To B or Not to B a Flower: The Role of *DEFICIENS* and *GLOBOSA* Orthologs in the Evolution of the Angiosperms

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Abstract

DEFICIENS (*DEF*) and *GLOBOSA* (*GLO*) function in petal and stamen organ identity in *Antirrhinum* and are orthologs of *APETALA3* and *PISTILLATA* in *Arabidopsis*. These genes are known as B-function genes for their role in the ABC genetic model of floral organ identity. Phylogenetic analyses show that *DEF* and *GLO* are closely related paralogs, having originated from a gene duplication event after the separation of the lineages leading to the extant gymnosperms and the extant angiosperms. Several additional gene duplications followed, providing multiple potential opportunities for functional divergence. In most angiosperms studied to date, genes in the *DEF/GLO* MADS-box subfamily are expressed in the petals and stamens during flower development. However, in some angiosperms, the expression of *DEF* and *GLO* orthologs are occasionally observed in the first and fourth whorls of flowers or in nonfloral organs, where their function is unknown. In this article we review what is known about function, phylogeny, and expression in the *DEF/GLO* subfamily to examine their evolution in the angiosperms. Our analyses demonstrate that although the primary role of the *DEF/GLO* subfamily appears to be in specifying the stamens and inner perianth, several examples of potential sub- and neofunctionalization are observed.

Introduction to the "Abominable Mystery" of the Angiosperms

The explosive diversification of the early angiosperm lineages evident in extant species and the fossil record was famously characterized by Darwin as "an abominable mystery" (Darwin 1903). The most obvious key innovation in angiosperms is the flower. As a reproductive structure, the flower is amazingly effective. In most flowers, the male-functioning stamens and female-functioning carpels are placed side by side, surrounded by an attractive perianth. The diversity of perianth architecture observed in flowering plants is immense, and advances in understanding of the developmental evolution of angiosperm reproduction have made the rapid and early diversification of flowers and flowering plants both more astonishing and somewhat less mysterious (Becker and Theissen 2003; Kim et al. 2005a; Kramer et al. 2004; Litt and Irish 2003; Soltis et al. 2005; Stellari et al. 2004). Over the past decade, the resolution of phylogenetic relationships among most major angiosperm clades including the basalmost lineages (Figure 1) (Soltis and Soltis 2004; Zanis et al. 2002) have paved the way for these and other comparative studies aimed at understanding the molecular mechanisms of early angiosperm diversification (Soltis et al. 2002a).

At the same time, an improved understanding of angiosperm relationships (Figure 1) and the timing of branching events (Chaw et al. 2004; Crane et al. 1995; Davies et al. 2004; Soltis et al. 2002b; Wikstrom et al. 2001) adds weight to the characterization of early angiosperm diversification as an abominable mystery. The basal angiosperms exhibit a wide variety of floral forms (Albert et al. 1998; Endress 1994, 2001; Soltis et al. 2005). *Amborella* produces male and female flowers with spirally arranged parts on separate plants. Perianth architecture in water lilies (Nymphaeaceae, Nymphaeales) varies in organization of parts (spiral versus whorled), differentiation of inner and outer parts (undifferentiated tepals versus outer sepals + inner petals) and number of perianth parts (three sepals and petals versus many undifferentiated). Both magnoliids and
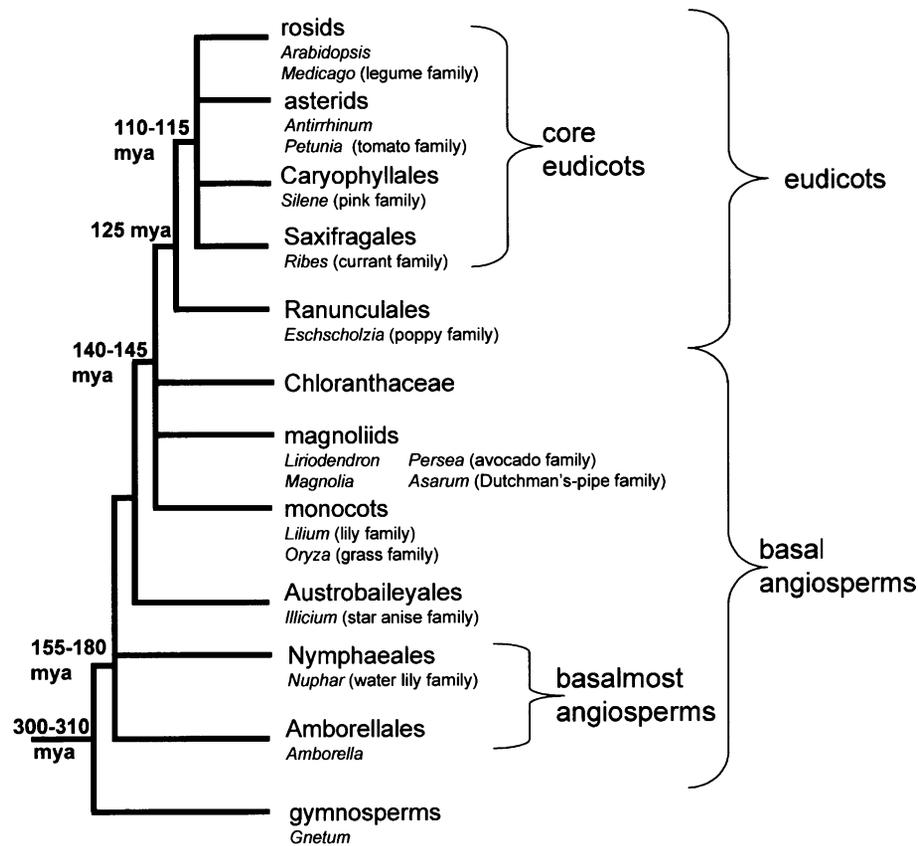
Figure 1. Angiosperm phylogeny. The identity of basal angiosperm lineages have been well established over the last decade. In addition, fossil evidence for the origin of eudicots (Crane et al. 1995) and molecular clock estimates reveal the timing of key diversification events in angiosperm history (see text for references).

monocots also show extreme variation in floral form, including bilaterally symmetric flowers with elaborately modified perianth parts. The organization of flower parts is a bit less variable in the core eudicots. Flowers in this most speciose group of angiosperms typically have a bipartite perianth and four distinct whorls of parts starting with sepals on the outside, then petals, stamens and an inner whorl of carpels (Figure 2). Our current understanding of how organ identity is determined in each whorl is based on pioneering experiments involving the eudicots *Antirrhinum* (Schwarz-Sommer et al. 1990; Trobner et al. 1992) and *Arabidopsis* (Coen and Meyerowitz 1991; Goto and Meyerowitz 1994; Jack et al. 1992; Ma 1994; Weigel and Meyerowitz 1994). The extent to which this understanding extends to basal eudicots (e.g., Ranunculales) and basal angiosperms is the focus of much recent research.

MADS-box Genes and Flower Development

MADS-box genes encode transcription factors with critical function in floral organ specification and development. Several MADS-box genes, identified in *Arabidopsis* and *Antirrhinum*, confer homeotic floral function and are often referred to as the floral MADS-box genes (Theissen 2001; Weigel and Meyerowitz 1994). As homeotic genes, similar to the *HOX*-gene family in animals, loss of function mutants among MADS-box genes cause changes in organ identity. Analyses of mutants in these MADS-box genes were used to develop the genetic ABC model. This model explains how specific functions alone or in combination initiate and specify the four major floral organ types—sepals, petals, stamens and carpels in *Arabidopsis* and *Antirrhinum* (Bowman and Meyerowitz 1991; Coen and Meyerowitz 1991; Ma, 1994; Weigel and Meyerowitz 1994).

