Hidden Variability of Floral Homeotic B Genes in Solanaceae Provides a Molecular Basis for the Evolution of Novel Functions

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B-class MADS box genes specify petal and stamen identities in several core eudicot species. Members of the Solanaceae possess duplicate copies of these genes, allowing for diversification of function. To examine the changing roles of such duplicate orthologs, we assessed the functions of B-class genes in \textit{Nicotiana benthamiana} and tomato (\textit{Solanum lycopersicum}) using virus-induced gene silencing and RNA interference approaches. Loss of function of individual duplicates can have distinct phenotypes, yet complete loss of B-class gene function results in extreme homeotic transformations of petal and stamen identities. We also show that these duplicate gene products have qualitatively different protein–protein interaction capabilities and different regulatory roles. Thus, compensatory changes in B-class MADS box gene duplicate function have occurred in the Solanaceae, in that individual gene roles are distinct, but their combined functions are equivalent. Furthermore, we show that species-specific differences in the stamen regulatory network are associated with differences in the expression of the microRNA miR169. Whereas there is considerable plasticity in individual B-class MADS box transcription factor function, there is overall conservation in the roles of the multimeric MADS box B-class protein complexes, providing robustness in the specification of petal and stamen identities. Such hidden variability in gene function as we observe for individual B-class genes can provide a molecular basis for the evolution of regulatory functions that result in novel morphologies.

INTRODUCTION

Specification of floral organ identities depends on the combined functions of a number of MADS box transcription factors. In \textit{Arabidopsis thaliana}, plants that lack these factors lose floral organ identities and instead develop leaf-like organs, while conversely, ectopic expression of such genes is sufficient to convert leaves into floral organs (Pelaz et al., 2000, 2001; Honma and Goto, 2001). The combinatorial action of these transcription factors is biochemically effected through the formation of higher-order transcriptional complexes that likely serve to bring both a transcriptional activation domain to the complex as well as facilitate protein complex stability and DNA binding (Riechmann et al., 1996; Egea-Cortínez et al., 1999; Honma and Goto, 2001; Immink et al., 2009; Melzer and Theissen, 2009; Melzer et al., 2009).

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Petal and stamen identities are specified by the combined activities of \textit{APETALA3 (AP3)} and \textit{PISTILLATA (PI)} in \textit{Arabidopsis} or their orthologs \textit{DEFICIENS (DEF)} and \textit{GLOBOSA (GLO)} in \textit{Antirrhinum majus} (Jack et al., 1992; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994). In both species, these B-class gene products form obligate heterodimers that in turn appear to act in conjunction with several other MADS box proteins to affect individual organ identities (Honma and Goto, 2001; Pelaz et al., 2001). Obligate heterodimerization of AP3 and PI in \textit{Arabidopsis} appears to be necessary for stable localization to the nucleus as well as for DNA binding (Krizek and Meyerowitz, 1996; McGonigle et al., 1996). Similarly, heterodimerization of DEF and GLO is also necessary for DNA binding, and interactions with the SQUA MADS box transcription factor can enhance DNA binding affinity of this heterodimer (Egea-Cortínez et al., 1999).

Comparisons between \textit{Arabidopsis} (a rosid) and \textit{A. majus} (an asterid) of petal and stamen identity specification suggested an overall strong conservation in the regulatory circuit that establishes the identity of these organs. However, both \textit{Arabidopsis} and \textit{A. majus} appear to represent a derived state from an ancestrally more complex regulatory system. Phylogenetic analyses have shown that a duplication in the DEF lineage occurred at the base of the core eudicots, resulting in the euAP3 and TOMATO MADS6 (TM6) lineages (Kramer et al., 1998). A duplication also occurred in the GLO lineage, such that core asterids possess two such genes as well (Viaene et al., 2009). Within the
asterids, the Solanaceae represent this ancestral state, in that tomato (*Solanum lycopersicum*) possesses all four B-class genes, as does petunia (*Petunia × hybrida*) (Vandenbussche et al., 2004; de Martino et al., 2006; Rijpkema et al., 2006) (Table 1).

