

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

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Studies of flower development in core eudicot species have established a central role for B class MADS-box genes in specifying petal and stamen identities. Similarly in maize and rice, B class genes are essential for lodicule and stamen specification, suggesting homology of petals and lodicules and conservation of B class gene activity across angiosperms. However, lodicules are grass-specific organs with a morphology distinct from petals, thus their true homology to eudicot and nongrass monocot floral organs has been a topic of debate. To understand the relationship of lodicules to the sterile floral organs of nongrass 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 outgroups *Joinvillea* and *Elegia*, which have a typical monocot floral plan. Our results support a conserved role for B function genes across the angiosperms and provide additional evidence linking the evolution of lodicules and second whorl tepal/petals of monocots. The expression data and morphological analysis suggest that the function of B class genes should be broadly interpreted as required for differentiation of a distinct second floral whorl as opposed to specifying petal identity *per se*.

flower development | MADS-box | petal evolution | B-function | *Streptochaeta*

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 (1, 2). 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 (3). 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 phenotypes similar to those observed in B and C class mutants of higher eudicot species like *Arabidopsis* and *Antirrhinum* (4–7). 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, but the lack of genetic knockout or knockdown data in many nonmodel species of angiosperms makes testing this model difficult.

The origin of the petal and the number of times it has evolved have long been of interest to botanists (8–10), as has the origin of novel structures in the grasses such as the palea, lemma, and lodicules (11–13). Current research has explored the 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. The term petal refers to organs with a combination of

morphological characteristics: (i) position, in a whorl just outside the stamens but internal to sepals; (ii) appearance, compared with the sepals, generally larger, colored or otherwise nongreen and more delicate; and (iii) epidermal cell morphology, characteristic conical cells (P. Endress, personal communication). However, these three characteristics do not always co-occur in angiosperms, making a general definition of petals difficult.

The original B class mutants described in *Arabidopsis* and *Antirrhinum* undergo homeotic transformations in second whorl (petal) and third whorl (stamen) organs into sepals and carpels, respectively (14, 15). *Antirrhinum* and *Arabidopsis* both have two B class genes that ultimately were shown to encode a pair of closely related MADS-box genes: *APETALA3* (*AP3*) (14) and *PISTILLATA* (*PI*) (15) in *Arabidopsis* and their *Antirrhinum* orthologs *DEFECIENS* (*DEF*) (16) and *GLOBOSA* (*GLO*) (17), respectively. The paralogous *AP3/DEF* and *PI/GLO* lineages are the result of a duplication that occurred near the base of the angiosperms (18). 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 within their C terminus (19). Rescue of the *Arabidopsis ap3* mutant with a chimeric AP3 protein containing a paleoAP3 C terminus from a basal eudicot resulted in stamen rescue but no rescue of petal identity (20). These results suggest that the paleo*AP3* functions primarily in stamen identity, and, perhaps, the eu*AP3* evolved a distinct role in petal identity in the core eudicots.

Further evidence that the eu*AP3* lineage evolved to specify core eudicot petal identity comes from a study in which expression of B class genes in noncore eudicots was shown to be weak or patchy in petals (21). This contrasts with core eudicots, where expression is strong throughout petal development. In light of morphological and anatomical evidence that petals evolved multiple times independently during the evolution of angiosperms (8–10), 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 (21, 22).

A critical test of this hypothesis would be to disrupt B class gene function in a noncore eudicot species. To date, such a disruption of B class gene function has been described only for the paleo*AP3* genes of the grass species maize and rice, which