The ABC model stipulates that A-function genes specify the development of the sepals, A + B the petals, B + C the stamens, and C alone the carpels (Coen and Meyerowitz 1991) (Figure 2). In *Arabidopsis APETALA1* (*AP1*) is an A-function gene that is necessary for sepal development in the first whorl; *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are B-function genes that along with *AP1* are necessary for petal development in whorl 2 and along with *AGAMOUS* (*AG*) are necessary for stamen development in whorl 3; and the *AG* gene is a C-function gene necessary for carpel development in whorl 4 (Bowman and Meyerowitz 1991; Coen and Meyerowitz 1991; Ma 1994; Weigel and
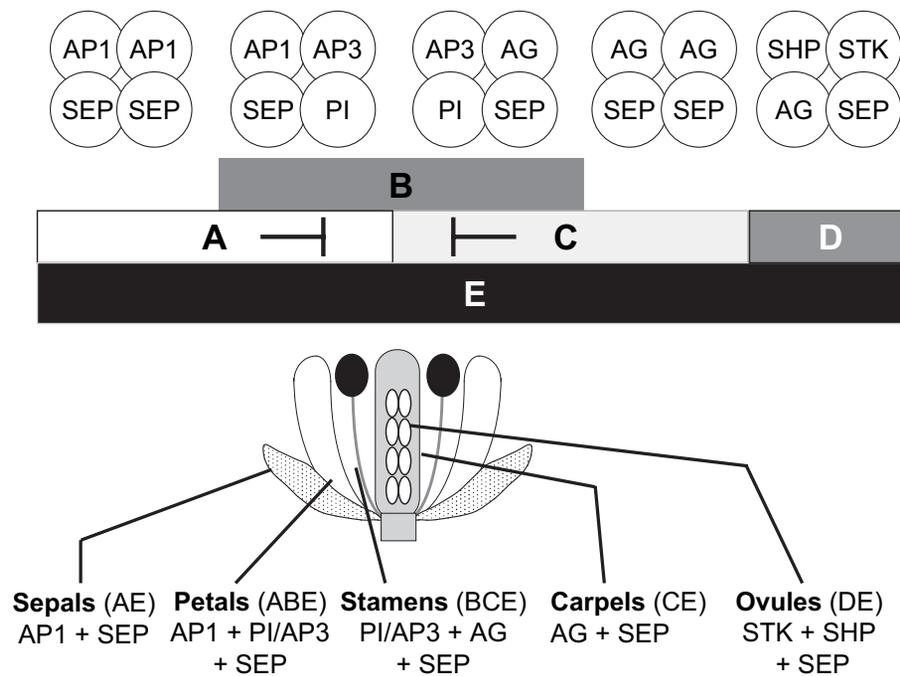
Figure 2. The genetic ABC + DE model and the quartet model. The ABC model states that A-function genes, such as *AP1* in*Arabidopsis*, are necessary for the formation of the sepals, B-function genes, which include *AP3* and *PI* in *Arabidopsis*, along with A-function genes, are necessary for the formation of the petals and B-function, along with C-function genes, which in *Arabidopsis* includes *AG*, are necessary for the formation of the stamens, and C-function genes alone are necessary for the formation of the carpels. This has been expanded to include class D- and E-function genes, which are necessary for the ovules and whorls of the flower, respectively. D-function genes in *Arabidopsis* include *SEEDSTICK* (*STK*) and *SHATTERPROOF1* and *SHATTERPROOF2* (*SHP1, SHP2*). E function *sensu lato* requires at least one of the four *SEPALLATA* (*SEP1, SEP2, SEP3*, and *SEP4*) genes. The floral quartet model expands on this idea using data from protein interaction studies (Theissen 2001; Theissen and Saedler 2001). In this figure the hypothesized quartets, based on experimentally determined dimeric or multimeric protein interactions, necessary for each floral organ are presented.

Meyerowitz 1994). In *Antirrhinum*, the functionally characterized *SQUAMOSA* (*SQUA*), *DEFICIENS* (*DEF*), *GLOBOSA* (*GLO*), and *PLENA* (*PLE*) are either orthologs or closely related paralogs of *AP1, AP3, PI*, and *AG*, respectively.

ABC genes are required but are not sufficient to specify floral organ identity. Therefore, the ABC model has been expanded to include D-function for ovule development (Angenent et al. 1995b; Colombo et al. 1995) and E-function for specifying each of the four types of floral organs (Ditta et al. 2004; Gutierrez-Cortines and Davies 2000; Theissen 2001; Theissen and Saedler 2001) (Figure 2). In *Arabidopsis* D-function requires *SHATTERPROOF1* or *SHATTERPROOF2* (*SHP1, SHP2*; formerly *AGL1* and *AGL5*) and *SEEDSTICK* (*STK*; formerly *AGL11*) (Favaro et al. 2003) and E-function *sensu stricto* requires one of three functionally redundant genes, *SEPALLATA1, 2, 3* (*SEP1, 2, 3;* formerly *AGL2, AGL4, AGL9*) that are coexpressed with the *PI, AP3*, and *AG* genes in the petals, stamens, carpel, and ovules (Pelaz et al. 2000). Recently yet another *SEP* gene, *SEP4* (formerly *AGL3*) has been defined, being a close relative of the other *SEP* genes (Ditta et al. 2004). *sep1 sep2 sep3 sep4*

quadruple mutants develop vegetative leaves rather than sepals, petals, stamens, or carpels (Ditta et al. 2004). These findings demonstrate that functionality of at least one of the four *SEP* genes ("E-function *sensu lato*") is required to superimpose sepal identity on vegetative leaf identity, and it appears likely that class A floral homeotic proteins plus any one of the four SEP proteins are sufficient to specify sepal identity (Figure 2).

Loss-of-function mutants among the floral MADS-box genes cause changes in organ identity. Ideal A-function mutants develop carpels rather than sepals and stamens instead of petals. B-function mutants develop sepals rather than petals and carpels instead of stamens. C-function mutants develop petals instead of stamens, and the carpels undergo a homeotic conversion into sepals. In D-function *shp1 shp2 stk* triple mutants the ovules are converted into leaf-like structures (Favaro et al. 2003), in E-function *sep1 sep2 sep3* triple mutants all whorls of the flower are converted into sepals (Pelaz et al. 2000), and in *sep1 sep2 sep3 sep4* quadruple mutants all whorls of the flower are converted into leaves (Ditta et al. 2004). Additionally, the ABC model hypothesizes that A- and C-function genes negatively regulate each other

such that the A-function *AP1* is expressed in floral whorls where the expression of the C-function *AG* gene is not detected and the C-function *AG* is expressed in floral whorls where the expression of the A-function *AP1* gene is not detected (Coen and Meyerowitz 1991; Ma 1994; Weigel and Meyerowitz 1994).

The floral quartet model is a molecular model that advances the genetic ABC model by integrating genetic studies of floral MADS-box genes and the molecular data demonstrating interactions between floral MADS-domain proteins. The quartet model hypothesizes that MADS-domain proteins form specific heteromeric complexes of different proteins for each floral organ (Theissen 2001; Theissen and Saedler 2001). This model is supported by observations that MADS-domain proteins form dimeric and multimeric complexes in yeast-2 and yeast-3-hybrid experiments (Davies et al. 1996b; Fan et al., 1997; Honma and Goto 2001; Immink et al. 2003; Moon et al. 1999) and that ectopic expression of AP1, AP3, PI, and SEP and AG, AP3, PI, and SEP proteins results in homeotic conversion of leaves into petals or stamens, respectively (Honma and Goto 2001; Pelaz et al. 2001). It has been postulated that these quaternary complexes of MADS-box genes may be involved in activating or repressing target genes by binding to their promoters (Theissen and Saedler 2001).

Our understanding of MADS-box gene function in *Arabidopsis* and other model plants has expanded dramatically in the past decade. However, understanding of the function of floral MADS-box genes outside of the model plants is much less clear. Compared to the extant gymnosperms, angiosperms have made significant advances, including the defining features of a second fertilization event that gives rise to a nutritive endosperm (Williams and Friedman 2002), the bisexual flower, the carpel and the presence of a perianth (Theissen and Becker 2004). Many of these traits are hypothesized to be affected by MADS-box genes, including members of the *SQUAMOSA/APETALA1*, *DEFICIENS/GLOBOSA* (also referred to as *APETALA3/PISTILLATA*), *AGAMOUS*, and *SEPALLATA* subfamilies (Becker and Theissen 2003; Kramer et al. 1998, 2004; Litt and Irish 2003; Zahn et al. 2005).