Although their DNA binding capabilities are unlikely to have changed considerably during evolution, analyses of some of these B-class genes suggested that their developmental roles have been parsed differently in different members of the Solanaceae. For instance, functional analyses of the DEF lineage genes in tomato demonstrated that these genes have distinct functions, with the *euAP3* gene *TOMATO APETALA3* conditioning both petal and stamen development, while the *TM6* gene functions predominantly in specifying stamen identity (de Martino et al., 2006). In *Petunia*, though, loss of function of the *euAP3* gene *GREENPETALS* (*GP*; also known as Ph DEF) only affects petal identity, whereas *TM6* is required for stamen identity (Tsuchimoto et al., 2000; Rijpkema et al., 2006). Furthermore, there are differences between tomato and *Petunia* in terms of the protein interaction specificities of the *euAP3* and *TM6* lineage gene products (de Martino et al., 2006; Rijpkema et al., 2006; Leseberg et al., 2008).

A comparative study of the DEF and GLO lineage functions in Solanaceae provides an interesting starting point to understand how a duplicate regulatory system evolves. Few studies are available that include functional information to understand the evolutionarily forces that act on duplicate gene lineages and the outcomes that ensue (Hittinger and Carroll, 2007; Des Marais and Rauscher, 2008). Here, we consider three main theoretical models to understand the evolution of B-class gene duplicates. These models capture aspects of functional diversification and are not mutually exclusive. They can be distinguished by the selective constraints that act upon the duplicate lineages throughout the main phases in the life history of a duplicate gene lineage: initial fixation, subsequent fate determination, and final preservation (Innan and Kondrashov, 2010). The classical model that first explained the preservation of duplicate gene lineages uses the idea of neofunctionalization (Ohno, 1970). In this model, relaxed selection on one copy because of the presence of a duplicate allows the evolution of novel functions in this selected copy, while an ancestral copy retains its function. However, this model has not been adequate in explaining the frequency with which duplicate lineages are retained (Force et al., 1999). A second model, which has been most often applied to MADS box gene evolution, is the duplicate degeneration complementation model. This extends the concept of Ohno’s neofunctionalization with the idea of subfunctionalization (Force et al., 1999). Key in this subfunctionalization model is that the fixation of the duplicate pair is the outcome of degenerate mutations in both copies and that, rapidly after duplication, both duplicate lineages partition the original ancestral function and thus acquire complementary roles. A third model explaining retention of duplicate lineages emphasizes the role of dosage balance (Freeling and Thomas, 2006; Birchler and Veitia, 2007). In this model, duplicate genes whose products are involved in protein complexes can be expected to be retained with increased probability to maintain overall dosage of the functional complex. Such a model may have importance in understanding B-class gene evolution because B-class genes act as members of transcriptional complexes (e.g., Honma and Goto, 2001). Finally, a fourth model, “escape from adaptive conflict,” describes two functions of an ancestral gene that both can be improved (Hughes, 1994; Des Marais and Rausher, 2008). This improvement becomes possible after duplication because the constraint of the two functions being performed by a single gene is relieved after duplication.

To comprehensively examine the diversification of functions of B-class genes in the Solanaceae and to understand which theoretical models may explain this system, we have examined the developmental roles of all four B-class genes in *Nicotiana benthamiana* and in tomato. We assessed the relative contributions of these gene products in specifying petal and stamen identities through single and double loss of function combinations, as well as by characterizing their protein interaction capabilities and cross-regulatory interactions in the respective species. Together, these observations suggest that orthologous B-class genes have acquired distinct functional roles in each of these Solanaceae species, yet the B-class multiprotein complex as a whole has retained a common function. Furthermore, phenotypic differences between *N. benthamiana* and tomato B-class mutants appear to be mediated by differential expression of the microRNA miR169. Because we find that the GLO lineage genes are undergoing positive selection, this suggests that there is a balance between rapid evolution of individual gene functions and maintenance of the roles of multimeric MADS box protein complexes in effecting organ identity.