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both have highly derived floral organs (5, 6). These mutants show transformation of stamens to carpels, as seen in higher eudicots, in addition to transformation of a grass-specific organ, the lodicule, into a lemma/palea-like organ. This phenotype is consistent with an interpretation of lodicules as modified grass petals and palea 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 (23). Other than position, however, little in the mature morphology of lodicules indicates homology with petals, raising the possibility that B class genes were recruited independently to specify lodicule fate in the grasses (24, 25). Remane's well known criteria for homology (26) include homology of position, homology of what he called "special characteristics" (color, texture, and cell structure), and homology via intermediate forms. The latter criterion suggests that we may view two structures as homologous if they are connected by a series of transitional forms. To help clarify the relationship between petals and lodicules, 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 nongrass outgroups *Joinvillea ascendens* and *Elegia elephas* that have a typical monocot floral plan. Our results are consistent with Remane's criteria for homology and indicate that lodicules indeed are modified second whorl organs and that B class genes of a basal grass species and nongrass outgroups 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 eudicots.

Results

Isolation of B Class Genes from Basal Grass Species and Outgroups.

Grass B class genes were isolated originally from maize (*Zea mays*) and rice (*Oryza sativa*) (5, 27–29). 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 (5, 6, 29). However, a previous analysis of the grass *PI*-like genes indicated 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* (29). 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 *Streptochaeta*, as well as from two closely related outgroup species, *Joinvillea* and *Elegia*. A Bayesian phylogenetic analysis of the grass *AP3* genes closely matches the consensus topology published by the Grass Phylogeny Working Group (30), and is in agreement with a single lineage for *AP3*-like genes in the grasses (Fig. 1B). This is consistent with a single *AP3* ortholog in the complete rice genome sequence and a nonredundant (i.e., strong) B class phenotype when this gene is disrupted in rice *spw1* and maize *si1* mutants. However, two *AP3*-like genes were isolated from *Elegia*, 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 *PIs*, named here *PI1* and *PI2*, are apparent with *Joinvillea ascendens PI* (*JaPI*) as sister to both clades (Fig. 1A). Each clade has a *PI* ortholog from each grass species, and the topology matches the Grass Phylogeny Working Group topology with only slight, unsupported variations. These results are consistent with a *PI* duplication event occurring at or near the base of the grass

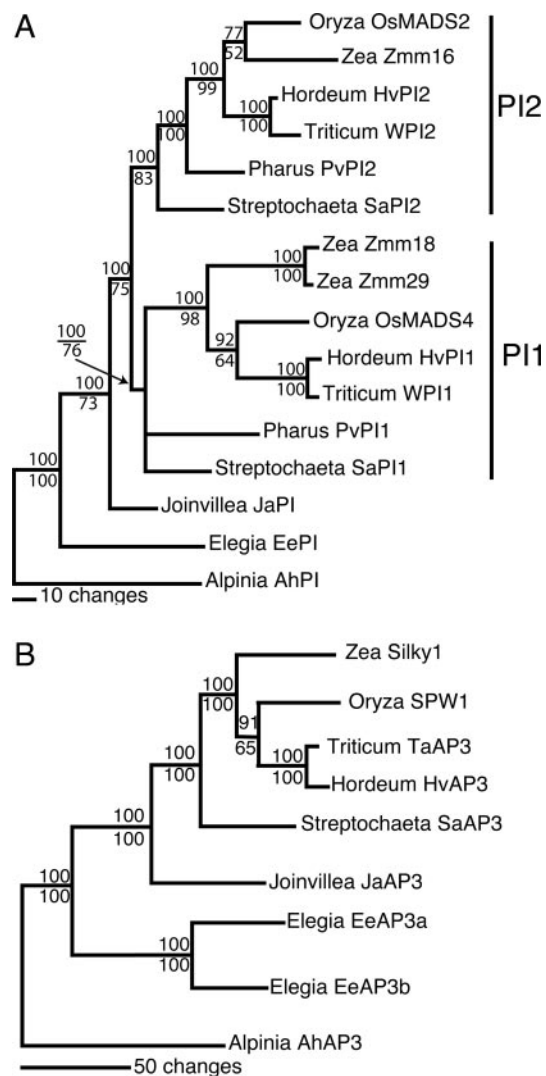


Fig. 1. Phylogenetic analysis of B class sequences from the Poaceae and close outgroups. Trees are 50% majority rule consensus, and phylogenetic analysis was performed as described in *Materials and Methods*. Bayesian posterior probabilities are indicated above the branches, with maximum likelihood bootstrap 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 *SPW1*, *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*; *EePI*, *EeAP3a*, and *EeAP3b*, *Elegia elephas* (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 are named *PI1* and *PI2* as indicated. (B) *AP3* orthologs from the grass family.

family, coinciding with a putative genomewide duplication event early in the evolution of the grass family (31). 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. 1A).