Gene and Genome-Wide Duplication Events in Angiosperm Evolution

Multiple gene duplications have occurred within floral MADS-box gene subfamilies, including *AP1, DEF/GLO, AG*, and *SEP*, and may have been important in the origin and diversification of the angiosperms (Becker and Theissen 2003; Irish 2003; Kramer et al. 1998, 2004; Zahn et al. 2005). Genome-wide duplication events are common in angiosperm history (Bowers et al. 2003; Irish 2003; Ku et al. 2000) and one (or more) might be responsible for multiple MADS-box gene duplications in the early eudicot lineages (Blanc and Wolfe 2004). Duplicate gene copies may have also resulted from genome-wide or segmental duplication events, leading to diversification within the *AP1, DEF, AG*, and *AGL2/3/4*

lineages within the eudicots (Becker and Theissen 2003; Kramer et al. 1998, 2004; Litt and Irish 2003; Zahn et al. 2005).

MADS-box gene duplications have been hypothesized as important in the origin and diversification of the early angiosperms (Becker and Theissen 2003; Theissen et al. 2000). This hypothesis has received support over the past year as gene sequences for the basalmost angiosperms have been added to phylogenetic analyses of the floral MADS-box genes (Aoki et al. 2004; Kim et al. 2004; Kim et al. 2005; Kramer et al. 2004; Stellari et al. 2004; Zahn et al. 2005). A genome-wide duplication has also been hypothesized to have occurred before the diversification of the extant angiosperm lineages (Bowers et al. 2003) and the possibility that such a duplication event spurred the origin and early diversification of angiosperms is quite intriguing.

Gene duplication has been postulated to be a source of variation for the evolution of new function, either through sub- or neofunctionalization (Force et al. 1999). It has been observed that genes within a single subfamily often retain similar functional capacities (Kramer et al. 2004; Litt and Irish 2003; unpublished data). However, among the floral MADS-box genes there can also be significant variation in expression and, most likely, function among closely related homologs (Kramer et al. 2004; Malcomber and Kellogg 2004; unpublished data). This is not surprising because duplicate genes with redundant function are not expected to persist (Force et al. 1999).

For the remainder of this article we will focus on functional studies, shifting expression patterns, and gene duplication in the *DEF/GLO* subfamily. Although many recent papers have identified *DEF/GLO* genes and in some cases described expression patterns of their orthologs from multiple angiosperm lineages, a comprehensive examination of the expression patterns of *DEF/GLO* genes in a phylogenetic perspective is lacking. *Arabidopsis* and *Antirrhinum* genes in the *DEF/GLO* subfamily are B-class genes specifying petal and stamen development (Figure 2). If these functions extend back to the earliest angiosperms, they may have played a central role in the origin and diversification of the angiosperms. Nevertheless, from any perspective, *DEF* and *GLO* orthologs have most certainly played an important role in floral evolution (Irish 2003; Theissen and Becker 2004).

The First Characterized B-Function Genes, *DEFICIENS, GLOBOSA, APETALA3*, and *PISTILLATA*

The *DEF/GLO* MADS-box subfamily (Theissen and Saedler 1995; Theissen et al. 1996) is named for the first B-function genes to be molecularly characterized: the *Antirrhinum DEF* (Sommer et al. 1990) and *GLO* genes (Trobner et al. 1992). Soon after *DEF* and *GLO* were described, their functional equivalents from *Arabidopsis*, *AP3* and *PI*, were also molecularly cloned (Goto and Meyerowitz 1994; Jack et al. 1992). *AP3, DEF, GLO*, and *PI* are all

MADS-box genes with critical function in floral organ specification and floral development. Early phylogenetic studies showed that *AP3* is a *DEF* ortholog and *PI* is a *GLO* ortholog, whereas *DEF* and *AP3* are paralogs of *GLO* and *PI*, respectively (Doyle 1994; Goto and Meyerowitz 1994; Jack et al. 1992; Purugganan et al. 1995).

Both *AP3* and *DEF* are expressed early during floral development, after the sepal primordia have just initiated from the floral meristem, in the regions that will develop into the petals and the stamens and continues in these organs as they form from the floral meristem (Jack et al. 1992; Schwarz-Sommer et al. 1992). At later developmental stages, low levels of transcript of *DEF* are detected at the base of the sepals (Schwarz-Sommer et al. 1992). In the fourth whorl, *DEF* is detected relatively early in the developing carpels, whereas *AP3* is detected during late floral development in the ovules (Jack et al. 1992; Schwarz-Sommer et al. 1992). *ap3* and *def* mutants result in homeotic changes of petals to sepals in the second whorl and stamens to carpels in the third whorl (Jack et al. 1992; Sommer et al. 1990).

Overall, *PI* and *GLO* are expressed similarly to *AP3* and *DEF* in whorls 2 and 3 and the corresponding mutants have similar phenotypes (Goto and Meyerowitz 1994; Trobner et al. 1992). Both *PI* and *GLO* are expressed at low levels in early carpel development (Goto and Meyerowitz 1994; Trobner et al. 1992). Constitutive ectopic expression of *AP3* in *Arabidopsis* flowers results in a homeotic replacement of the carpels with stamens (Jack et al. 1994). This phenotype can be explained by the fact that the simultaneous presence of both AP3 and PI protein appear to be necessary for the formation of the petals and stamens (Jack et al. 1992). Because *PI* is normally found in the carpels alone in early stages of development, the addition of *AP3* in this organ results in a homeotic transformation of the carpels to stamens, due to autoregulation of these genes (see later discussion).

The functions of *DEF* and *GLO* (and also *AP3* and *PI*) appear to be tightly coordinated based on the overlap of their expression and similar mutant phenotypes. The early expression of *GLO* (*PI*) is not dependent on *DEF* (*AP3*) and vice versa (Goto and Meyerowitz 1994; Honma and Goto 2000; Trobner et al. 1992). However, studies of expression of *GLO* (*PI*) in *def* (*ap3*) mutants and *DEF* (*AP3*) in *glo* (*pi*) mutants demonstrate that the levels of transcript of the nonmutated gene are reduced in single mutants (Goto and Meyerowitz 1994; Trobner et al. 1992). Therefore higher levels of expression require both DEF and GLO (AP3 and PI) proteins, demonstrating that *DEF* (*AP3*) and *GLO* (*PI*) regulate the other's expression (Trobner et al. 1992). These observations are supported by the evidence that GLO and DEF (AP3 and PI) proteins form heterodimeric complexes with which they upregulate their own transcription (Goto and Meyerowitz 1994; Jack et al. 1994; Samach et al. 1997; Schwarz-Sommer et al. 1992; Trobner et al. 1992). AP3 and PI heterodimers bind to specific DNA sites in vitro, including putative regulatory elements of both *AP3* and *PI* (Riechmann et al. 1996b; Honma and Goto 2000). This autoregulation may be a mechanism by which the cell ensures

that there is sufficient protein product available for gene function (Davies and Schwarz-Sommer 1994; Schwarz-Sommer et al. 1992; Trobner et al. 1992). Therefore the loss of the function of a single gene (i.e., either *DEF* or *GLO*) significantly reduces the ability of the other paralog to function properly.

B-Function Beyond *Arabidopsis* and *Antirrhinum*

The only functionally characterized core eudicot *DEF* ortholog outside of *Arabidopsis* and *Antirrhinum* is *pMADS1* (also known as *GREEN PETALS* and *PhDEF*) from petunia. Similar to *DEF* and *AP3*, *pMADS1* is expressed weakly in the sepals and carpels, strongly in the petals, and moderately in the stamens (Tsuchimoto et al. 2000; van der Krol et al. 1993). However, reported expression of *pMADS1* in the sepals and carpels was not supported in later studies (Immink et al. 2003). Northern blot analyses demonstrated no expression in the sepals or carpel for this gene, suggesting that any expression here is very low and transient, if it is expressed at all in these organs (Immink et al. 2003). *pMADS1* also seems to function similarly to *AP3* and *DEF* in that it was able to activate the *GLO* orthologs, *pMADS2* and *FBP1* in the first whorl when ectopically expressed (Halfter et al. 1994).

Nevertheless, in *pmads1* mutants or cosuppression transformants, the petals undergo homeotic transformation and are converted to sepals in *pmads1* mutants (van der Krol et al. 1993; van der Krol and Chua 1993), similar to *ap3* and *def*. However, in the third whorl the stamens have petaloid cells on their filaments and no homeotic transformation is observed. This phenotype is significantly different from the third whorl of the *def* or *ap3* mutants. When *pMADS1* is ectopically expressed it results in a homeotic conversion of the sepals to petals, and in the second whorl, there is extra petaloid tissue resulting in an inside-out orientation (Halfter et al. 1994). It has been suggested that *DEF, pMADS1, AP3*, and *PTD* (a *DEF* ortholog from poplar) may control cell proliferation which may explain this phenotype (Halfter et al. 1994; Sablowski and Meyerowitz 1998; Sheppard et al. 2000).

