

identifying these genes will provide additional information regarding the evolutionary modifications associated with lodicule evolution.

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Figures and Figure Legends

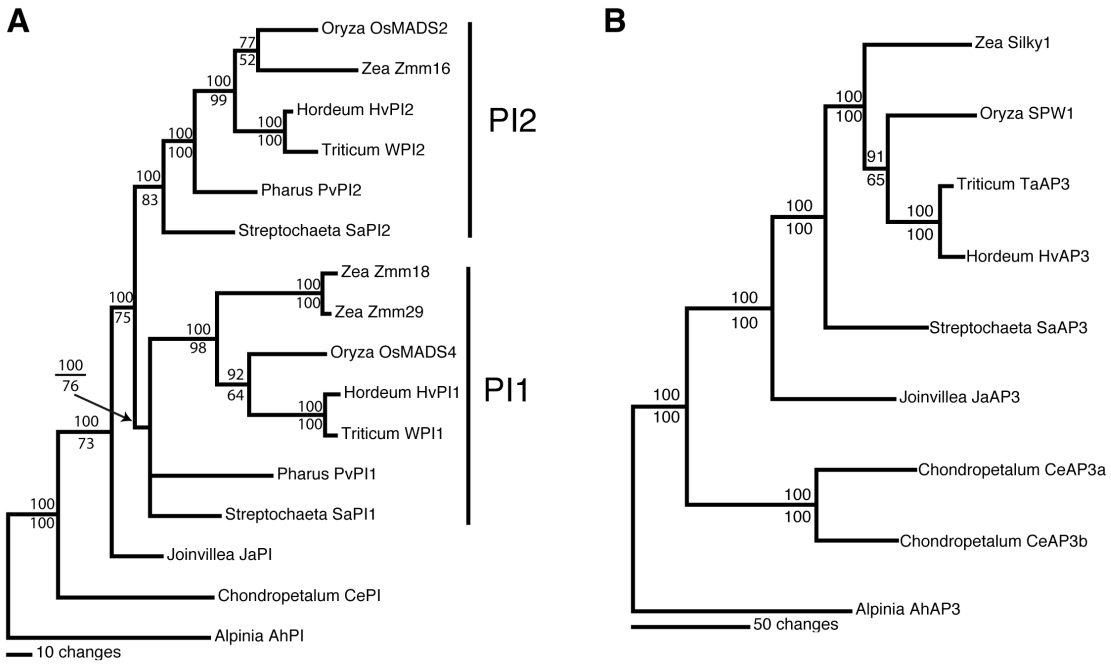

Figure 3.1 Bayesian phylogenetic analysis of B class sequences from the Poaceae and close outgroups

Trees are 50% majority rule consensus, and phylogenetic analysis was performed as described in Methods. Bayesian posterior probabilities are indicated above the branches, with Maximum Likelihood bootstrap values below. Taxa from which the genes were isolated are as follows: *HvPI1*, *HvPI2*, and *HvAP3 Hordeum vulgare* (barley), *WPI1*, *WPI2*, and *TaAP3 Triticum aestivum* (wheat), *OsMADS2*, *OsMADS4*, and *SPW Oryza sativa* (rice), *Zmm16, Zmm18, Zmm29,* and *Si1 Zea mays* (corn), *PvPI1, PvPI2,* and *PvAP3 Pharus virescens*, *SaPI1, SaPI2,* and *SaAP3 Streptochaeta angustifolia*, *JaPI* and *JaAP3 Joinvillea ascendens, CePI, CeAP3a, and CeAP3b Chondropetalum elephantinum* (cape rush), *AhPI* and *AhAP3 Alpinia hainanensis* (ginger).
A. *PI* orthologs from the grass family. Two well supported clades of grass *PI* orthologs exist, and named PI1 and PI2 as indicated.
B. *AP3* orthologs from the grass family.

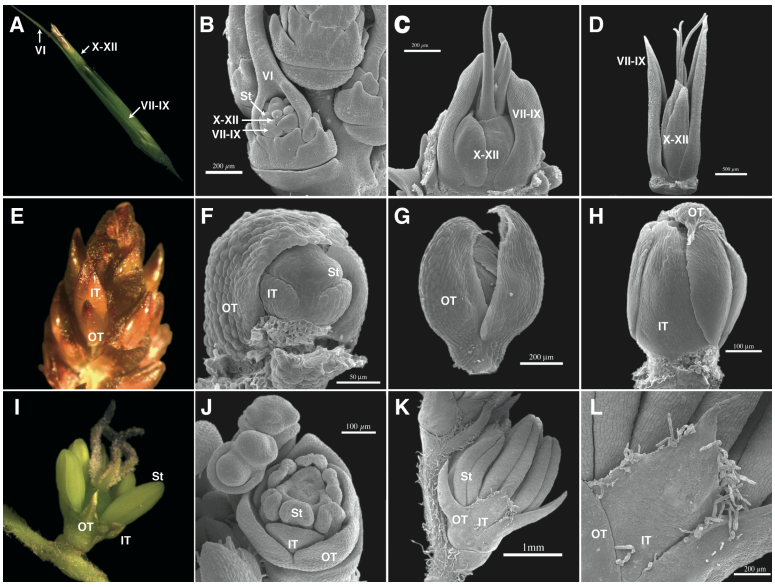

Figure 3.2 Early floral development in *Streptochaeta*, *Chondropetalum*, and *Joinvillea*

A-D. *Streptochaeta* spikelet development. (A) Mature spikelet with anthers beginning to emerge from the overlapping whorl of bracts X-XII, which are distinct in shape and size from the pointed bracts VII-IX. (B) Early floral development showing the long awned bract VI, initiation of the the 'outer tepal' bracts VII-IX, one of the 'inner tepal' bracts X-XII, as well as stamen and carpel primordia. (C) The entire spikelet was removed from the inflorescence, the large enclosing bract VI was removed, and the flower is viewed here from behind. Three stigmas are emerging from the overlapping whorl of bracts X-XII. Outside of this whorl, two of the bracts from the VII-IX whorl are developing their pointed tips, while the third has apparently aborted. (D) Later stage flower dissected from the inflorescence as in (C) showing the distinct development of the pointed 'outer tepal' whorl VII-IX and the overlapping 'inner tepal' whorl X-XII.

E-H. *Chondropetalum* floral development. (E) a mature inflorescence containing flowers subtended by bracts just before anthesis. Labeled flower has the bract removed, showing the inner tepals (it) are longer than the outer tepals (ot) and each is morphologically distinct. (F) young floral meristem with one outer tepal removed showing the initiation of inner tepal and stamen (st) primordia. (G) maturing flower showing hooded outer tepals. (H) as in (G) but with two outer tepals removed showing the inner tepals as more laminar in shape than the young outer tepals in (F) and (G).

I-L. *Joinvillea* floral development. (I) Mature flower showing the apparently similar morphology of the inner and outer tepals. (J) Early floral development clearly showing characteristic monocot floral morphology. (K) Later developmental stage showing distinct shape of the inner and outer tepal whorls. (L) Close view of the inner tepal in (K) showing flat broad triangular shape with a papillate margin as opposed to the narrowly pointed, curved outer tepal with a smoother margin.

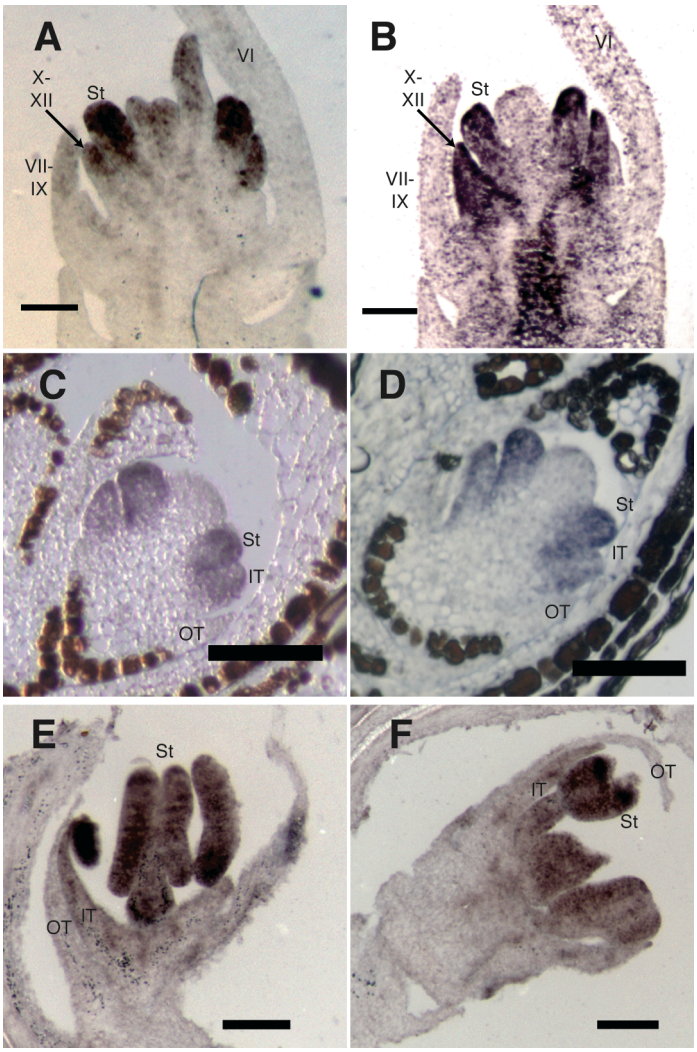

Figure 3.3 *In situ* RNA hybridization of B class genes in *Strepotochaeta*, *Chondropetalum*, and *Joinvillea*
A. *SaAP3* is strongly expressed in the developing stamens (st) and the whorl just outside the stamens containing bracts X-XII. Weaker expression is evident in the carpel and possibly the VII-VIII whorl.
B. *SaPI2* is strongly in the stamens whorl and the X-XII whorl as is *SaAP3*.
C. *CeAP3a* is strongly expressed in the emerging stamens and inner tepals (it), but absent from the outer tepals (ot).
D. *CeAP3b* expression is very similar to *CeAP3a*: strong in stamens and inner tepals, but absent from outer tepals.
E. *JaAP3* expression can be seen in the stamens and inner tepal, but the adjacent outer tepal shows little *JaAP3* expression.
F. *JaPI* expression, like *JaAP3* is seen in the stamens and inner tepals, but apparently absent from the outer tepals. Scale bars in A-F represent 100μm.