Morphology of Second Whorl Organs in *Streptochaeta*, *Joinvillea*, and *Elegia* Is Distinct. 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 (13). The lodicule is such an organ unique to the grasses that occurs in a whorl just outside the stamens. Lodicules can be fleshy or

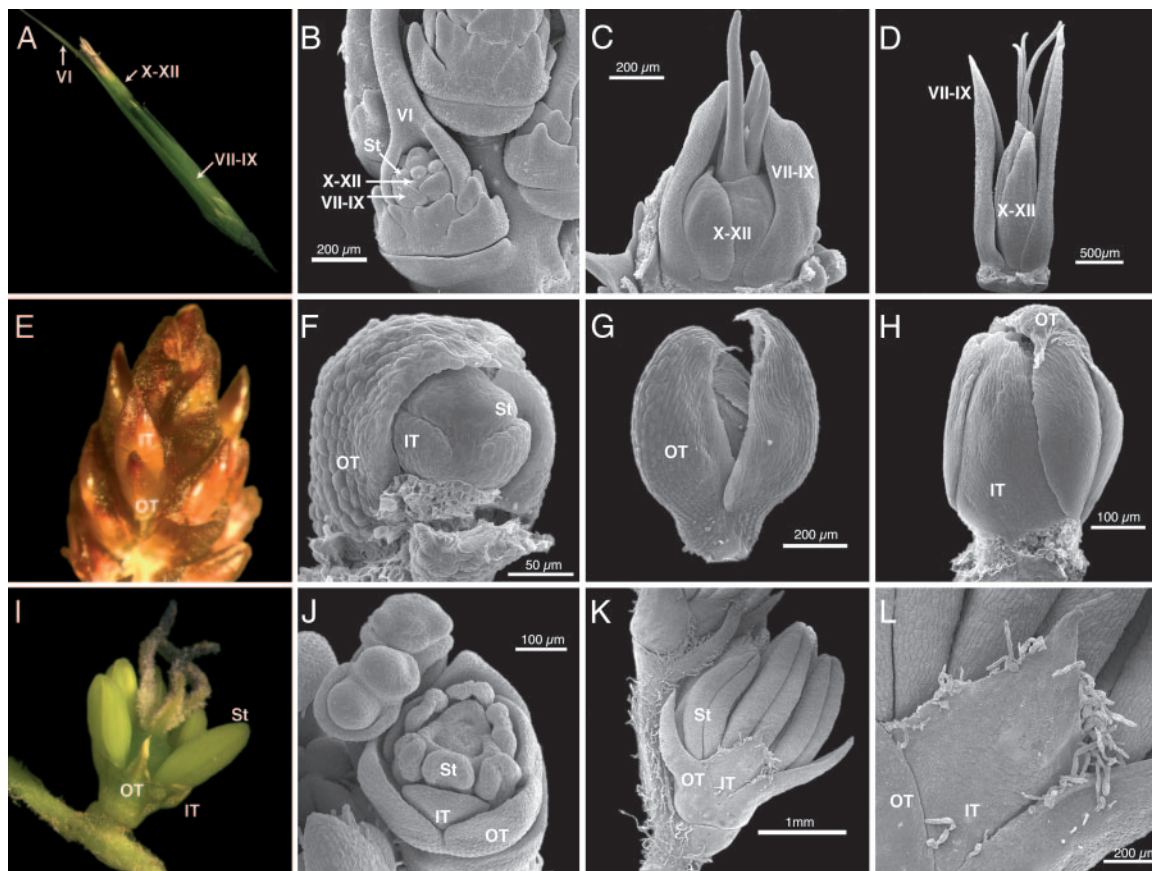


Fig. 2. Early floral development in *Streptochaeta*, *Elegia*, and *Joinvillea*. (A–D) *Streptochaeta* spikelet equivalent development. (A) Mature spikelet equivalent with anthers emerging from the overlapping whorl of bracts X–XII, which are distinct in shape and size from the pointed bracts VII–IX. (B) Early floral development showing the long-awned bract VI, initiation of the “outer tepal” bracts VII–IX, one of the “inner tepal” bracts X–XII, as well as stamen (St) and carpel primordia. (C) The entire spikelet equivalent was detached from the inflorescence, the large enclosing bract VI was removed, and the flower was viewed 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, whereas the third apparently has 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) *Elegia* floral development. (E) A mature inflorescence containing flowers subtended by bracts just before anthesis. Labeled flower has the bract removed, showing that 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 St primordia. (G) Maturing flower showing hooded outer tepals. (H) Same 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.

scale-like and generally swell at anthesis to allow the stamens to extend and the lemma and palea to separate. The two grasses *Anomochloa* and *Streptochaeta* are sisters to all other grasses and do not have lodicules (32, 33). In *Streptochaeta*, three leaf or bract-like organs surround the stamens, whereas in *Anomochloa*, a hairy “perigonian annulus” surrounds the stamens, indicating that lodicules evolved after the lineages for *Streptochaeta* and *Anomochloa* diverged (34). 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 observed the floral ontogeny of *Streptochaeta*, *Joinvillea*, and *Elegia* (Restionaceae) to understand the morphology of their second whorl organs.