On the basis of these results it has been suggested that *pMADS1* is a petal organ identity gene and probably has overlapping expression with another *DEF* homolog in petunia, possibly *PhTM6*, that controls stamen identity (Tsuchimoto et al. 2000; Vandenbussche et al. 2004; van der Krol et al. 1993; van der Krol and Chua 1993). *PhTM6* may have a primary function in the stamens because it is expressed at high levels in these organs and is expressed at lower levels in the petals and sepals and in the carpels in the developing placenta, ovules, and seedpod (Immink et al. 2003; Vandenbussche et al. 2004). Unlike other *DEF* homologs studied to date, *PhTM6* does not appear to be regulated by *GLO* orthologs and thus may have a different mechanism of regulation than what has been observed in other *DEF/GLO* genes (Vandenbussche et al. 2004).

In petunia there are two distinct *GLO* homologs that have been functionally characterized, *FBP1* and *pMADS2*. *FBP1* is transcribed in petals and sepals although FBP1 protein is only detected in the petals (Angenent et al. 1992, 1995a; Greco et al. 1997; Immink et al. 2003; van der Krol et al. 1993; van der Krol and Chua 1993). *pMADS2* expression is similar to that of *FBP1*, strong in the petals and moderate in the stamens (Immink et al. 2003; Tsuchimoto et al. 2000; van der Krol et al. 1993; van der Krol and Chua 1993). In *fbp1* mutants only the petals are affected, and in *pmads2* mutants only the anthers are affected (Angenent et al. 1992, 1995a; Vandenbussche et al. 2004). When both genes are defective, a complete loss of B-function occurs as indicated by a complete homeotic transformation of petals to sepals and stamens to carpels similar to the phenotype in *glo* or *pi* mutants (Vandenbussche et al. 2004).

These data show that interactions between petunia *DEF/GLO* genes are more complex than in *Arabidopsis* and *Antirrhinum*. The distinctive null mutant phenotype for both *fbp1* and *pmads2* suggests that sub- or neofunctionalization has occurred among the duplicate *DEF* and *GLO* orthologs consistent with their expression patterns. This is further supported by observations that the pMADS1 protein interacts with both FBP1 and pMADS2 proteins, whereas PhTM6 interacts with pMADS2 strongly and only weakly with FBP1, if at all (Immink et al. 2003; Vandenbussche et al. 2004).

The Phylogeny of the *DEF/GLO* Subfamily

DEF and *GLO* play an important role in specifying the petal, a major organ type that is unique to the angiosperms. Therefore the changes these genes underwent in their evolution may be one of the most important components in understanding how MADS-box genes have changed in the evolution of the angiosperms (Sundstrom and Engstrom 2002; Theissen and Becker 2004). Since the discovery of the *Antirrhinum DEF* and *GLO* and *Arabidopsis AP3* and *PI* genes, it has been suggested that these genes with highly conserved protein sequences and similar expression patterns are closely related (Jack et al. 1992).

Several gymnosperm genes with homology to members of the *DEF/GLO* subfamily in the angiosperms have been identified to date representing the major evolutionary lineages of the nonflowering seed plants (Becker et al. 2000; Becker and Theissen 2003; Fukui et al. 2001; Mouradov et al. 1999; Shindo et al. 1999; Sundstrom et al. 1999; Winter et al. 1999, 2002a). These genes are consistently placed at the base of the lineage leading to the angiosperm *DEF* and *GLO* clades in phylogenetic analyses as sister to both the angiosperm *DEF* and *GLO* lineages (Aoki et al. 2004; Becker et al. 2000; Becker and Theissen 2003; Fukui et al. 2001; Kim et al. 2005a; Mouradov et al. 1999; Shindo et al. 1999; Stellari et al. 2004; Sundstrom et al. 1999; Winter et al. 2002a; Theissen and Becker 2004). The phylogenetic placement of these gymnosperm genes do not always follow organismal phylogeny, and duplication events may have occurred on the lineage leading to the extant gymnosperms

after the separation of the angiosperm and gymnosperm lineages (Sundstrom et al. 1999; Winter et al. 2002a).

It has been hypothesized that the clades containing *AP3* and *PI* arose from a duplication event before the diversification of the extant angiosperm lineages (Doyle 1994; Kramer et al. 1998; Kramer and Irish 2000; Munster et al. 2001; Purugganan 1997; Purugganan et al. 1995; Theissen et al. 1996, 2000), and recent studies including basalmost angiosperm lineages support this hypothesis (Aoki et al. 2004; Kim et al. 2005a; Stellari et al. 2004). In the angiosperms, both *DEF* and *GLO* genes have been identified from the basalmost lineages *Amborella* and the Nymphaeales, the next most basal lineage, the Austrobaileyales, and a number of basal angiosperm lineages, including magnoliids and monocots (Kramer and Irish 2000; Kim et al. 2005a,b; Stellari et al. 2004) (see Figure 3). Additionally, *DEF* and *GLO* genes have been isolated in many monocots representing most major lineages, including the grass, asparagus, and lily families (Caporali et al. 2000; Mena et al. 1995; Munster et al. 2001; Tzeng and Yang 2001). In the eudicots, a large number of genes have been isolated in the basalmost lineages, including the poppy and buttercup families (Kramer et al. 1998; Kramer and Irish 2000; Skipper 2002). Within the core eudicots lineages there has been extensive sampling in the two largest groups, the asterids and rosids, and less so in the other core eudicot lineages including the pink family (Hardenack et al. 1994; Matsunaga et al. 2003; Sheppard et al. 2000; Yao et al. 1999; Yu et al. 1999). This dense sampling among species at almost every hierarchical level in the angiosperms makes the *DEF/GLO* MADS-box subfamily one of the best represented plant nuclear genes.

Given the large amount of sampling, many phylogenetic reconstructions have been performed on the *DEF/GLO* MADS-box genes. Several major duplication events have occurred in the history of these genes and the duplication of *DEF/GLO* genes is apparently an ongoing process (Aoki et al. 2004; Fukui et al. 2001; Kim et al. 2005a; Kramer et al. 1998, 2003; Kramer and Irish 1999, 2000; Mouradov et al. 1999; Purugganan et al. 1995; Stellari et al. 2004). It has been proposed that various taxa may have retained (or regained) the potential for developmental flexibility through these gene duplication events in the *DEF* and *GLO* lineages (Ferrario et al. 2003; Kim et al. 2004; Kramer et al. 2003; Munster et al. 2001). *DEF/GLO* MADS-box genes have also been shown to evolve at a faster rate than other MADS-box genes in the core eudicots (Purugganan 1997). This rapid evolutionary rate, or additional duplication events that may become apparent with increased sampling, may explain why some *DEF/GLO* relationships and the placement of individual genes may not follow the organismal phylogeny of known monophyletic groups. Nevertheless, the phylogeny of the *DEF/GLO* subfamily in angiosperms has been shown to generally track organismal phylogeny (Figure 3), allowing for the clear identification of duplication events in their evolutionary history (Aoki et al. 2004; Kim et al. 2004; Stellari et al. 2004).