### RESULTS

#### Duplication in the GLO Lineage Predates the Origin of Core Lamiids and Is Possibly Older

The Solanales contain multiple copies of DEF lineage and GLO lineage genes (Table 1). The DEF duplication that gave rise to the *euAP3* and *TM6* lineages occurred early in the radiation of

<table>
<thead>
<tr>
<th>Orthologous Gene Lineage Names</th>
<th><em>Petunia × hybrida</em></th>
<th><em>S. lycopersicum</em></th>
<th><em>N. benthamiana</em></th>
</tr>
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<tr>
<td>GLO1</td>
<td>Ph GLO1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI GLO1 = TPIB = Le P&lt;sub&gt;f&lt;/sub&gt;</td>
<td>Nb GLO1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GLO2</td>
<td>Ph GLO2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SI GLO2 = TPI&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Nb GLO2&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>TM6</td>
<td>Ph TM6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>TM6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Nb TM6&lt;sup&gt;bc&lt;/sup&gt;</td>
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<tr>
<td><em>euAP3</em></td>
<td>GP = Ph DEF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TAP3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Nb DEF&lt;sup&gt;e&lt;/sup&gt;</td>
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As reported in (a) Vandenbussche et al. (2004), (b) Rijpkema et al. (2006), (c) Leseberg et al. (2008), (d) de Martino et al. (2006), and (e) this work.
the core eudicots, considerably predating the origin of the Solanales (Kramer et al., 1998). To identify the likely origin of the GLO lineage duplication, we performed phylogenetic analyses of GLO lineage genes from representatives of all euasterid orders (except Garryales). Maximum likelihood, parsimony, and Bayesian analyses all provide support for the duplication event at least predating the origin of core lamiales, before the joint origin of extant Solanales, Lamiales, and Gentianales species (Figure 1; see Supplemental Data Set 1 online). However, the duplication may have occurred even earlier, before the origin of euasterids (Viaene et al., 2009). Our current sampling of GLO sequences has not allowed us to confidently resolve the exact timing of the inferred duplication, and it may even have occurred early in core eudicot evolution, coincident with the duplication in the DEF lineage (Kramer et al., 1998). It will require the addition of more taxa to obtain conclusive support for any one of these three possibilities. All three scenarios imply minimal inferences of reciprocal gene loss, with the apparent loss of the GLO1 lineage in Gentianales and loss of the GLO2 lineage in Lamiales. The availability of more whole-genome sequences will allow further testing of this pattern. For the purpose of this study, though, the phylogeny demonstrates that the Solanaceae genes being characterized in this study are orthologous (Table 1).

Silencing of \textit{N. benthamiana} GLO1, GLO2, or TM6

We used tobacco rattle virus–mediated virus-induced gene silencing (TRV-VIGS) to characterize the functions of B-class genes in \textit{N. benthamiana}. This method has been previously demonstrated to generate a dramatic knockdown of transcript and protein levels of DEF, the \textit{N. benthamiana} euAP3 lineage gene (Liu et al., 2004). DEF-silenced plants displayed a marked transformation of petals into sepals and stamens into carpelloid structures (Liu et al., 2004). Using gene-specific fragments (see Methods), we silenced GLO1, GLO2, and TM6 individually and in various combinations. Silencing was demonstrated to be strong and gene specific, in that transcripts of the other B-class genes were still detectable in loss of function of individual \textit{Nb} genes (see Supplemental Figure 1 online).

Wild-type \textit{N. benthamiana} sepals have an epidermis that is characterized by the presence of puzzle-shaped cells, the stomata are more densely spaced on the abaxial surface than adaxially, and many trichomes are present (Figures 2A to 2G). \textit{NbGLO1-VIGS} plants show a number of defects in the development of petals and stamens (Figures 2H to 2M). Most flowers of these plants develop sepaloid tissues in petals and carpelloid tissues in stamens. This results in the bending and shortening of the petal tube in most flowers. In the most extreme cases, the

![Figure 1. Maximum Likelihood Phylogeny of Euasterid GLO Lineage Genes Used to Test for Adaptive Evolution.](image-url)

Euasterid representatives from the orders Solanales, Gentianales, Lamiales, Asterales, Apiales, Dipsacales, and Aquifoliales (Garryales was not sampled). Node support values indicate maximum likelihood bootstrapping parsimony bootstrapping/Bayesian posterior probabilities. Branch lengths are in expected number of changes per site.
Figure 2. Floral Phenotypes of N. benthamiana Wild-Type and Individually Silenced B-Class Genes.