The *Streptochaeta* spikelet equivalent has been described as a complex arrangement of twelve bracts (I–XII) that initiate before the reproductive organs (32). Bracts I–V initiate in a spiral, are small, and occasionally can 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, is 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. 2 A–C). Bracts X–XII initiate as a whorl outside the stamens and inside the VII–VIII–(IX) whorl, and because of their position often are interpreted as lodicules (12), although they have none of the other 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) bracts (Fig. 2 C and 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 (35). 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 nonshowy, “sepaloid.” The closest extant relatives to the grasses include *Joinvillea* and the Restionaceae, which have two similar whorls of nonshowy tepals. However, the development of these flowers shows that, although similar, the first and second whorls do have distinct morphologies (Fig. 2 E–L). In *Elegia*, the outer tepals initiate sequentially, rather than in a whorl, and are hooded (Fig. 2 F and G), whereas the inner tepals are more laminar and develop as an overlapping whorl that surrounds the stamens (Fig. 2H). As the flower matures, the inner tepals elongate and continue to enclose the reproductive organs (Fig. 2E). In *Joinvillea*, the outer tepal whorl is hooded and elongated with a smooth margin, whereas the inner tepal whorl is laminar and triangular with a papillate margin (Fig. 2 K and L).

Thus, in both *Joinvillea* and *Elegia*, the inner and outer tepals are distinct in morphology, although the second whorl does not have the characteristic showy features of petals. A distinct morphology for the first and second whorl also has been described for other taxa in the same family as *Elegia* (36). Additionally, the *Streptochoeta* inner whorl (interpreting bracts VII–VIII–IX as the outer whorl) has a distinct morphology, as do lodicules in the grasses. Although it is not clear what organs should be interpreted as the grass first whorl (13), some evidence suggests the palea and/or lemma could be (5, 6). The position and distinct morphology of the second whorl in *Streptochoeta*, *Joinvillea*, and *Elegia*, 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 *Streptochoeta* may be a transitional form (*sensu* Remane, ref. 26) in the evolution of lodicules.