In the monocots there have been several duplication events within the *GLO* clade but not in the *DEF* clade
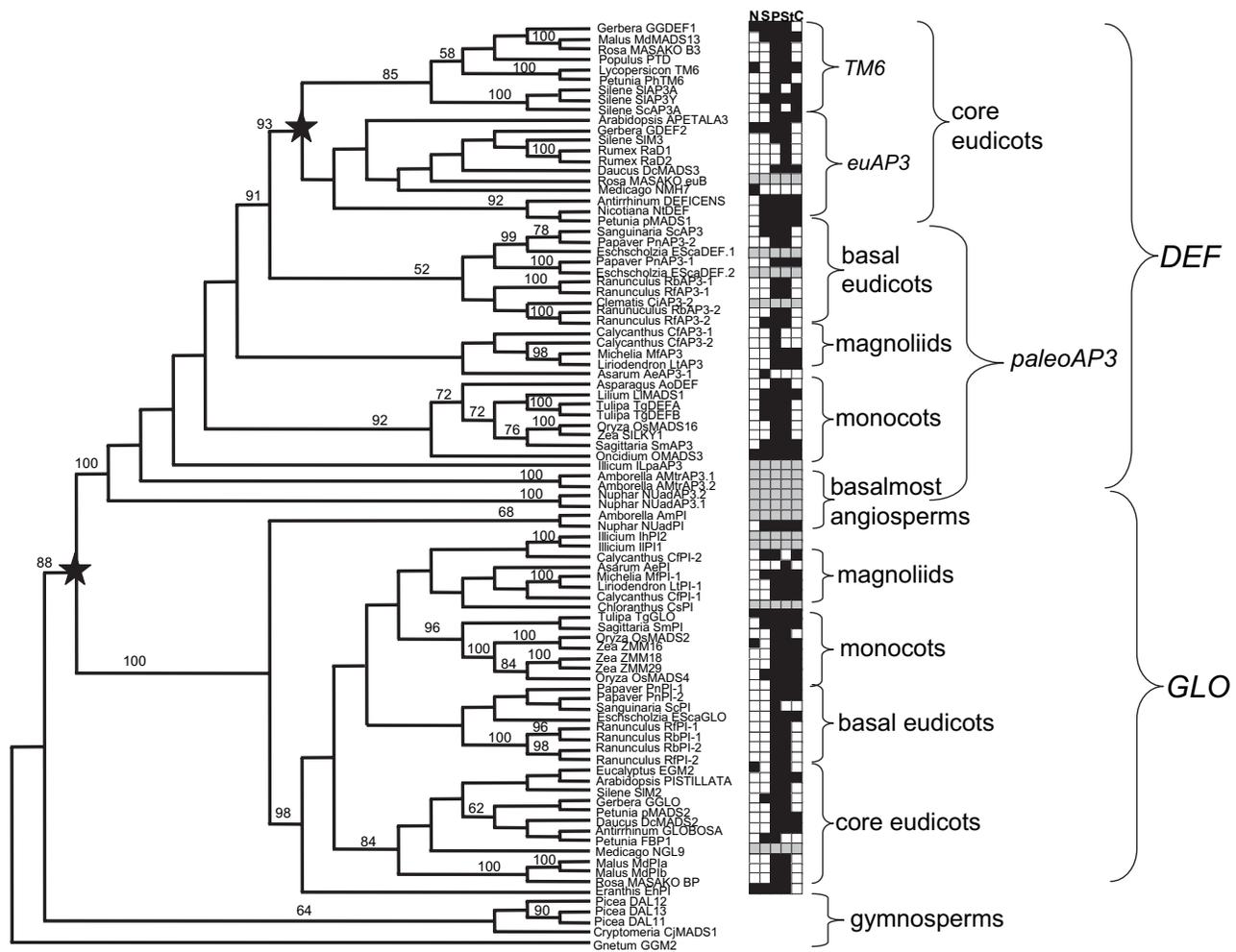
Figure 3. A summary of expression pattern for *DEF/GLO* homologs in a phylogenetic context. Genes with known expression from the *euAP3, TM6, paleoAP3,* and *GLO* clades are denoted on a maximum likelihood tree based on manual alignment of the entire gene. Bootstrap values greater than 50 are noted. An early gene duplication in the *DEF/GLO* subfamily that preceded the diversification of all living angiosperm lineages is indicated with a star. Boxes are placed next to terminal taxa and shaded black to denote expression or white to denote no recorded expression in nonfloral organs (N), the sepals or outer perianth (S), the petals or inner perianth (P), the stamens (St), and the carpel (C). Gray boxes denote taxa where experimental evidence is not yet available. Brackets denote the phylogenetic placement of genes from the major angiosperm lineages.

(Munster et al. 2001; Winter et al. 2002b). This is especially remarkable given that each major clade in the *AP1, AG,* and *SEP* subfamilies has at least two copies in rice and maize (Litt and Irish 2003; Kramer et al. 2004; LMZ, JL-M, CWD, HM, unpublished data). This suggests that the *DEF* lineage in the grasses, and perhaps the monocots as a whole, may have been evolutionarily constrained (Munster et al. 2001; Winter et al. 2002b).

In basal eudicots there have been multiple duplication events in both the *DEF* and *GLO* clades (Kramer et al. 1998). Many of the *GLO* duplication events are relatively shallow and most likely occurred fairly recently in specific lineages within families before the split of closely related genera (Kramer et al. 1998; Kramer and Irish 1999, 2000). However, the *DEF* lineage has retained gene copies from at least one and possibly two duplication events that apparently occurred early in the evolution of the Ranunculales (Kramer et al. 1998; Kramer and Irish 1999, 2000; unpublished data). Although lacking strong bootstrap support, three clades, AP3-I, AP3-II, AP3-III, have been identified in the Ranunculales and represent at least one gene duplication event that occurred near the base of this lineage (Kramer and Irish 2000), based on the hypothesized phylogeny of the ranunculids (Hoot and Crane 1995).

In addition to the duplication event that gave rise to the *DEF* and *GLO* clades, a second major duplication event occurred in the *DEF* lineage at the base of the core eudicots giving rise to the *TM6* and *euAP3* lineages (Kramer et al. 1998; Kramer and Irish 2000). Of note is the fact that the *Arabidopsis* lineage has apparently lost its *TM6* gene copy,

although *TM6* orthologs have been obtained from several rosid and asterid species.

Novel Function and Expression among *DEF/GLO* Orthologs

We undertook a survey of expression in the *DEF/GLO* subfamily to investigate the extent of conservation and divergence between orthologs and paralogs. Expression of the *GLO* homolog, *EScaGLO* from California poppy (*Eschscholzia californica*), as demonstrated by *in situ* hybridization, is typical of many *DEF/GLO* genes and is shown in Figure 4. In our survey, we focused on function when data is available, but there is a lack of functional studies outside of well-studied model organisms and we are limited to use mRNA expression patterns to estimate function. We are aware that there appears to be a separation of low-level expression from function among members of the *DEF/GLO* subfamily as supported by the fact that *DEF* and *AP3* demonstrate expression in the sepals and carpels but no protein nor mutant phenotype is detected in these organs (Goto and Meyerowitz 1994; Jack et al. 1992, 1994; Trobner et al. 1992). Therefore one cannot conclusively assign function to a *DEF/GLO* gene on the basis of expression data alone. Nonetheless, some expression, sometimes even low levels, are most definitely correlated with function in the *DEF/GLO* subfamily, and the diversity of expression patterns among orthologs and paralogs still aids us in our understanding of shifting expression, which may reflect function, that occurred over the course of this family's evolution.

We surveyed the detection of transcripts among *DEF/GLO* orthologs and found many intriguing patterns (Figure 3). It has been hypothesized that *DEF* and *GLO* genes in the basal angiosperms specified male function (Irish 2003). This theory also proposes that following the duplication of the *DEF* lineage in the core eudicots, genes in the *euAP3* clade were recruited for the specification of petals (Irish 2003). However, gene expression studies show that *DEF* and *GLO* orthologs may have been involved in perianth specification well before the origin of the eudicots (Figure 3). Moreover, we see in this example that although there is some phylogenetic signal in gene expression patterns (and presumably function), tissue-specific gene expression varies greatly among orthologs. This result underscores the importance of distinguishing between ancestry as inferred in gene phylogenies (e.g., *DEF* or *GLO* orthologs) and function (e.g., B-class function) as inferred from expression or functional studies (Theissen, 2002).