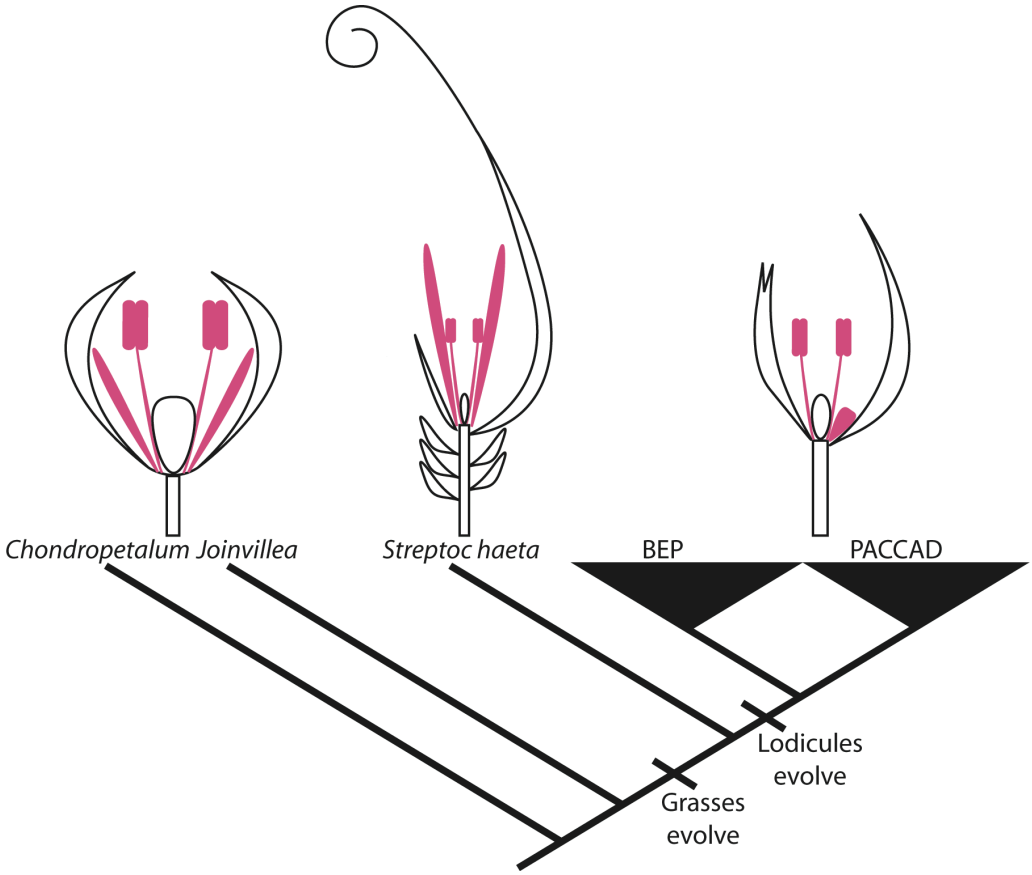

Figure 3.4 Evolution of lodicules as indicated by B class gene expression

A schematic of the grass family phylogeny with the position of the outgroups examined in this study as described by the Grass Phylogeny Working Group (GPWG). Lodicules evolve in the grasses after the divergence of the basal grass *Streptochaeta*. B-class genes are consistently expressed in stamens and the organ whorl just outside the stamens. This whorl just outside the stamens comprises the inner tepals of *Chondropetalum* and *Joinvillea*, the bracts X-II of *Streptochaeta* , and the lodicules of the grasses. Both position and B-class gene expression indicate lodicules are modifications of the inner tepals, with bracts X-XII of *Streptochaeta* being an intermediate step in this process.

References

Albert, V.A., Gustafsson, M.H.G. and Di Laurenzio, L. (1998). Ontogenetic systematics, molecular developmental genetics, and the angiosperm petal. *In* "Molecular Systematics of Plants II" (D. E. Soltis, P. S. Soltis and J. J. Doyle, eds.), pp. 349-373. Kluwer. New York.

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F. and Schmidt, R.J. (2000). Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. *Molecular Cell*. **5**, 569-579.

Arber, A. (1929). Studies in the Gramineae. VI. 1. Streptochaeta. 2. Anomochloa. 3. Ichnanthus. *Annals of Botany*. **43**, 35-53.

Baum, D.A. and Whitlock, B.A. (1999). Plant development: Genetic clues to petal evolution. *Curr Biol*. **9**, R525-527.

Chung, Y.-Y., Kim, S.-R., Kang, H.-G., Noh, Y.-S., Chul, P.M., Finkela, D. and An, G. (1995). Characterization of two rice MADS box genes homologous to GLOBOSA. *Plant Science*. **109**, 45-56.

Clifford, H.T. (1987). Spikelet and Floral Morphology. *In* "Grass Systematics and Evolution" (T. R. Soderstrom, K. W. Hilu, C. S. Campbell and M. E. Barkworth, eds.), pp. 21-30. Smithsonian Institution Press. Washington D. C.

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

Decraene, L.P.R., Linder, H.P. and Smets, E.F. (2002). Ontogeny and evolution of the flowers of South African Resionaceae with special emphasis on the gynoecium. *Plant Systematics and Evolution*. **231**, 225-258.

Fukui, M., Futamura, N., Mukai, Y., Wang, Y., Nagao, A. and Shinohara, K. (2001). Ancestral MADS box genes in Sugi, Cryptomeria japonica D. Don (Taxodiaceae), homologous to the B function genes in angiosperms. *Plant Cell Physiol*. **42**, 566-575.

Goto, K. and Meyerowitz, E.M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes Dev*. **8**, 1548-1560.

Goto, K. and Meyerowitz, E.M. (1994). Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. *Genes Dev*. **8**, 1548-1560.

Group, G.P.W. (2001). Phylogeny and subfamilial classification of the grasses (Poaceae). *Ann Missouri Botanical Garden*. **88**, 373-457.

Irish, V.F. (2000). Variations on a theme: flower development and evolution. *Genome Biol*. **1**, REVIEWS 1015.1-1015.4.

Irish, V.F. (2003). The evolution of floral homeotic gene function. *BioEssays*. **25**, 637-646.

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

Judziewicz, E.J. and Soderstrom, T.R. (1989). Morphological, anatomical, and taxonomic studies in *Anomochloa* and *Streptochaeta* (Poaceae: Bambusoideae). *Smithsonian Contributions to Botany*. **68**, 1-52.

Kanno, A., Saeki, H., Kameya, T., Saedler, H. and Theissen, G. (2003). Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (Tulipa gesneriana). *Plant Molecular Biology*. **52**, 831-841.

Kellogg, E.A. (2001). Evolutionary history of the grasses. *Plant Physiol*. **125**, 1198-1205.

Kramer, E.M., Dorit, R.L. and Irish, V.F. (1998). Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. *Genetics*. **149**, 765-783.

Kramer, E.M. and Irish, V.F. (1999). Evolution of genetic mechanisms controlling petal development. *Nature*. **399**, 144-148.

Kramer, E.M. and Irish, V.F. (2000). Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *Int J Plant Sci*. **161**, S29-S40.

Kramer, E.M. and Jaramillo, M.A. (2005). Genetic basis for innovations in floral organ identity. *J Exp Zoolog B*. **304**, 526-535.

Lamb, R.S. and Irish, V.F. (2003). Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. *Proc Natl Acad Sci U S A*. **100**, 6558-6563.

Long, J.A., Moan, E.I., Medford, J.I. and Barton, M.K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature*. **379**, 66-69.

Moon, Y.H., Jung, J.Y., Kang, H.G. and An, G. (1999). Identification of a rice APETALA3 homologue by yeast two-hybrid screening. *Plant Mol Biol*. **40**, 167-177.

Mouradov, A., Hamdorf, B., Teasdale, R.D., Kim, J.T., Winter, K.U. and Theissen, G. (1999). A DEF/GLO-like MADS-box gene from a gymnosperm: Pinus radiata contains an ortholog of angiosperm B class floral homeotic genes. *Dev Genet*. **25**, 245-252.

Münster, T., Wingen, L.U., Faigl, W., Werth, S., Saedler, H. and Theissen, G. (2001). Characterization of three GLOBOSA-like MADS-box genes from maize: evidence for ancient paralogy in one class of floral homeotic B-function genes of grasses. *Gene*. **262**, 1-13.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H. and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. *Development*. **130**, 705-718.