(A) to (G) Wild type.
(H) to (M) GLO1-VIGS.
(N) to (P) GLO2-VIGS.
(Q) to (W) TM6-VIGS.

(A) Wild-type corolla tube with anthers attached through short filaments.
(B) Wild-type abaxial petal tube epidermis with fusiform cells.
(C) Wild-type corolla throat abaxial petal epidermis with puzzle-shaped cells.
(D) Wild-type petal tube adaxial epidermis with rectangular cells.
(E) Wild-type corolla throat adaxial petal epidermis showing round cells.
(F) Wild-type abaxial sepal epidermis.
(G) Wild-type adaxial sepal epidermis.
(H) NbGLO1-VIGS, crumpled corolla tube with greenish midveins.
(I) NbGLO1-VIGS, abaxial corolla epidermis has several cell types, some shorter than in the wild type.
(J) NbGLO1-VIGS, adaxial corolla cells become crenelated.
petal tube does not lengthen because of many shallow folds, resulting in a wrinkled appearance. This seems to be the consequence of the strong transformation of the petal midveins into sepal midveins that thus become shorter, forcing the second whorl tissue to fold (Figure 2H). Stomata can occasionally be found on the surface of transformed petals, and the outline of many epidermal cells shows crenellations, which start to resemble the puzzle-shaped cells of wild-type sepal epidermal cells (Figures 2I and 2J). Adnation of stamens to the corolla tube is often lost, resulting in free stamens (Figure 2L). These stamens can also show partial transformation to carpelloid (Figure 2M). Several flowers in which GLO1 was silenced showed the separation of the petal tube into individual petals (Figure 2K). We did not observe this effect in TRV-NbGLO2-treated plants (Figure 2N). The strongest effect results in stamens that are replaced by carpels. Remarkably, a single transformed stamen consistently developed two short styles and stigmas, rather than one (Figure 2P). Together, these results suggest that GLO1 has an additional specific role in the fusion of the petals into a tube.

NbGLO2-VIGS plants show some similarities and some differences compared with NBGL1-VIGS plants. Although NBGL2-VIGS plants do not show separations of the petal tube, they still often have lost the adnation of the stamen filaments to the corolla tube (Figures 2N and 2O). Other effects of downregulation of GLO2 are the development of short styles and stigmas between the locules of the anthers or the presence of ovules inside the anthers (Figures 2O and 2P). The strongest effect results in stamens that are replaced by carpels. Remarkably, a single transformed stamen consistently developed two short styles and stigmas, rather than one (Figure 2P). Together, these results suggest that GLO1 and GLO2 are not functionally redundant and that both genes are required for development of petals and stamens.

Similar to what can be observed in GLO1− or GLO2-silenced flowers, the petal tube bends or dents in TM6-silenced flowers (Figure 2Q). This was accompanied by changes in epidermal cell identity (Figures 2R and 2S). These flowers show only weak phenotypes in stamen development. However, in some flowers, stigmatic tissue developed in the stamen anther, and the filament of these stamens was not fused to the adaxial side of the petal tube (Figures 2T and 2U). In N. benthamiana NbTM6-VIGS plants, we also observed several flowers that develop fruits with no or few seeds, suggesting that ovule abortion occurs more often in flowers with downregulated TM6 levels than in wild-type flowers (Figures 2V and 2W). Although stamens of these flowers always produce pollen, ovule abortion could be a consequence of reduced pollen viability.