Among the members of the *DEF/GLO* lineages examined, only one gene, a *DEF/euAP3* ortholog in *Medicago*, *NMH7*, has no floral expression. *NMH7* is only expressed in root nodules at the beginning of the symbiotic zone and the central tissue of the nodule in inoculated roots (Heard and Dunn 1995). Although NMH7 can be easily aligned for most of its sequence with other euAP3 proteins, it has an 18-amino-acid extension at the C-terminal end. Expression in the root is hypothesized to always be a result of
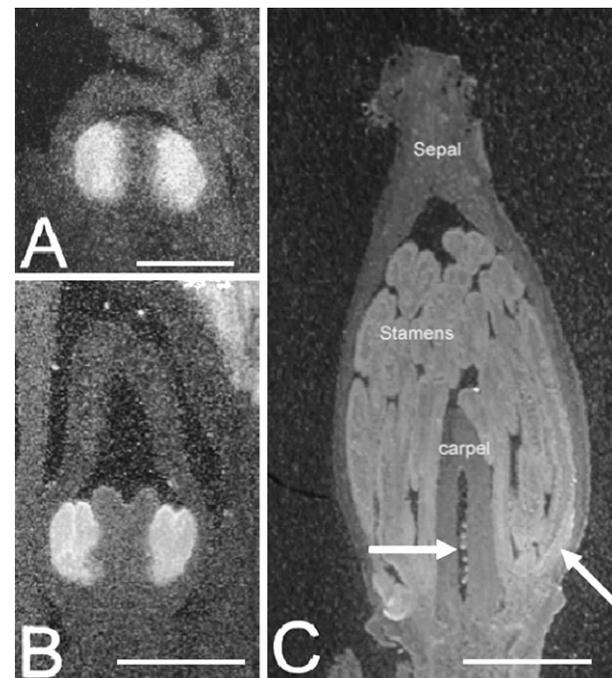
Figure 4. An *in situ* hybridization experiment using *EScaGLO*, a *GLO* ortholog from *Eschscholzia californica* (California poppy), a member of the basal eudicot ranunculid clade. (**A**) Expression at the stage corresponding to stage 3 in *Arabidopsis* (Smyth et al. 1990) when the sepals have differentiated from the rest of the floral meristem. Signal is high in the regions of the floral primordia that will become the petals and stamens. The white line denotes 0.5 mm. (**B**) Expression at a later stage corresponding to *Arabidopsis* stage 5 (Smyth et al. 1990), demonstrates high level of signal in the stamen primordia but not in the carpel primordia. Not shown is that strong expression at this stage is also in the small petal primordia. The white line denotes 1 mm. In the upper right of this image there is a bright area corresponding to the bottom of an older bud with signal in the petals and stamens. (**C**) Expression at the stage corresponding ~ to stage 9 in *Arabidopsis* (Smyth et al. 1990) shows high levels of signal in the petal primordia (white arrow) and stamens and a moderate level of expression in the developing ovules. The white line denotes 5 mm. This high expression in the ovules is unique in that in most ranunculids the expression in the carpel is too weak to detect with *in situ* hybridization techniques (Kramer and Irish 1999).

neofunctionalization in MADS-box genes (Ricker personal communication), which appears especially plausible for a function in root nodules rather than ordinary root tissue. A *GLO* ortholog has also been identified from *Medicago*, but its expression is not known. Further studies of this sequence and function of these genes along with the identification of additional genes from *Medicago*, especially any *TM6* or more *euAP3* orthologs, if present, may add to our understanding of the changes in function that have occurred among the *DEF/GLO* lineages.

Besides *NMH7*, several other *DEF* and *GLO* orthologs may have novel functions outside of the flower, on the basis of their expression. The function of *TM6* (also *TDR6*), a *DEF* paralog in the *TM6* lineage from tomato, is not known, but it has low levels of expression in the leaves, meristems, petals, stamens, carpels, and the seed and higher levels of expression in the developing fruit (Busi et al. 2003; Pnueli et al. 1991). On the basis of these studies it has been speculated that *TM6* may function both in flower and fruit development (Busi et al. 2003). The *GLO* ortholog from apple, *MdPI*, is expressed solely in flowers but may have a role in regulating fruit development. When *MdPI* expression is removed flowers lack both petals and stamens and parthenocarpic fruits develop (Yao et al. 2001). The monocot orchid *DEF* ortholog, *OMADS3* is expressed in all four floral whorls as well as in the leaves (Hsu and Yang 2002). *OMADS3* is hypothesized to have a meristematic effect on the basis of experiments that ectopically expressed *OMADS3* in *Arabidopsis*. These transgenic *Arabidopsis* plants flowered earlier than wild type, lost meristem indeterminacy, and produced terminal flowers having two to three carpels (Hsu and Yang 2002).

Other *DEF/GLO* genes exhibit expression in both the floral and nonfloral organs, although the low levels of expression detected may not reflect function. The eucalyptus *GLO* ortholog *EGM2* is expressed very weakly in leaves, along with varying intensities of expression among the floral organs (Southerton et al. 1998). In gerbera, a member of the sunflower family, the *DEF* orthologs, *GDEF1* and *GDEF2* are expressed in the leaves, bracts, and scapes and in the floral head and roots (Yu et al. 1999). *GDEF2* is more strongly expressed in all these tissues, although expression was significantly higher in the floral head than in any nonfloral organ (Yu et al. 1999). Additionally, the *DEF* ortholog *NtDEF* from tobacco is expressed in the entire floral meristem, although later in development strong expression of this gene becomes restricted to the flower in the sepal, petal, stamen, and carpel primordia (Davies et al. 1996a). In the basal eudicots, the winter aconite *Eranthis EhAP3* and *EhPI* genes were expressed in the vasculature of the plant and therefore are expressed in the leaves, stems, and within the flower in the tepals, honey-leaves, and stamens along with the floral organs (Skipper 2002). The monocot maize *GLO* ortholog, *ZMM16*, is expressed in the lodicules, stamens, and carpels but is also expressed weakly in the leaves, root, and young seedlings (Munster et al. 2001).

Besides floral specific development, sex-specific development appears to potentially have evolved in the *TM6 DEF* lineage. In the dioecious *Silene latifolia* and its close relatives *S. diocia* and *S. diclinis* there are two genes, *SlAP3Y* and *SlAP3A*, one of which (*SlAP3Y*) has been mapped to the Y chromosome (Matsunaga et al. 2003). *SlAP3Y* is only expressed in male buds at low levels in the rudimentary gynoecium, at moderate levels in the petal, and strongly in the stamens (Matsunaga et al. 2003). In male buds *SlAP3A* is expressed highly in the developing petals, whereas in female buds it is expressed strongly in the petals and moderately in the style primordia and ovary (Matsunaga et al. 2003).

Interestingly *SlAP3Y* apparently has no X chromosome homolog, which would be expected if it is involved in sex determination (Matsunaga et al. 2003). A closely related hermaphrodite species, *S. conica*, was found to have only a single gene, *ScAP3A*, which is expressed similarly to *SlAP3A* (Matsunaga et al. 2003). These data suggests that the Y chromosome specific *SlAP3Y* gene may have been involved in the transition from hermaphroditism to dioecy (Matsunaga et al. 2003).

Although these examples of subfunctionalization are limited to the eudicots, the mapping of expression patterns in the basal eudicots and basal angiosperms does not support the hypothesis that ancestrally *DEF* and *GLO* orthologs functioned only in the stamens and were recruited into the petals only in the core eudicots (Figure 3). Instead, it has been shown in the caryophyllid *Silene* that a novel recruitment occurred in a *TM6* homolog from the petal into the stamen. Furthermore, expression in a representative of the basalmost taxa, *Nuphar*, suggests that the *GLO* homolog is expressed in all floral organs and is highest in the second and third whorl, similar to that observed in *Arabidopsis* and *Antirrhinum* (Kong and Hu personal communication).

The expression of *DEF/GLO* genes tend to be floral and localized to the flower, suggesting that there were multiple ancestral functions of these genes and that their expression is not constrained. We noted a spectrum of expression patterns among diverse taxa, suggesting that gene function among *DEF* and *GLO* orthologs is somewhat labile, most likely a result of gene duplication. The range of expression patterns, even among clearly orthologous sequences, is both dramatic and inconsistent with the expectation that these genes have maintained a single conserved function in the evolution of the angiosperms. Based on our results, we hypothesize that both *GLO* and *DEF* are expressed in both the perianth and stamens in the basalmost lineages, suggesting that this trait is ancestral to the extant angiosperms. Furthermore, we hypothesize that multiple gene duplication events among taxa have led to multiple incidents of sub- and neofunctionalization that resulted in the patterns observed today.