Page, V.M. (1951). Morphology of the Spikelet of Streptochaeta. *Bull Torrey Botanical Club*. **78**, 22-37.

Park, J.H., Ishikawa, Y., Ochiai, T., Kanno, A. and Kameya, T. (2004). Two GLOBOSA-like genes are expressed in second and third whorls of homochlamydeous flowers in Asparagus officinalis L. *Plant Cell Physiol*. **45**, 325-332.

Park, J.H., Ishikawa, Y., Yoshida, R., Kanno, A. and Kameya, T. (2003). Expression of AODEF, a B-functional MADS-box gene, in stamens and inner tepals of the dioecious species Asparagus officinalis L. *Plant Molecular Biology*. **51**, 867-875.

Paterson, A.H., Bowers, J.E. and Chapman, B.A. (2004). Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. *Proceedings of the Nationall Academy of Sciences of the United States of America*. **101**, 9903-9908.

Piper, C.V. (1906). The terminology of the parts of the Grass spikelet. *Science*, **23**, 789-790.

Rudall, P.J. and Bateman, R.S. (2004). Evolution of zygomorphy in monocot flowers: iterative patterns and developmental constraints. *New Phytologist*. **162**, 25-44.

Schwarz-Sommer, Z., Hue, I., Huijser, P., Flor, P.J., Hansen, R., Tetens, F., Lonnig, W.E., Saedler, H. and Sommer, H. (1992). Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. *EMBO J*. **11**, 251-263.

Soltis, D.E., Soltis, P.S., Albert, V.A., Oppenheimer, D.G., dePamphilis, C.W., Ma, H., Frohlich, M.W. and Theissen, G. (2002). Missing links: the genetic architecture of flowers [correction of flower] and floral diversification. *Trends Plant Sci*. **7**, 22-31; dicussion 31-24.

Sommer, H., Beltran, J.P., Huijser, P., Pape, H., Lonnig, W.E., Saedler, H. and Schwarz-Sommer, Z. (1990). Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. *EMBO J*. **9**, 605-613.

Stellari, G.M., Jaramillo, M.A. and Kramer, E.M. (2004). Evolution of the APETALA3 and PISTILLATA lineages of MADS-box-containing genes in the basal angiosperms. *Mol Biol Evol*. **21**, 506-519.

Sundstrom, J., Carlsbecker, A., Svensson, M.E., Svenson, M., Johanson, U., Theissen, G. and Engstrom, P. (1999). MADS-box genes active in developing pollen cones of Norway spruce (Picea abies) are homologous to the B-class floral homeotic genes in angiosperms. *Dev Genet*. **25**, 253-266.

Sundstrom, J. and Engstrom, P. (2002). Conifer reproductive development involves B-type MADS-box genes with distinct and different activities in male organ primordia. *Plant J*. **31**, 161-169.

Takhtajan, A. (1991). "Evolutionary Trends in Flowering Plants". Columbia University Press, New York.

Trobner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P., Lonnig, W.E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1992). GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. *EMBO J*. **11**, 4693-4704.

Tzeng, T.Y. and Yang, C.H. (2001). A MADS box gene from lily (Lilium Longiflorum) is sufficient to generate dominant negative mutation by interacting with PISTILLATA (PI) in Arabidopsis thaliana. *Plant Cell Physiol*. **42**, 1156-1168.

van Tunen, A.J., Eikelboom, W. and Angenent, G.C. (1993). Floral organogenesis in *Tulipa*. *Flowering Newsletter*. **16**, 33-37.

Weigel, D. and Meyerowitz, E.M. (1994). The ABCs of floral homeotic genes. *Cell*. **78**, 203-209.

Whipple, C.J., Ciceri, P., Padilla, C.M., Ambrose, B.A., Bandong, S.L. and Schmidt, R.J. (2004). Conservation of B-class floral homeotic gene function between maize and Arabidopsis. *Development*. **131**, 6083-6091.

Winter, K.U., Becker, A., Münster, T., Kim, J.T., Saedler, H. and Theissen, G. (1999). MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. *Proc Natl Acad Sci U S A*. **96**, 7342-7347.

Zanis, M.J., Soltis, P.S., Qiu, Y.L., Zimmer, E. and Soltis, D.E. (2003). Phylogenetic Analyses and perianth evolution in basal angiosperms. *Ann Missouri Botanical Garden*. **90**, 129-150.

CHAPTER IV

Protein subfunctionalization and the maize C class genes *Zag1*, *Zmm2* and *Zmm23*

Abstract

The ABC model of floral development holds that C class function is required to specify stamen and carpel identity. In addition, C class function promotes determinacy of the floral meristem. Two maize C class genes, *Zag1* and *Zmm2* have been reported, but a loss of function mutant has only been described for *Zag1*. These *zag1* mutants show a partial loss of floral meristem determinacy and disrupted carpel development, but no clear loss of reproductive organ identity. Expression differences between *Zag1* and *Zmm2* led to the hypothesis that they are partially redundant and that C class function is partitioned in maize, with *Zag1* playing a more important role in carpel development and *Zmm2* playing a more prominent role in stamen development. Here we describe rescue of *Arabidopsis ag* mutants with the maize *Zag1* and *Zmm2*, and show that their protein functions have diverged following the gene duplication event that created these parologous lineages. Mutant alleles were isolated for *Zmm2* and its duplicate *Zmm23*. Although these alleles produce no phenotype on their own or together, they do significantly enhance the *zag1* phenotype. The *zag1 zmm2 zmm23* triple mutant provides insight into the maize C class function and the consequences of gene duplication in a crucial floral homeotic gene.

Introduction

The angiosperm flower is a determinate structure that consists of four distinct organ types occurring in concentric whorls. The outermost whorl is composed of sepals, followed by the petals, then stamens, and finally a central carpel (or carpels)

terminates the floral axis. Each organ represents a modified leaf, and the identity of each floral organ type is determined by the expression, either alone or in combination, of three distinct classes of genes: A, B, and C (Weigel and Meyerowitz, 1994). This ABC model of flower development was determined by genetic studies in the model species *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz, 1991). Genes in each class function in two adjacent whorls, with A class function active in whorls one and two, B class function in whorls two and three, and C class in whorls three and four. Among the first of the ABC genes to be cloned was the C class gene *AGAMOUS* from *Arabidopsis*, whose product was shown to belong to a conserved group of DNA-binding proteins termed MADS-box proteins, including members from both yeast and mammals (Yanofsky et al., 1990). According to the ABC model, C class function is necessary for the identity of the reproductive organs in whorls three and four. C class function alone is necessary for carpel development in whorl four, while C and B class gene functions combine to provide stamen identity in whorl three. The model also proposes that A and C class functions are mutually exclusive and that C activity is required for determinacy of the floral meristem. Consequently, in the C class mutant *agamous*, A class function replaces C in whorls three and four, leading to a flower that reiterates a pattern of sepals, petals, petals, new flower (composed of sepals, petals, petals, new flower) (Bowman et al., 1989).

Since the initial cloning and description of the C class genes *AG* in *Arabidopsis* and *PLENA* (*PLE*) in *Antirrhinum* (Bradley et al., 1993), C class homologs have been isolated from a diverse group of angiosperms and gymnosperms

(Kramer et al., 2004). In all cases where it has been analyzed, C class gene expression is correlated with the reproductive structures of both angiosperm and gymnosperm species (Bradley et al., 1993; Kapoor et al., 2002; Kyozuka and Shimamoto, 2002; Pnueli et al., 1994; Rutledge et al., 1998; Schmidt et al., 1993; Tandre et al., 1998; Yu et al., 1999). However, due to the difficulty of working with non-model species, there is little evidence for C class function outside of the core eudicots. An exception is the model grass species maize (*Zea mays*) and rice (*Oryza sativa*). Initially, a single maize C class gene *ZeaAgamous1* (*Zag1*) was isolated from maize, and shown to be expressed early throughout the floral meristem and later in both stamen and carpels (Schmidt et al., 1993). Isolation of a *Mutator* transposon insertion in *Zag1* resulted in a partial C class phenotype (Mena et al., 1996). These *zag1-mum1* plants had a loss of floral meristem determinacy, most prominently in the ear (female florets) where the carpel was replaced by an indeterminate reiteration of carpel-like structures. The male florets of the *zag1-mum1* tassel showed no effect on stamen identity, although careful examination of the aborting carpel by SEM revealed a loss of determinacy (Ambrose, 2000). Consequently the maize C class gene *Zag1* has a role in floral meristem determinacy, but no clear, non-redundant role in organ identity. Subsequently, another maize C class gene *ZeaMaysMADS2* (*Zmm2*) was isolated (Mena et al., 1996; Theissen et al., 1995). Expression analysis of *Zag1* and *Zmm2* showed that they had overlapping but non-identical expression domains (Mena et al., 1996). Specifically, *Zag1* was strongly expressed in the ear and carpels, but weakly in the tassel and ear, while *Zmm2* was strongly expressed in the tassel and stamens, but weakly in the ear

and carpels. This expression difference suggested that the partial C class phenotype of *zag1-mum1* was due to partial redundancy with *Zmm2* and led to the hypothesis that the C class of maize has been partitioned with *Zag1* playing a more important role in carpel development, and *Zmm2* a more important role in stamen development.