B Class MADS-Box Genes Mark the Second and Third Whorls of *Streptochoeta*, *Elegia*, and *Joinvillea*. Our observations of the distinct morphology of the second whorl organs of *Streptochoeta* and nongrass outgroups, in addition to genetic data from maize and rice showing that B class genes control the identity of the second whorl lodicules, suggest that B class genes control second whorl organ identity in a broader sense than just petal identity, as seen in eudicots or lodicule identity in the grasses. If true, one would expect to see B class gene expression in the developing second whorl organ primordia of *Streptochoeta* and grass outgroups. Such expression would further support interpretation of lodicules as modified second whorl organs. Consequently, we performed RNA *in situ* hybridization on developing flowers of these species by using probes derived from the B class genes we had isolated.

In *Streptochoeta*, we examined expression of the *AP3* ortholog *SaAP3* and the *PI* ortholog *SaPI2*. *SaAP3* was expressed strongly in the stamens and second whorl primordia. Additionally, weaker expression was observed in the developing carpel and ovules (Fig. 3A). Such expression in the fourth whorl is not uncommon and has been reported for *PI* and *DEF* at early stages of floral development (15, 37) as well as for the maize *PI* orthologs at later stages (29). For *SaPI2*, expression was observed only in the stamen whorl and the second whorl (Fig. 3B). In *Elegia*, we performed *in situ* hybridizations with both of the *AP3* orthologs, *EeAP3a* and *EeAP3b*. For *EeAP3a*, we observed strong expression in the developing stamen and second whorl primordia (Fig. 3C). A similar result was seen with *EeAP3b* (Fig. 3D), although expression possibly was weaker overall compared with *EeAP3a* (data not shown). We were able to obtain tissue of