DEF/GLO Genes and Their Protein–Protein Interactions

In both *Arabidopsis* and *Antirrhinum, DEF/GLO* proteins must form a heterodimer to function properly as transcriptional regulators. As discussed previously, these heterodimers regulate both *AP3* and *PI* (or *DEF* and *GLO*) transcription (Riechmann et al. 1996a; Schwarz-Sommer et al. 1992). Although heterodimerization appears to be the rule for the proper function, via DNA binding, of the core eudicot DEF/GLO proteins (Goto and Meyerowitz 1994; Honma and Goto 2000; Immink et al. 2003; Riechmann et al. 1996a; Schwarz-Sommer et al. 1992), homodimerization has been observed in vitro, and AP3/AP3 homodimers have been identified in yeast and immunoprecipitation experiments (Riechmann et al. 1996a,b; Sundstrom and Engstrom 2002). It has recently been suggested that the C-terminus of the *AP3* and *PI* genes confer specific functionality (Lamb

and Irish 2003), and this region is known to be important in the assembly of ternary protein complexes required for proper MADS-box gene function (Egea-Cortines et al. 1999; Ferrario et al. 2003; Honma and Goto 2001; Theissen and Saedler 2001).

Unlike the apparent constraint of heterodimerization among the core eudicots, some monocots have the ability to either heterodimerize and/or homodimerize. The orchid *DEF* homolog *OMADS3* forms homodimers in vitro and appears to be unable to form heterodimers with either AP3 or PI in *Arabidopsis* (Hsu and Yang 2002). *GLO* orthologs from tulip and lily demonstrated the ability to form homodimers and bind to DNA (Kanno et al. 2003; Winter et al. 2002b). However, this ability to homodimerize may not be conserved among the monocots as the *DEF* ortholog *SILKY1* only forms heterodimers with the maize *GLO* homolog (Ambrose et al. 2000; Whipple et al. 2005).

Obligate heterodimerization may be a relatively recent development. It has been suggested, based on differences in amino acid residues at key locations in the protein coding region, that the *Amborella* Am.tr.AP3 protein may be less able to dimerize with the Am.tr.PI protein than the AP3/PI complex in *Arabidopsis* (Kim et al. 2004). It has also been suggested that *DEF* and *GLO* homologs in *Amborella*, with their relatively undifferentiated C-terminal ends, may also have retained the ability to homodimerize, although experimental evidence is currently lacking (Kim et al. 2004; Soltis et al. 2005; Stellari et al. 2004). If Am.tr.AP3 and Am.tr.PI are functionally indistinguishable, it may mean that the range of multimer complexes is greater in *Amborella* and possibly other basal angiosperms (Kim et al. 2005a).

Homologous DEF/GLO homodimers have been identified in several gymnosperm genes (Hsu and Yang 2002; Sundstrom et al. 1999; Tzeng and Yang 2001; Winter et al. 2002a). The gymnosperm GGM2 protein was demonstrated to preferentially homodimerize in vitro rather than heterodimerize with AP3, DEF, or GLO through gel retardation assays (Winter et al. 2002b). Furthermore, GGM2 does not interact with AP3, PI, or another *Gnetum DEF/GLO* homolog GGM15 in yeast-2-hybrid studies (Winter et al. 2002b). It is possible that the proper partner for GGM2 heterodimerization has not yet been identified, but GGM2 homodimers have demonstrated proper DNA binding function, which suggest that it is able to function properly on its own (Winter et al. 2002b). Although the data support that homodimerization is the ancestral state in the gymnosperms, this is not necessarily so. The gymnosperm DAL11 and DAL13 proteins are able to form heterodimers with each other and with AP3 but not PI (Sundstrom and Engstrom 2002). It is important to note that the ability to heterodimerize may have arose multiple times among *DEF/GLO* genes, as both *DAL11* and *DAL13* are recent paralogs and may have only evolved the ability to heterodimerize quite recently, and maize may have recently re-evolved obligate heterodimerization (Whipple et al. 2005). Based on these studies it has been postulated that homodimerization is ancestral and the homodimerization observed in the monocots may represent a transitory state between obligate

homodimerization, as observed in the gymnosperms, and obligate heterodimerization, observed in the core eudicots (Theissen and Becker 2004; Winter et al. 2002b). A model of the evolution of binding patterns of DEF and GLO proteins is presented in Figure 5.

To B or Not to B in the Seed Plants

It has been suggested that the fern *CRM3* protein from *Ceratopteris* (Munster et al. 1997) has a sequence similar to many members of the *DEF/GLO* subfamily (Kramer et al. 1998). Nam et al. (2003) used molecular clock estimates to calculate that the age of the *DEF/GLO* lineage originated ∼ 600 million years ago (MYA), which well predates the fern–seed plant split at about 400 MYA (Savard et al. 1994). This supports the hypothesis that the *DEF/GLO* subfamily has a deep ancestry. However, extensive gene sampling and phylogenetic analyses have provided no significant support placing any fern gene as basal to the rest of the *DEF/GLO* subfamily. Further identification and phylogenies using fern, gymnosperm, and angiosperm genes will be needed to determine if there are indeed true *DEF/GLO* homologs within the ferns.

Studies of the *DEF/GLO* subfamily from the extant gymnosperms demonstrate that they are less diverse in their expression patterns than angiosperms in that they are only expressed in the male reproductive units (the microsporophylls). However, expression patterns do vary among duplicate gymnosperm *DEF/GLO* genes (Becker et al. 2003; Fukui et al. 2001; Mouradov et al. 1999; Sundstrom et al. 1999; Sundstrom and Engstrom 2002; Winter et al. 1999). Some gymnosperm genes partially complement *ap3* or *pi* mutants when ectopically expressed in *Arabidopsis* (Winter et al. 2002a). When *DAL11* and *DAL12* are expressed in *Arabidopsis*, the resulting phenotype is similar to the ectopic expression phenotype of *PI* (Sundstrom and Engstrom 2002). These findings strongly suggest that gymnosperm and angiosperm B genes have potentially conserved interaction partners and equivalent functions in the male reproductive organs (Albert et al. 1998; Theissen and Becker 2004). It has been suggested, therefore, that microsporangia in the angiosperms and gymnosperms are homologous and that B-function is conserved in the specification of organ identity for pollen-bearing organs (Albert et al. 1998; Sundstrom et al. 1999; Sundstrom and Engstrom 2002; Theissen and Becker 2004). These data suggest that B-function probably specified male reproductive organs in the most recent common ancestor of both the extant gymnosperms and angiosperms 300 MYA (Theissen and Becker 2004; Winter et al. 1999).

However, some gymnosperm *DEF/GLO* homologs appear to have a significantly different function from their angiosperm counterparts. Ectopic expression of *DAL13* in *Arabidopsis* did not phenocopy the ectopic expression of *AP3* or *PI* and produced flowers with stamens in whorls one, two, and/or four (Sundstrom and Engstrom 2002). Furthermore, when *GGM2* was expressed in wild-type *Arabidopsis*, it apparently disrupted proper B-function. The resulting flowers had stretched out sepals with petaloid cells along the margins, carpels with reduced amounts of stigmatic tissue
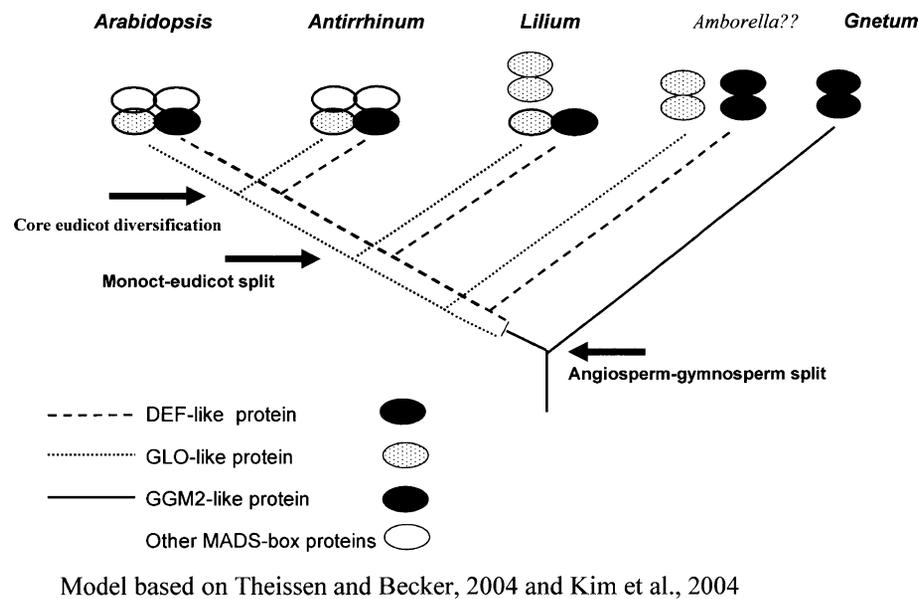
Figure 5. A model of the evolution of protein–protein interactions between DEF and GLO proteins as demonstrated in *Arabidopsis*, *Antirrhinum*, *Lilium*, and *Gnetum* and as hypothesized by Kim et al. (2005a) in *Amborella*. This model assumes that in the core eudicots *Arabidopsis* and *Antirrhinum* heterodimerization is obligate, in the monocot *Lilium* heterodimerization occurs between DEF and GLO proteins, and homodimerization occurs among GLO proteins. In the basalmost angiosperm *Amborella*, it is hypothesized that homodimerization occurs preferentially between GLO and DEF proteins; in the gymnosperm *Gnetum*, proteins only homodimerize.

and poorly developed valves, mosaics of carpel and filament cells, and sterile stamens unable to develop properly (Winter et al. 2002a). This disruption of function suggests that compatibility between GGM2 and the *Arabidopsis* floral proteins may have been lost through evolution or that neofunctionalization may have occurred.