Further confirmation of the partitioning of C class function has come from analysis of the rice C class genes. Like maize, rice has two C class genes *OsMADS3* and *OsMADS58,* which are orthologous to the *Zmm2* and *Zag1* respectively (Yamaguchi et al., 2006). Insertions in *OsMADS3* result in stamens transformed into lodicules, and production of extra carpels indicating a weak loss of floral determinacy, while RNAi of *OsMADS58* results in a strong loss of floral meristem determinacy and disruption of carpel development. Thus it appears that, as was proposed for the corresponding maize genes, *OsMADS3* is more important for stamen identity and *OsMADS58* is more important for carpel identity and floral meristem determinacy, although both genes have partially overlapping functions. The double mutant created by RNAi silencing of *OsMADS58* in the hypomorphic *osmads3-2* allele results in a mutant flower very similar to C class mutants described in eudicots, suggesting that the C class roles of controlling reproductive organ identity and floral meristem determinacy defined in the eudicots is conserved in the monocots.

These two orthologous C class genes in rice and maize appear to be the result of a duplication event in the lineage leading to the grass family (Paterson et al., 2004), as all other monocot C class genes are sister to the *Zag1*/*OsMADS58* and *Zmm2*/*OsMADS3* clade (Kramer et al., 2004). Gene duplication events create two

identical copies that should theoretically be entirely redundant, at least initially. Two processes, neofunctionalization and subfunctionalization, have been proposed to explain how these duplicate copies could be maintained (Force et al., 1999; Ohno, 1970). By neofunctionalization, one of the gene copies mutates to obtain a novel function that provides a selective advantage. This is likely to be a rare occurrence since most mutational events are deleterious. In order to explain the prevalence of duplicate gene copies present in genomes, Force et al. (1999) proposed subfunctionalization. In this process, both gene copies mutate to loose part of their function, however each individual copy looses a distinct aspect of the ancestral gene function. Consequently, both gene copies are compromised in different ways, but together they recapitulate the ancestral function and thus both will be maintained. In this scenario, it was proposed that the most likely mutations would be in the cis-regulatory regions creating an expression subfunctionalization. Indeed this is exactly what is seen for the expression of the maize C class duplicates *Zag1* and *Zmm2*. Interestingly, constitutive expression of *Zag1* and *Zmm2* in *Arabidopsis* results in distinct phenotypes, with *Zag1* capable of promoting ectopic carpel development but not ectopic stamens, and just the converse true for *Zmm2* (Ambrose, 2000). Since these overexpression studies used the viral 35S promoter, the differential function should be due to amino acid differences in the proteins themselves. Thus *Zag1* and *Zmm2* appear to have subfunctionalized at the level of protein function in addition to expression.

In order to more fully characterize the maize C class function, we have undertaken a further functional analysis of the maize C class genes. Here we describe the isolation of a third maize C class gene, *Zmm23*, which is itself a recent duplicate of *Zmm2*. We also confirm the protein subfunctionalization of ZAG1 and ZMM2 by rescuing the *Arabidopsis ag* mutant with the maize genes using the native *Arabidopsis AG* regulatory sequence. Finally we describe the isolation of a *Mutator* insertion in *Zmm2* that, in combination with a naturally occurring polymorphism in *Zmm23,* enhances the previously described *zag1* phenotype.

Results

Isolation of *Zmm23*, a duplicate copy of *Zmm2*

Southern hybridizations to maize genomic DNA using the *Zmm2* cDNA clone (Mena et al., 1996; Theissen et al., 1995) consistently showed an additional weakly hybridizing band (Fig. 4.1A). This suggested the presence of a third maize C class gene. In order to clone this gene, a maize genomic BAC library was screened with a *Zmm2* probe. From these BACs the genomic sequence for the *Zmm2* duplicate was obtained, allowing isolation of a complete cDNA, which was independently isolated and mapped by another group as *Zmm23* (Münster et al., 2002). The predicted protein of *Zmm23* was found to be very similar to ZMM2 (88% identical), although the original *Zmm23* cDNAs isolated had a 4 bp insertion near the C-terminus that created a frame shift disrupting a highly conserved motif found in C class genes as distant as the gymnosperms (Fig. 4.1D and E). Sequence of *Zmm23* from several maize inbreds

showed that this 4 bp insertion was a naturally occurring polymorphism present in some but not all inbreds. For example, *Zmm23* from the OH43 inbred contained the insertion and this allele was designated *zmm23-OH43*. Northern expression analysis of *Zmm23* showed a similar pattern similar to that described for *Zmm2*, with high expression in stamens and tassels, and comparatively weak expression in carpels and ears (Fig. 4.1B).

Rescue of *Arabidopsis ag* mutants by the maize C class genes *Zag1* and *Zmm2*

Zag1 and *Zmm2* were previously shown to have overlapping, yet distinct expression patterns indicating subfunctionalization had occurred, with *Zag1* expression maintained primarily in the developing carpel, and *Zmm2* maintained in the stamens (Mena et al., 1996). Overexpression of these maize genes in *Arabidopsis* produced results consistent with the expression subfunctionalization, and further suggested that the protein products themselves had diverged such that ZAG1 was better able to promote carpel identity, and ZMM2 was better able to promote stamen identity (Ambrose, 2000). In order to further verify these overexpression results, we created constructs in which the maize cDNAs were fused to the regulatory sequences of the *Arabidopsis AG* gene. Since it is known that sequences in the second intron are crucial for the proper regulation of *AG* (Busch et al., 1999; Deyholos and Sieburth, 2000; Hong et al., 2003; Sieburth and Meyerowitz, 1997), the rescue constructs were created to contain 6 kb of 5' promoter region, the first two exons and first two introns of *AG*, which was then fused to the corresponding 3' region of both *Zag1* and *Zmm2*

cDNAs (Fig. 4.2A). MADS box proteins are composed of five distinct domains NMIKC: N-domain, for a short N-terminal sequence that is often absent, M-domain for the MADS-box, important for DNA binding, I-domain is an intervening sequence between the MADS-box and the K-domain, a keratin-like coiled coil domain thought to mediate protein-protein interactions, and finally the C- domain for a variable region at the C-terminus that may have a role in mediating higher order complex formation. Our constructs were created to produce a protein containing the *Arabidopsis* N and M domains of AG, and the I, K and C domains of ZAG1 and ZMM2. It was reasoned that since MADS domains are nearly identical between the maize and *Arabidopsis* proteins (compared to the 56 a.a. AG MADS domain, ZAG has 2 substitutions, and ZMM2 has one), and the N domain is not considered to have a crucial function (Mizukami et al., 1996), that the AG:ZAG1 and AG:ZMM2 constructs would behave functionally like the maize proteins. Because *Zmm2* and *Zmm23* were so similar, and their expression profiles were indistinguishable, we felt that analysis of either gene would provide identical results. Consequently, we compared only the functions of ZAG1 and ZMM2 in this manner.

The constructs were transformed into wt *Arabidopsis* and then individual T1 transformant lines were crossed to plants heterozygous for the *ag-2* allele, (a strong *ag* allele due to a T-DNA insertion (Yanofsky et al., 1990)). Progeny from these crosses containing the transgene and heterozygous for *ag-2* were selfed, and the F2 segregating families were analyzed. For both *AG:Zag1* and *AG:Zmm2* rescued *ag-2* plants there was a range of phenotypes depending on the line. Some lines showed no

apparent rescue and were indistinguishable from *ag*, while others showed complete rescue of both stamen and carpel development (Fig. 4.2D and F). Both strong rescue and lack of rescue were seen in both the *AG*:*Zag1* and *AG*:*Zmm2* lines. However, there were multiple intermediate lines where *AG*:*Zag1* and *AG*:*Zmm2* had consistently different phenotypes. The intermediate *AG*:*Zag1* lines showed a partial to strong rescue of carpel identity and meristem determinacy, but little to no rescue of stamen identity (Fig. 4.2E). While the intermediate *AG*:*Zmm2* lines showed a strong rescue of stamen identity and little to no rescue of carpel identity or floral meristem determinacy (Fig. 4.2G).

The strongest rescuing lines show that both *Zag1* and *Zmm2* are capable of promoting stamen and carpel development. However, the intermediate lines suggest that in some cases *Zag1* does better at promoting carpel identity, and *Zmm2* does better at promoting stamen identity. A likely explanation for the difference seen between the strongest lines and intermediate lines is that the intermediate lines have weaker expression of the transgenes, and consequently even though ZAG1 and ZMM2 are both partially compromised in promoting stamen and carpel development respectively, they can each do both when expressed at high enough levels.