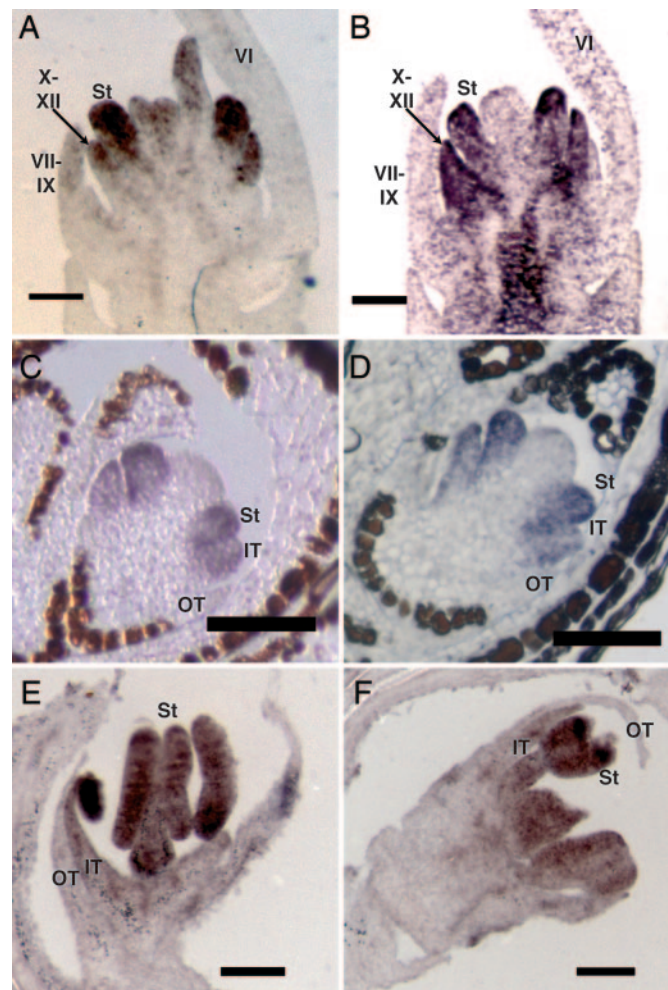


Fig. 3. *In situ* RNA hybridization of B class genes in *Streptochoeta*, *Elegia*, and *Joinvillea*. (A) *SaAP3* is strongly expressed in the developing stamens (St) and the whorl containing bracts X–XII. Weaker expression is evident in the carpel and possibly the VII–VIII whorl. (B) *SaPI2* is expressed strongly in the stamen whorl and the X–XII whorl, as is *SaAP3*. (C) *EeAP3a* is strongly expressed in the emerging stamens and inner tepals (IT) but absent from the outer tepals (OT). (D) *EeAP3b* expression is very similar to *EeAP3a*: 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 is relatively lacking in *Joinvillea* expression. (F) *JaPI* expression, like *JaAP3*, is seen in the stamens and inner tepals but relatively absent from the outer tepals. (Scale bars: 100 μ m.)

Joinvillea, although the floral stages were not as young as for *Streptochoeta* and *Elegia*, and fresh tissue was not available for fixing. For these reasons, *in situ* hybridization of the *Joinvillea ascendens AP3* (*JaAP3*) and *PI* (*JaPI*) was not as robust. Nevertheless, for both *JaAP3* and *JaPI*, expression was observed in the stamen whorl and the second whorl, but apparently absent from the first whorl (Fig. 3 E and F).

Discussion

B Class Gene Expression Supports a Second Whorl Origin for the Grass Lodicule. B class genes in angiosperms are expressed consistently 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 (38–42). In the core eudicots, B class genes also have a role in specifying the sterile organs of the second floral whorl, the petal. Similarly, in grasses, these same genes specify the second whorl lodicule. We have isolated B class genes from a basal grass without lodicules

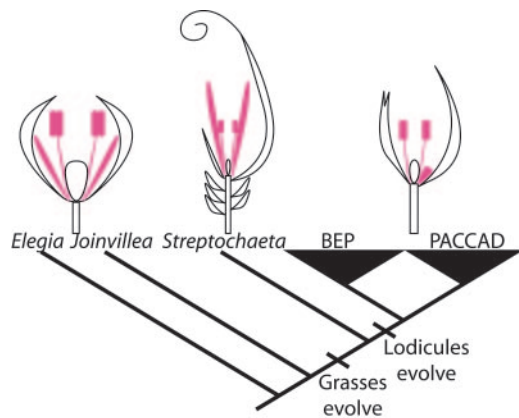


Fig. 4. Evolution of lodicules as indicated by B class gene expression. A schematic of the grass family phylogeny with the position of the outgroups examined in this study as described by the Grass Phylogeny Working Group. Lodicules evolve in the grasses after the divergence of the basal grass *Streptochoaeta*. B class genes are consistently expressed in stamens and the organ whorl just outside the stamens. This whorl just outside the stamens is the inner tepals of *Elegia* and *Joinvillea*, the bracts X–XII of *Streptochoaeta*, 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 *Streptochoaeta* being an intermediate step in this process. BEP clade, Bambusoideae, Ehrhartoideae, and Pooideae; PACCAD clade, Panicoideae, Arundinoideae, Centothecoideae, Chloridoideae, Aristidoideae, and Danthoioideae.

and from outgroups to the grasses, and we find that B class genes are consistently expressed in stamens and the organ whorl just outside the stamens in these species. In most grasses, the whorl outside the stamens is composed of lodicules, whereas in the basal grass *Streptochoaeta*, the whorl is composed of bracts X–XII, and in outgroups to the grasses like *Joinvillea* and *Elegia*, the whorl is the inner tepals. In *Asparagus officinalis*, another nongrass monocot with nonshowy tepals, the *AP3* and *PI* orthologs are expressed similarly in stamens and inner tepals (43, 44). Taken together, these data strongly suggest that bracts X–XII of *Streptochoaeta* and lodicules of other grasses evolved by modification of the inner tepal or second whorl of a typical monocot flower (Fig. 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 second whorl identity. However, the *sil* and *spw1* mutants clearly show that the *AP3* ortholog in grasses is necessary for lodicule identity. If B class genes were recruited independently to specify lodicule identity in the grasses, then we would not expect B class expression in the second whorl of *Streptochoaeta* and the grass outgroups. To explain our observations, we then would have to hypothesize that B class expressing second whorl organs in *Streptochoaeta* and the outgroups were lost over evolutionary time and were replaced later in grass evolution with lodicules in the same position and whose identity came under control of the same regulatory genes. We feel this is unlikely. The much simpler hypothesis, that B class genes specify a second whorl identity in many angiosperms, that the details of second whorl morphology are variable, 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 nongrass 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, whereas 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 is not brightly colored or showy. Likewise, *Asparagus* has two whorls of sepaloid tepals, but B class genes still are expressed in the second whorl.