The timing of the split between the *DEF* and *GLO* lineages has been difficult to estimate as these genes evolve at an accelerated rate relative to other MADS-box genes (Kramer and Irish 2000; Nam et al. 2003; Purugganan et al. 1995; Purugganan 1997). Recent studies that attempt to accommodate the variable evolutionary rates observed in the *DEF/GLO* subfamily have estimated that the *DEF* and *GLO* clades diverged ~ 260 MYA with a range between 145–316 MYA (Aoki et al. 2004; Kim et al. 2005b). It is therefore possible that this event occurred near the hypothesized divergence of angiosperms from extant gymnosperms around 300 MYA (Goremykin et al. 1997; Savard et al. 1994; Wolfe et al. 1989).

The function and expression of *DEF/GLO* genes in most lineages of angiosperms are still being studied in many taxa. The function of *DEF* and *GLO* homologs in the basalmost angiosperms is not yet thoroughly known, although these genes in *Amborella*, *Nuphar* (a member of the water lily family) and *Illicium* (Austrobaileyales) are expressed broadly across the flower (Kong and Hu personal communication; Kim and Soltis personal communication). Expression studies are available in the magnoliid and monocot lineages, and there are a few functional studies within the grasses. These studies demonstrate that most *DEF/GLO* genes are expressed strongly in the inner perianth and stamens, although some genes in the Calycanthaceae (allspice family) and Aristolochiaceae (Dutchman's pipe or wild ginger family) are not expressed in the stamens (Kramer and Irish 2000) (Figure 3). Expression in the sepals and carpels among the noncore eudicots is highly variable, although common. The expression of *DEF/GLO* genes among the basal eudicots is primarily in the stamens and petals and is also highly variable in expression in the sepals and carpels (Kramer and Irish 2000).

Despite varying expression in many floral whorls, the *paleoAP3* genes might confer stamen but not petal identity (Lamb and Irish 2003). This hypothesis was supported by an experiment where a construct of a C-terminal *euAP3* region was substituted with a *paleoAP3* C-terminal region which was able to rescue the stamens in an *ap3* mutant background but not the petals, which remained sepalloid (Lamb and Irish 2003). However, expression of *SILKY*, the putative *DEF* ortholog from maize, not only restored stamen development but also petal development in the *Arabidopsis ap3* mutant, even though *SILKY* encodes a *paleoAP3* rather than a *euAP3* motif (Whipple et al. 2005). This demonstrates that a *paleoAP3* motif can be sufficient for specifying petals. Given the large number of *DEF* gene duplications within the Ranunculales, which includes *Dicentra* from which the *paleoAP3* gene was a donor gene for this experiment, one cannot rule out that this particular case represents a more recent specialization that occurred following gene duplication events. Alternatively, a stronger expression of *SILKY* in transgenic plants compared to wild-type *AP3* expression might have compensated for functional deficiencies implied by the presence

of the *paleoAP3* rather than the *euAP3* motif (Whipple et al. 2005). Similar experiments from other species may help elucidate if divergence among duplicate copies results in different functions within the *euAP3* and *paleoAP3* lineages.

The consequence of *PI* and *GLO* expression in the ovules is not understood because there is no fourth whorl effect on *pi* or *glo* mutants, suggesting that these genes have little or no effect on ovule development (Goto and Meyerowitz 1994; Trobner et al. 1992). Viable seeds are produced in *ap3* mutants when pollinated by normal pollen, suggesting that *AP3* has either no or a minimal role in ovule and seed development (Jack et al. 1992). Given that almost no fourth whorl effects on organ identity have been observed in mutants of any *DEF/GLO* genes observed to date, it may be that this ancestral function was lost but the expression of these genes in the carpals has been retained. There may be previously unidentified proteins that interact with DEF, GLO, or both in the fourth whorl. Alternatively, one could suggest that expression in the fourth whorl reflects leakiness in gene regulation. However, given the importance of these genes and their widespread occurrence in the fourth whorl of many angiosperm lineages, we hypothesize that the expression of *DEF/GLO* genes in the ovule reflects a hitherto unidentified function in the development of the fourth whorl that is apparently conserved among many taxa. This function must be significantly less critical than *DEF* and *GLO* function in the petals and stamens of the core eudicots. Interestingly, the gymnosperm *DAL11* gene was cloned from a seed cone library and has been hypothesized to be expressed at very low levels and nonfunctional (Sundstrom et al. 1999). Consequently, we suggest that the expression of *DEF/GLO* orthologs in the female reproductive organs may reflect an ancestral function in the gymnosperms. It thus might not be just by chance that the putative sister genes of the *DEF/GLO* (B) genes, the B$_{sister}$ genes, are almost exclusively expressed in female reproductive organs, especially ovules, where also their function is focused (Becker et al. 2002; Nesi et al. 2002).

From the accumulation of data about floral MADS-box genes in a variety of core eudicot species, we are beginning to observe patterns that suggest a relationship between changes in MADS-box gene number and morphological evolution. Although these relationships are not fully understood at this time, we can use them to make predictions about the changes the floral MADS-box genes have undergone in their evolution. It is clear that novel function(s) in the formation of the petals were developed between the diversification of the angiosperms and gymnosperms and are most likely due to the establishment of novel function within the *DEF/GLO* subfamily (Kim et al. 2005a; Theissen and Becker 2004), perhaps as a result of gene duplication. Furthermore, the *AGAMOUS* subfamily has undergone similar gene duplication events, and its function is equally important in another angiosperm specific character, the carpel. It may be that gene expression among the floral MADS-box genes has always been highly variable, as observed among *AGAMOUS* homologs (unpublished data) and *DEF/GLO* homologs. We suggest that this nonfixed expression coupled with additional gene copies of cofactors allowed for a diversity of protein–protein interactions that may have led to the formation of novel organs rather rapidly, through ectopic expression of novel gene combinations. This is not a far-fetched idea, as novel function and increased complexity as a result of gene duplication events and overlapping has been observed in the animals in the *HOX* gene family of transcription factors, as reviewed in Hughes and Kaufman (2002). However, this flexibility in B gene expression domains may have been significantly reduced in the core eudicots on the establishment of obligate heterodimerization of DEF and GLO proteins, which may have contributed to the canalization of the core eudicot flower structure (Winter et al. 2002b).

In conclusion, although the expression and function of members of the *DEF/GLO* subfamily are not as variable as reported for the *AGAMOUS* subfamily (Kramer et al. 2004; unpublished data), the function of *DEF/GLO* genes are highly complex. The *DEF/GLO* MADS-box subfamily is not simply a family of genes that specify male reproductive organs and the petals in the angiosperms, although they may have been major components of their evolution. We demonstrate in this review the importance and need for more functional studies of these genes, especially to determine if they have function in the forth whorl and to understand why expression boundaries are so variable. Additionally, we demonstrate the importance of establishing model organisms of basal lineages with transformation ability. With a better understanding of the changes that occurred throughout gymnosperm and angiosperm evolution in the expression and function of these genes we may piece together the genetic basis of the puzzle of Darwin's abominable mystery of how the angiosperms originated and diversified.

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