Mutator insertion allele of *Zmm2*

Considering the partial C class phenotype displayed by *zag1* mutants, and the apparent protein and expression subfunctionalization of *Zag1* and *Zmm2*, we were interested in functionally characterizing *Zmm2*. A previous screen for *Mu* transposon insertions in

Zmm2 had produced numerous intronic insertions, one of which (*zmm2-mum4*) had an effect on *Zmm2* transcript levels (Ambrose, 2000). However, these plants had no phenotype, and the transcript reduction was variable among homozygous *zmm2-mum4* lines. Further screening produced an exonic insertion in the MADS-box of *Zmm2*, and was designated *zmm2-mum5* (Fig. 4.3B). Plants homozygous for the *zmm2-mum5* insertion had a severe and consistent reduction in transcript levels as indicated by a northern (Fig. 4.3A). The residual hybridization seen in these *zmm2-mum5* plants could be due to presence of the very similar *Zmm23* transcript. In order to determine if the *zmm2-mum5* insertion produced an RNA null, we performed RT PCR with primers specific to *Zmm2*, 3' of the *Mu* insertion (Fig. 4.3C). These results showed that, even in homozygous *zmm2-mum5* lines some full-length *Zmm2* transcript was still being produced, presumably by somatic excision of the *Mu* transposon (Levy et al., 1989). *zmm2-mum5* mutant plants had no discernable phenotype. This could be due to presence of the putatively redundant *Zmm23* gene, which was sequenced from these lines and did not contain the 4 bp insertion found in some inbreds (described above). We created a *zmm2-mum5 zmm23-OH43* double mutant by crossing to the OH43 inbred, which contains the 4 bp insertion in *Zmm23*. F2 progeny homozygous *zmm2-mum5* and *zmm23-OH43* were indistinguishable from wild type. Considering the dramatic phenotype seen in the rice *osmads3* mutant, we expect a phenotype when the maize *OsMADS3* orthologs, *Zmm2* and *Zmm23* are functionally disrupted. While it is likely that the *zmm2-mum5* allele is sufficient to disrupt *Zmm2* function, it is

possible that the *zmm23-OH43* allele has no significant effect, or that any mild effect is redundant with *Zag1*.

zmm2-mum5 and *zmm23-OH43* enhance the *zag1-mum1* phenotype

Although *zmm2-mum5* and *zmm23-OH43* had no effect on flower development on their own or together, it was still possible that they could enhance the *zag1* phenotype. Consequently we created triple mutants by crossing *zag1-mum1* mutants with the *zmm2-mum5 zmm23-OH43* double mutants and screening for individuals homozygous for *zag1-mum1*, *zmm2-mum5*, and *zmm23-OH43* in the F2 generation. The triple mutants isolated showed a dramatic enhancement of the *zag1* phenotype (Fig. 4.3D-L). In the ear, carpel identity was severely affected, and bract-like, or bract-carpel fusion organs grew indeterminately in the place of carpels (Fig. 4.3J). Often, after producing a number of bract and/or bract-carpel like organs, the floral meristem would revert to an indeterminate inflorescence meristem identity (Fig. 4.3K), indicating that floral meristem identity was completely lost. A similar loss of floral meristem identity was also seen in the tassel florets (Fig. 4.3F), additionally, some florets showed a partial loss of stamen identity, with lodicules or stamen-lodicule chimeras growing in the place of some stamens (Fig. 4.3G and H). It is important to note that only a small number of florets showed this loss of stamen identity. Additionally, some triple mutants had a less severe loss of carpel identity and floral meristem determinacy. The reason for this variation in the phenotype is not entirely clear. One possibility is that the *zmm2-mum5* and/or *zag1-mum1* are suppressible by *Mu* activity, as has been

described for other *Mu*-tagged genes (Martienssen and Baron, 1994). Another possibility is that the triple mutants were segregating other loci with an effect on C class function. While there was variability in the triple mutant phenotype, all triple mutants analyzed (>15) showed at least some enhancement of the *zag1-mum1* phenotype.

Discussion

Our examination of the maize C class genes *Zag1*, *Zmm2* and *Zmm23* further confirms the subfunctionalization of maize C class activity proposed earlier (Mena et al., 1996) and also seen in rice (Yamaguchi et al., 2006). Here we show that *Zmm23*, a recent duplicate of *Zmm2*, is expressed in a similar manner to *Zmm2*, suggesting functional redundancy. Rescue of the *Arabidopsis ag* mutant with *Zag1* and *Zmm2*, shows that their protein functions, in addition to their expression domains, have subfunctionalized. Attempts to determine *Zmm2* and *Zmm23* function were not entirely successful. While an insertion in *Zmm2* severely affected its expression, these plants had no phenotype, nor did a double mutant with a naturally occurring insertion in *Zmm23*. As the *zmm23-OH43* insertion created a frame shift that only altered the sequence of the last 10 a.a., it may still be functional. Considering the dramatic phenotype of the rice *osmads3* mutant, it is expected that loss of *Zmm2* and *Zmm23* function would have an obvious effect on maize floral development. Seeing this phenotype may require the creation of a double mutant with a more severe *zmm23* mutant allele. In spite of this lack of phenotype for *zmm2-mum5* and *zmm23-OH43*,

these alleles did significantly enhance the *zag1-mum1* mutant phenotype, providing direct evidence that *Zmm2* and *Zmm23* have a role in the maize C class function.

While there is good reason to believe that the *zag1 zmm2 zmm23* triple mutant phenotype described here does not represent a complete loss of the maize C class function, this "partial" C class phenotype does provide insight into how C class function is conserved between the eudicots and the grasses. As is seen for all other eudicot C class mutants, loss of maize C class function leads to loss of reproductive organ identity, and a loss of determinacy in the floral meristem suggesting that these aspects of C class function were present in the common ancestor of monocots and eudicots. A potential difference is the loss of floral meristem identity seen in the maize triple mutant, whereas *ag* and *ple* simply show a loss of floral meristem determinacy. However, under certain circumstances (e.g. in short day conditions or in a *leafy*/+ background), *ag* mutants can loose floral meristem identity (Okamuro et al., 1996). Additionally, silencing of the *Petunia* C class gene *pMADS3* results in loss of floral meristem identity (Kapoor et al., 2002). Consequently C class genes play a clear role in floral meristem identity in maize and at least one eudicot, but in *Arabidopsis* that role can be masked by redundancy with other factors.

The subfunctionalization observed between *Zag1* and *Zmm2* is particularly interesting in light of recent work on the C class genes of the core eudicots. While the ABC model holds that a single gene controls C class activity in both *Arabidopsis* (*AG*) and *Antirrhinum* (*PLE*), these genes are not actually orthologous (Causier et al., 2005). In fact, a duplication event in the eudicot C class lineage created two eudicot "C" class

lineages: the *AG/FAR* lineage and the *PLE/SHP* lineage. In *Arabidopsis AG* controls C class activity, while the redundant *SHP1* and *SHP2* play a specialized role in fruit development (Liljegren et al., 2000). In *Antirrhinum* it is *PLE* that controls C class activity while the parologous *FAR* has a specialized role in stamen development (Davies et al., 1999). Interestingly, the main C class activity was delegated to paralogous lineages in *Antirrhinum* vs. *Arabidopsis*. Furthermore, Causeir et al. (2005) show that both in *Arabidopsis* and *Antirrhinum* ectopic *PLE* results in ectopic carpel identity, but not stamen, while ectopic *FAR* promotes stamen identity, but not carpel.

Why would two independent duplications (*Zag1/Zmm2* and *PLE/FAR*) have subfunctionalized in a similar way? This represents an interesting case of parallel evolution at the molecular level, and suggests that certain protein functions are more easily lost than others. One possibility is that loss of protein-protein interactions could have played a role. It is known that MADS-box proteins form higher order complexes important for their function. The quartet model has been proposed to explain how a complex four ABC MADS-box genes, including at least one SEPALLATA protein, is necessary to specify each floral organ (Theissen, 2001). For example, the model proposes that an AG/AP3/PI/SEP quartet will specify stamen identity, while an AG/AG/SEP/SEP quartet would specify carpel identity. Assuming this model holds for maize and *Antirrhinum*, if *Zag1* and *PLE* lost the ability to interact with the AP3/PI dimmer, then they would naturally loose the ability to promote stamen identity. In the case of *Zmm2* and *FAR*, it is not immediately clear from the quartet model which type

of interaction loss would lead to the inability to promote carpel identity, and perhaps in this case another mechanism is involved.

Materials and Methods

Isolation of *Zmm23*

A P^{32}-labelled PCR probe of the *Zmm2* cDNA (3' of the MADS box) was used to screen maize ZMMBBb B73 BAC library, purchased from Clemson University Genomics Institute (CUGI). Positive clones were isolated, prepped and digested with restriction enzymes to reveal two separate classes of clones. These two classes represented genomic clones of *Zmm2* and *Zmm23*. Four SacI fragments of a *Zmm23* BAC clone (211N6) were isolated that hybridized to the full length *Zmm2* cDNA probe. These fragments were subcloned in pBLUESCRIPT (Stratagene), and sequenced. From this genomic sequence, PCR primers were created to amplify the *Zmm23* cDNA by RT-PCR on cDNA created from emerging tassels of the A619, W23, OH43, B73, and Mo17 inbred lines.

Arabidopsis transformation

Rescue constructs were created based on the pAG-I::GUS plasmid that contains the regulatory sequences of *AG* with the GUS reporter fused to the third exon (Sieburth and Meyerowitz, 1997). A BamHI digest removed the third exon/GUS fusion and nos 3' terminator sequence. A new nos 3' terminator sequence was cloned into the BamHI site using a BglII cohesive end at the 3' to destroy the second BamHI site, leaving

only a single BamHI site in the second intron of *AG*. This region from the BamHI site of the second intron of through the beginning of the third *AG* exon was PCR amplified as was the corresponding 3' region of the *Zag1* and *Zmm2* cDNAs, and these individual PCR products were fused in a second round PCR to create the *AG:Zag1* and *AG:Zmm2* fusions. These fusion PCRs were created with BamHI sites in order to clone back into the BamHI site of the modified pAG-I::GUS plasmid. The completed constructs were transformed into *Agrobacterium tumefaciens* and *Arabidopsis* plants were transformed by the floral dip method (Clough and Bent, 1998).