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 simply could be the result of this B class petal program shifting to new organ whorls (45, 46). Expression data, functional genetic data (5, 6, 23), and morphological data taken together suggest that lodicules represent one of multiple possible sorts of second whorl organs, others being 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 nongrass monocots, the fascinating question remains how lodicules evolved their distinct morphology. Identifying the genetic targets of B class MADS-box proteins will help illuminate the evolutionary modifications distinguishing second whorl organs.

Materials and Methods

Plant Material. Inflorescence primordia were collected from *Streptochoaeta angustifolia*; plants were grown from seed in a growth room at 22°C under constant light conditions. *Joinvillea ascendens* tissue was collected from plants at the National Tropical Botanical Garden in Kalaheo, HI. *Elegia elephas* 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 by using the SuperScript First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA). A polyT primer with a 5' adapter sequence was used in the cDNA synthesis step (5'-CCGGATCCTCTAGAGCGGCCGCTTT-TTTTTTTTTTTTTT-3'). PCR of B class genes from grass species and outgroups was performed with a degenerate MADS-box sequence forward primer (5'-ATGGGBMGGVAR-KATHGAGA-3') and the polyT adapter primer. These PCR products were subcloned into pGEM-Teasy (Promega, Madison, WI) or TOPO-TA (Invitrogen) and sequenced. Isolation of *AP3* and *PI* orthologs from some species required a second round of PCR with internal primers: Grass *PIrev* (5'-YTCTGBARRT-TGGRTG-3'), and Grass *AP3rev* (5'-YYARCCSAGGCG-SAGGTCTGTG-3'). After isolation of a partial sequence, complete cDNA coding sequence was obtained by using 5' and/or 3' RACE. DNA sequences were submitted to GenBank with the following accession numbers: *PvPI1*, DQ662243; *PvPI2*, DQ662242; *SaPI1*, DQ662244; *SaPI2*, DQ662241; *JaPI*, DQ662245; *EePI*, DQ662246; *SaAP3*, DQ662237; *JaAP3*, DQ662238; *EeAP3a*, DQ66662239; and *EeAP3b*, DQ662240.

Phylogenetic Analysis. Initial DNA sequence alignment was performed with ClustalX, followed by manual adjustments with MacClade4. ModelTest then was used to determine the optimal model of evolution. Based on results from Akaike Information Criterion (AIC), the GTR + G model was selected and Bayesian phylogenetic analysis was performed by 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; *HvP11*, BU996044; *WPI2*, AB107992; *OsMADS4*, L37527; *ZMM18*, AJ292960; *ZMM29*, AJ292961; *HvP12*, AY541066; *WPI1*, AB107991; *AhPI*, AY621156; *SPW1*, AF454259; *Si1*, AF181479; *TaAP3*, AB107993; *HvAP3*, AY541065; and *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-100. 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.

RNA *in Situ* Hybridization. Freshly collected samples (except for *Joinvillea*, which was collected 24–48 h before fixation) were

fixed overnight at 4°C in FAA, then embedded, sectioned, hybridized, washed, and exposed as described in ref. 47; 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, La Jolla, CA). Antisense, digoxigenin-labeled UTP probe was synthesized by using T7 polymerase with either a PCR-amplified DNA template or a linearized plasmid.

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