Southern blot, Northern blot and RT-PCR

Hybridizations for the Southern and Sorthern blots were performed as previously described (Mena et al., 1995). P32-labelled probes were created by PCR amplification of the IKC domains from *Zag1*, *Zmm2*, and *Zmm23*. For northern blots and RT-PCR, total RNA was isolated from emerging tassels Plant RNA-Easy mini kit (Qiagen). Northern blots were performed using IKC probes also used in southerns. For RT-PCR, cDNA was made using the SuperScript First-Strand cDNA Synthesis kit (Invitrogen). Primers specific to *Zmm2* (Zmm2a_RTF: aaatcagcgctatacctggcgg, and Zmm2a_RTR: ctggccggagggtacaatagta) and *Zmm23* (Zmm2b_RTF: aatcttcggccaacaatccatg, and Zmm2b_RTR: ggccaaatgctgctgcaaac) were then used to PCR amplify the cDNA.

Maize and *Arabidopsis* genotyping

The *zag1-mum1*, *zmm2-mum5*, and *zmm23-OH43* alleles were genotyped by Southern hybridization to identify triple mutant individuals. Genomic DNA preps were digested with HindIII, run on a 0.6% agarose gel, and blotted to a nylon membrane. This was then hybridized with a P^{32}-labelled PCR probe containing the ICK domains of the *Zag1* cDNA, revealing an ~11kb band for wt *Zag1* allele and two bands for the *zag1-mum1* allele at ~10.5kb and ~2kb. Hybridization of a P^{32}-labelled PCR product from intron I of *Zmm2* resulted in a ~10.5 kb band for the wt *Zmm2* allele and a ~12kb band for *zmm2-mum5*. An XhoI fragment from the 3' end of the *Zmm23* cDNA was P^{32}-labelled and hybridized resulting in a ~7.5 kb band for wt *Zmm23* and two bands at ~7.3 kb (weak intensity) and ~2.8 kb (strong intensity) for the *zmm23-OH43* allele. Presence of the *ag-2* allele was confirmed by an *AG* specific primer (AG-X1: ttgtgatcatccatcctccattgt), and a primer specific to the T-DNA left boarder (TLFT: gatgcactcgaaatcagccaatttagac).

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Figures and Figure Legends

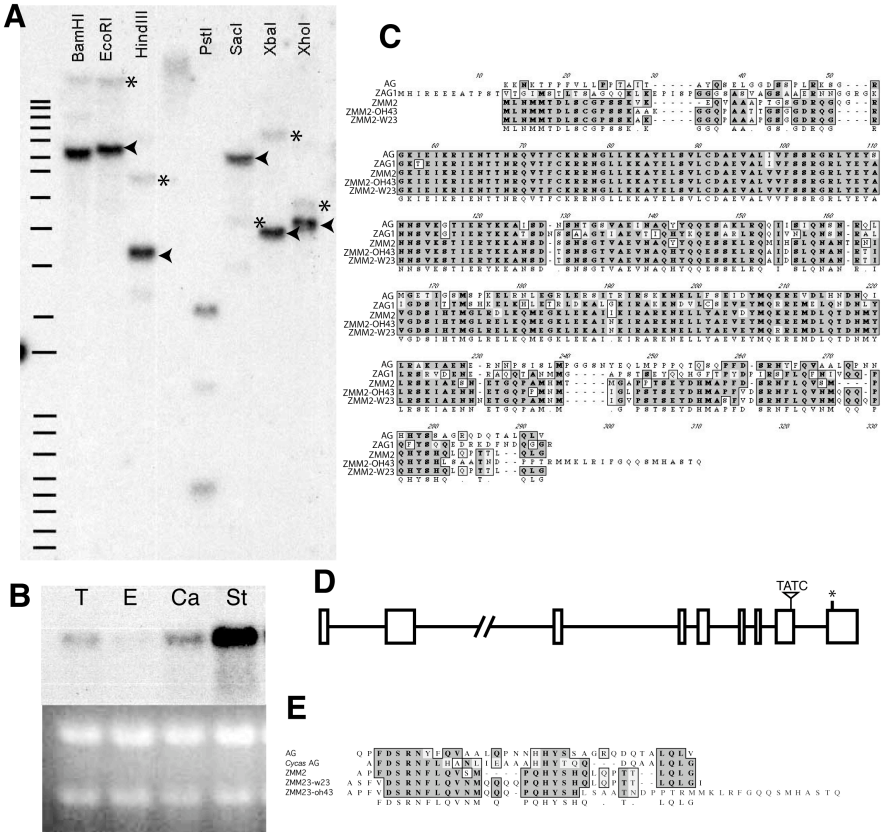

Figure 4.1 Isolation and molecular characterization of *Zmm23*

A) Southern blot probed at low stringency (55ºC), with a *Zmm2* probe shows a strongly hybridizing band (arrowheads), as well as a weakly hybridizing band (asterisk) that represents *Zmm23*.
B) Northern blot with a *Zmm23* specific probe (3' UTR), on tRNA from maize tissues. T= 2cm tassel primordia, E= 2cm ear primordia, C= immature carpel, St= immature stamens. Note that *Zmm23* is more highly expressed in tassel than ear, and its transcript more abundant in stamens than carpels.
C) ClustalW protein sequence alignment for maize C class genes and AG.
D) Gene model for *Zmm23*. Asterisk indicates the stop codon, and the position of the naturally occurring TATC insertion is indicated. This insertion was found in the A619 and OH43 inbreds, but not in B73, W23, or Mo17.
E) Alignment of the C terminal region of *Zmm23-OH43* with other C class genes, shows a highly conserved motif LQLG, is disrupted by a frame shift caused by the 4 bp insertion.

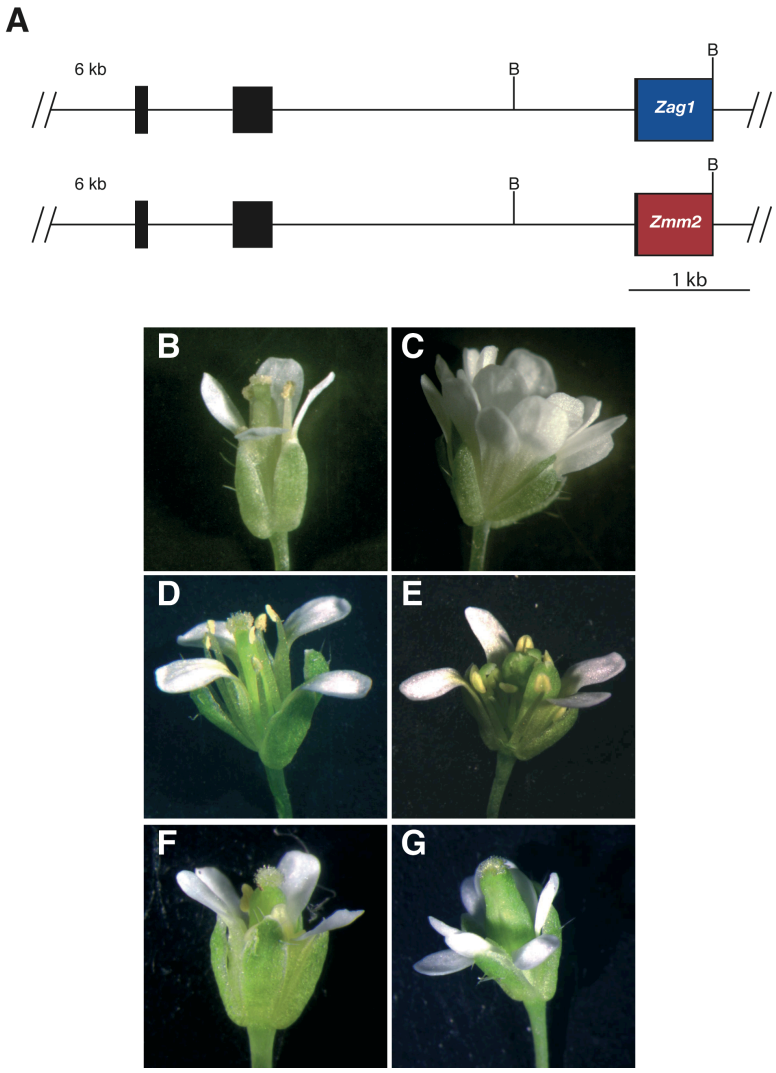

Figure 4.2 Rescue of the *Arabidopsis ag* mutant with maize C class genes

A) Constructs transformed into *Arabidopsis* and crossed to *ag* heterozygous plants, for rescue experiments. B indicates the position of BamHI site used to clone in the fusion PCR products (See Materials and Methods).
B) Wild type *Arabidopsis* flower
C) *ag* mutant flower
D) *ag* mutant strongly rescued by AG:ZMM2
E) *ag* mutant showing intermediate rescue by AG:ZMM2, note the restoration of stamen identity, but weak rescue of carpel identity.
F) *ag* mutant strongly rescued by AG:ZAG1
G) *ag* mutant with intermediate rescue by AG:ZAG1 has strong rescue of carpel identity, but no rescue of stamen identity.

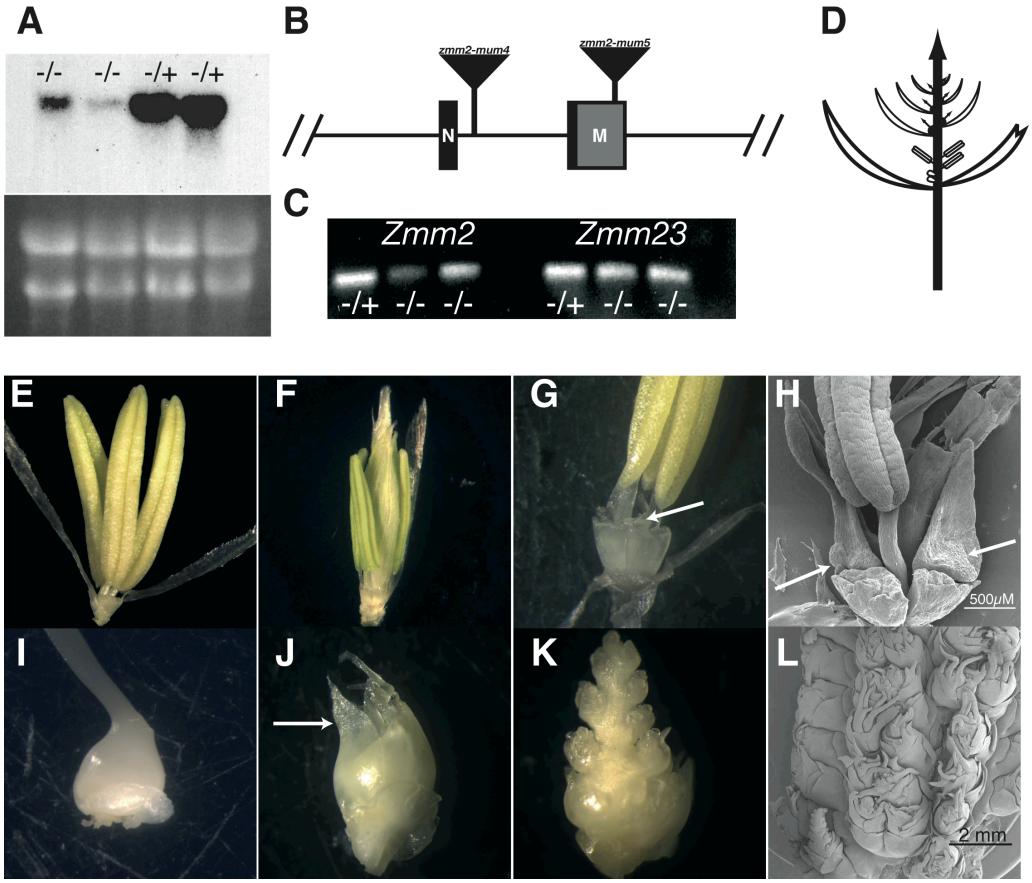

Figure 4.3 Loss of maize C class function

A) Northern blot on tRNA from emerging tassels of *zmm2-mum5* homozygous mutants (-/-) and heterozygous sibs (-/+). Residual expression could be due to cross hybridization of the *Zmm2* probe with *Zmm23*. However, RT-PCR (**C**) using cDNA made from the same individuals as in (**A**) with primers specific to *Zmm2* 3'UTR show that some *Zmm2* transcript 3' of the insertion is being produced in homozygous *zmm2-mum5* mutants RT PCR with primers specific to *Zmm23* served as a control.
B) Gene model showing the location of *Mu*-transposon insertions in *Zmm2*.
D) Floral diagram showing the progressive loss of floral meristem identity seen in both ear and tassel florets of *zag1-mum1 zmm2-mum5 zmm23-OH43* triple mutants.
E-L) Phenotype of *zag1-mum1 zmm2-mum5 zmm23-OH43* triple mutant florets. (**E**) is a wild type tassel floret. (**F**) is a mutant floret with stamen identity maintained, but a loss of floral meristem identity. (**G** and **H**) show the conversion of stamens into lodicule like structures (arrow) seen in some mutant flowers. (**I**) is a wild type ear floret. (**J**) is a mutant ear floret showing conversion of carpels to a bract-like identity. (**K** and **L**) show conversion of the floral meristem to an inflorescence identity indicating loss of floral meristem identity in many mutant ear florets.

References

Ambrose, B.A. (2000). Floral organ specification in *Zea mays*. University of California - San Diego. Ph.D. Dissertation.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1989). Genes directing flower development in Arabidopsis. *Plant Cell*. **1**, 37-52.

Bradley, D., Carpenter, R., Sommer, H., Hartley, N. and Coen, E. (1993). Complementary floral homeotic phenotypes result from opposite orientations of a transposon at the plena locus of Antirrhinum. *Cell*. **72**, 85-95.

Busch, M.A., Bomblies, K. and Weigel, D. (1999). Activation of a floral homeotic gene in Arabidopsis. *Science*. **285**, 585-587.

Causier, B., Castillo, R., Zhou, J., Ingram, R., Xue, Y., Schwarz-Sommer, Z. and Davies, B. (2005). Evolution in action: following function in duplicated floral homeotic genes. *Curr Biol*. **15**, 1508-1512.

Clough, S.J. and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J*. **16**, 735-743.

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

Davies, B., Motte, P., Keck, E., Saedler, H., Sommer, H. and Schwarz-Sommer, Z. (1999). PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. *EMBO J*. **18**, 4023-4034.

Deyholos, M.K. and Sieburth, L.E. (2000). Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. *Plant Cell*. **12**, 1799-1810.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L. and Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics*. **151**, 1531-1545.

Hong, R.L., Hamaguchi, L., Busch, M.A. and Weigel, D. (2003). Regulatory elements of the floral homeotic gene AGAMOUS identified by phylogenetic footprinting and shadowing. *Plant Cell*. **15**, 1296-1309.

Kapoor, M., Tsuda, S., Tanaka, Y., Mayama, T., Okuyama, Y., Tsuchimoto, S. and Takatsuji, H. (2002). Role of petunia pMADS3 in determination of floral organ and meristem identity, as revealed by its loss of function. *Plant J.* **32**, 115-127.

Kramer, E.M., Jaramillo, M.A. and Di Stilio, V.S. (2004). Patterns of gene duplication and functional evolution during the diversification of the AGAMOUS subfamily of MADS box genes in angiosperms. *Genetics*. **166**, 1011-1023.

Kyozuka, J. and Shimamoto, K. (2002). Ectopic expression of OsMADS3, a rice ortholog of AGAMOUS, caused a homeotic transformation of lodicules to stamens in transgenic rice plants. *Plant and Cell Physiology*. **43**, 130-135.

Levy, A.A., Britt, A.B., Luehrsen, K.R., Chandler, V.L., Warren, C. and Walbot, V. (1989). Developmental and genetic aspects of Mutator excision in maize. *Dev Genet*. **10**, 520-531.

Liljegren, S.J., Ditta, G.S., Eshed, Y., Savidge, B., Bowman, J.L. and Yanofsky, M.F. (2000). SHATTERPROOF MADS-box genes control seed dispersal in Arabidopsis. *Nature*. **404**, 766-770.

Martienssen, R. and Baron, A. (1994). Coordinate suppression of mutations caused by Robertson's mutator transposons in maize. *Genetics*. **136**, 1157-1170.

Mena, M., Ambrose, B.A., Meeley, R.B., Briggs, S.P., Yanofsky, M.F. and Schmidt, R.J. (1996). Diversification of C-function activity in maize flower development. *Science*. **274**, 1537-1540.

Mena, M., Mandel, M.A., Lerner, D.R., Yanofsky, M.F. and Schmidt, R.J. (1995). A characterization of the MADS-box gene family in maize. *Plant J.* **8**, 845-854.

Mizukami, Y., Huang, H., Tudor, M., Hu, Y. and Ma, H. (1996). Functional domains of the floral regulator AGAMOUS: characterization of the DNA binding domain and analysis of dominant negative mutations. *Plant Cell*. **8**, 831-845.

Münster, T., Deleu, W., Wingen, L.U., Ouzunova, M., Cacharrón, J., Faigl, W., Werth, S., Kim, J.T.T., Saedler, H. and Theissen, G. (2002). Maize MADS-box genes galore. *Maydica*. **47**, 287-301.

Ohno, S. (1970). "Evolution by Gene Duplication". Springer-VerlagNew York

Okamuro, J.K., den Boer, B.G., Lotys-Prass, C., Szeto, W. and Jofuku, K.D. (1996). Flowers into shoots: photo and hormonal control of a meristem identity switch in Arabidopsis. *Proc Natl Acad Sci U S A*. **93**, 13831-13836.

Paterson, A.H., Bowers, J.E. and Chapman, B.A. (2004). Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. *Proc Natl Acad Sci U S A*. **101**, 9903-9908.

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

Rutledge, R., Regan, S., Nicolas, O., Fobert, P., Cote, C., Bosnich, W., Kauffeldt, C., Sunohara, G., Seguin, A. and Stewart, D. (1998). Characterization of an AGAMOUS homologue from the conifer black spruce (Picea mariana) that produces floral homeotic conversions when expressed in Arabidopsis. *Plant J*. **15**, 625-634.

Schmidt, R.J., Veit, B., Mandel, M.A., Mena, M., Hake, S. and Yanofsky, M.F. (1993). Identification and molecular characterization of ZAG1, the maize homolog of the Arabidopsis floral homeotic gene AGAMOUS. *Plant Cell*. **5**, 729-737.

Sieburth, L.E. and Meyerowitz, E.M. (1997). Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. *Plant Cell*. **9**, 355-365.

Tandre, K., Svenson, M., Svensson, M.E. and Engstrom, P. (1998). Conservation of gene structure and activity in the regulation of reproductive organ development of conifers and angiosperms. *Plant J*. **15**, 615-623.

Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. *Curr Opin Plant Biol*. **4**, 75-85.

Theissen, G., Strater, T., Fischer, A. and Saedler, H. (1995). Structural characterization, chromosomal localization and phylogenetic evaluation of two pairs of AGAMOUS-like MADS-box genes from maize. *Gene*. **156**, 155-166.

Weigel, D. and Meyerowitz, E.M. (1994). The ABCs of floral homeotic genes. *Cell*. **78**, 203-209.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G. and Hirano, H.Y. (2006). Functional Diversification of the Two C-Class MADS Box Genes OSMADS3 and OSMADS58 in Oryza sativa. *Plant Cell*. **18**, 15-28.

Yanofsky, M.F., Ma, H., Bowman, J.L., Drews, G.N., Feldmann, K.A. and Meyerowitz, E.M. (1990). The protein encoded by the Arabidopsis homeotic gene agamous resembles transcription factors. *Nature*. **346**, 35-39.

Yu, D., Kotilainen, M., Pollanen, E., Mehto, M., Elomaa, P., Helariutta, Y., Albert, V.A. and Teeri, T.H. (1999). Organ identity genes and modified patterns of flower development in Gerbera hybrida (Asteraceae). *Plant J.* **17**, 51-62.

CHAPTER V

Concluding Thoughts and Future Directions

As described in Chapter I, two basic questions are of interest in plant evo-devo: 1. what developmental processes are conserved? and 2. what is responsible for differences in morphology? Flowers and flower development represent an excellent system to address these two questions. Flowers have an amazing diversity of shape and form (Endress, 1994), while at the same time a basic plan is present (with a few interesting exceptions (Ambrose et al., 2006)). Additionally, the elegant ABC model was established early, and provides a conceptual framework in which to investigate conservation or diversity of floral developmental pathways. Since the ABC model was proposed based on work in higher eudicot species, understanding how well it is conserved across the angiosperms more broadly requires analysis of species more distant phylogenetically. Maize is an excellent genetic model, and as a monocot provides a comparison point to the eudicots that spans the majority of flowering plant diversity (important exceptions are the basal angiosperm lineages that, while less speciose, are also critical for understanding floral evo-devo). Keeping in mind the two basic question of evo-devo (i.e. what developmental pathways are conserved vs. which developmental pathways explain diversity?), what have we learned about the development and evolution of flower development from our studies of maize B and C class MADS-box genes?

Previous work on maize B and C class genes had already indicated that at least some aspects of the ABC model are conserved between eudicots and monocots (Ambrose et al., 2000; Mena et al., 1996). The work presented here further

substantiates those initial conclusions. In particular we show that the maize B class genes are capable of functionally replacing their *Arabidopsis* orthologs indicating a conservation of biochemical function. We show that in spite of the considerably specialized morphology of the grass lodicule, it represents a modified second whorl organ, and as such it is under the control of B class genes. We also show that the maize C class genes have a role in stamen and carpel identity, in addition to the previously established role in floral meristem determinacy. This is entirely in line with what has been described for eudicot C class genes. Consequently, it would seem that at least as far as B and C functions are concerned, the ABC model holds for maize, and by inference all monocots and eudicots. These conclusions have been verified by functional studies of B and C class genes in another informative monocot model, rice (Nagasawa et al., 2003; Yamaguchi et al., 2006).

A function is a bit more problematic. It has been argued (with good reason) that A function may actually have more to do with floral meristem identity than floral organ identity *per se* (Litt and Irish, 2003). Unfortunately, although candidate A class MADS-box orthologs have been cloned from maize and other grass species, none have been analyzed functionally. Additionally, radiations in the grass A class genes (the *AP1/FUL* lineage) complicate functional characterization (see Chapter I). Maize florets have outer sterile organs (the palea and possibly the lemma), but it is not clear what establishes their identity. Reverse genetic approaches can be used to isolate knockouts of maize A class orthologs, and thus determine their role, if any, in maize floral organ identity. Forward genetic screens may turn up other regulators of palea

and/or lemma identity. Considering the lack of data for an A class function outside of *Arabidopsis*, such studies will help untangle the current inconsistencies regarding the A class, and may lead to a reevaluation of the ABC model.

Beyond the general conservation of function, several intriguing questions remain regarding the maize B and C class genes. One immediate question is what makes a lodicule so distinct from petals that morphologists have been arguing about its identity for over a century (Clifford, 1987)? B class genes are clearly necessary for lodicule identity (Ambrose et al., 2000), but are changes that occurred in the grass B class proteins themselves responsible for the morphological differences between a lodicule and its petal-like ancestor? This could be tested by determining if maize B class mutants (only *si1* is available now, but presumably others will be isolated in the future) can be rescued with B class genes from species without lodicules such as *Streptochaeta* or *Joinvillea*. It is possible that changes in genes downstream of the B class genes are responsible for the derived morphology of lodicules. In this case, identifying the genes would be more difficult, but perhaps careful expression analysis using cDNA arrays of *si1* mutants compared to wt would provide clues to the target genes downstream of the B function organ identity genes themselves.

While there is good reason to believe that subfunctionalization is responsible for the maintenance of *Zag1* and *Zmm2* (Mena et al., 1996), it is not yet clear why the *Zmm16/Zmm18-29* duplicates have been maintained. The duplication of these *PI* orthologs appears to date to the same genomic duplication event that created *Zag1* and *Zmm2*. Clearly not all duplicate copies from this event were maintained since the *AP3*

ortholog is single copy in both maize and rice, suggesting that either subfunctionalization or neofunctionalization is involved in maintaining the duplicate grass *PI* orthologs. Some hints at subfunctionalization in the grass *PI* lineage have come from rice where *OsMADS2* (the *Zmm16* ortholog) RNAi lines result in loss of lodicule identity but not stamen identity (Prasad and Vijayraghavan, 2003). Do expression differences in these *PI* duplicates corroborate this finding? What is the loss of function phenotype for *Zmm16, Zmm18,* and *Zmm29*? While the tandem duplication of *Zmm18* and *Zmm29* make creating the double mutant difficult, an RNAi approach could be used to silence both simultaneously.

We now have evidence that ZAG1 and ZMM2 proteins have subfunctionalized, opening the question of what kind of protein changes are responsible for their different functions. Using the approach described in Chapt IV to examine protein subfunctionalization, domain swaps between these two genes could be used to identify the protein region responsible for their respective contributions to C class activity. If their functional differences are caused by differential protein-protein interactions, such as ZAG1 losing the ability to interact with B class proteins, this could be tested by yeast two-hybrid assays.

While we have some idea how maize flowers are patterned, there still is a lot to be learned. Reverse genetics with maize and rice ABC MADS-box genes has, and likely will continue to shed light on how this model of flower development applies to diverse angiosperms. What remains unclear is how the unique morphologies (e.g. lemma, palea, and lodicules) of the grass floret evolved. These are considerably more

difficult questions to address, but are at the same time questions of considerable interest. In the case of the unique morphology of lodicules, some avenues present themselves as discussed above. As demonstrated in Chapter III, careful comparison with basal grasses and outgroups can provide clues to the homology of grass floral organs. Careful forward genetic screens in maize and rice are likely to continue to uncover new and interesting players. Integrating this molecular and genetic information from maize and rice with comparative studies on phylogenetically informative taxa, such as *Streptochaeta* and *Joinvillea*, will be essential to understanding how the grass floret evolved. Some of the groundwork has been laid, but much of the interesting work still lies ahead.

References

Ambrose, B.A., Espinosa-Matias, S., Vazquez-Santana, S., Vergara-Silva, F., Martinez, E., Marquez-Guzman, J. and Alvarez-Buylla, E.R. (2006). Comparative developmental series of the Mexican triurids support a euanthial interpretation for the unusual reproductive axes of Lacandonia schismatica (Triuridaceae). *Am J Bot*. **93**, 15-35.

Ambrose, B.A., Lerner, D.R., Ciceri, P., Padilla, C.M., Yanofsky, M.F. and Schmidt, R.J. (2000). Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. *Molecular Cell*. **5**, 569-579.

Clifford, H.T. (1987). Spikelet and Floral Morphology. *In* "Grass Systematics and Evolution" (T. R. Soderstrom, K. W. Hilu, C. S. Campbell and M. E. Barkworth, eds.), pp. 21-30. Smithsonian Institution Press. Washington D. C.

Endress, P.K. (1994). "Diversity and evolutionary biology of tropical flowers". Cambridge University PressCambridge

Litt, A. and Irish, V.F. (2003). Duplication and diversification in the APETALA1/FRUITFULL floral homeotic gene lineage: implications for the evolution of floral development. *Genetics*. **165**, 821-833.

Mena, M., Ambrose, B.A., Meeley, R.B., Briggs, S.P., Yanofsky, M.F. and Schmidt, R.J. (1996). Diversification of C-function activity in maize flower development. *Science*. **274**, 1537-1540.

Nagasawa, N., Miyoshi, M., Sano, Y., Satoh, H., Hirano, H., Sakai, H. and Nagato, Y. (2003). SUPERWOMAN1 and DROOPING LEAF genes control floral organ identity in rice. *Development*. **130**, 705-718.

Prasad, K. and Vijayraghavan, U. (2003). Double-stranded RNA interference of a rice PI/GLO paralog, OsMADS2, uncovers its second-whorl-specific function in floral organ patterning. *Genetics*. **165**, 2301-2305.

Yamaguchi, T., Lee, D.Y., Miyao, A., Hirochika, H., An, G. and Hirano, H.Y. (2006). Functional diversification of the two C-Class MADS box genes OSMADS3 and OSMADS58 in Oryza sativa. *Plant Cell*. **18**, 15-28.