In the Absence of N. benthamiana B-Function, Complete Homeotic Conversions of Petals and Stamens Occur and Additional Sepal-Like Organs Develop

To assess the complete loss of GLO lineage gene function in N. benthamiana, we downregulated both GLO1 and GLO2 using a concatenated construct. In the double NbGLO1− NbGLO2-VIGS plants, we observed flowers showing a strong conversion of petals into sepalis and stamens into carpels (Figure 3A). The petal-to-sepal transformation is also evident in that the abaxial and adaxial epidermal cells of the second whorl are morphologically similar to those of wild-type sepalis (Figures 2B to 2E, 2B, 3B, and 3C). In addition, we observed that carpelloid third-whorl organs that result from the transformed stamens are often fused with the gynoecium along the ovary and style and that this results in a single, but lobed, stigma (Figure 3D). Fusions between transformed stamens that were not fused to the central gynoecium were also occasionally observed, suggesting that the capacity to fuse is organ type specific, rather than whorl specific. For each of the transformed stamens that fused with the central gynoecium, two small leaf-like organs developed at the base of the style (Figures 3D and 3E). The surface of this organ displays crenellated epidermal cells, similar to those of wild-type sepalis (Figure 3F).

We also generated a complete loss of DEF lineage function by coordinately silencing DEF in combination with TM6, which produced essentially identical phenotypes to those produced by NbGLO1− NbGLO2-VIGS plants. These phenotypes included a full conversion of petals into sepalis (Figure 3G), a full conversion of third-whorl stamens into carpels as evidenced by epidermal cell transformations (Figures 3H and 3I), and the development of small sepal-like organs either between the whorl of sepaloid petals and the multilocular gynoecium (Figure 3J) or attached to the gynoecium (Figure 3K). Interestingly, we found two of these sepal-like organs developing per fused third-whorl organ. The identity of these organs is more sepal-like than petal-like, as evidenced by the presence of stamata, numerous trichomes, and puzzle-shaped epidermal cells (Figures 3L and 3M).
In addition, it appears that relative to wild-type sepals, chlorophyll levels are somewhat reduced in these curled organs (Figure 3O).

To test whether the combined silencing of TM6 with either GLO gene or of DEF with either GLO gene would result in additional phenotypes compared with the silencing effects of single or lineage-specific knockdowns, we used concatenated constructs to silence various N. benthamiana genes in combination. In NbTM6-NbGLO1-VIGS plants, flowers develop petals that are crumpled and have greenish sepaloid veins (Figure 4A). Stamen filaments lose adnation to the corolla tube (Figures 4A and 4B) or are more completely transformed into carpels. Ovules abort acropetally after an initial period of normal development (Figure 4C). NbTM6-NbGLO1-VIGS or NbTM6-NbGLO2-VIGS plants develop flowers with phenotypes that are similar to those downregulated in GLO1, GLO2, or TM6 alone (Figures 2Q to 2W and 4D to 4F).

In NbDEF-NbGLO1-VIGS plants, we observed a complete transformation of petals into sepals and stamens into carpels (Figures 4G to 4J). Again in this case, for every carpelloid third-whorl organ that fuses with the central gynoecium, two small organs develop with sepaloid identity (Figures 4I and 4J). We observed the same phenotypic effects in NbDEF-NbGLO2-VIGS plants (Figures 4K to 4N). Together, these observations indicate that when DEF is silenced in combination with GLO1 or GLO2, complete homeotic transformations of second and third whorls are observed but that when individual B-class genes or specific lineages of B-class genes are silenced, only a subset of phenotypic alterations are observed.

**Figure 3.** Floral Phenotypes Produced by VIGS Silencing of Gene Lineages Using Concatenated Constructs. NbGLO1-NbGLO2-VIGS ([A] to [F]) and combined NbDEF-NbTM6-VIGS silencing ([G] to [N]). Bars in μm, B=35, C=26, F=21, I=211, J=49, M=66, n = 76. (A), (D), (G), (J), (K), and (N) are stereomicroscopy photographs, and (B), (C), (E), (F), (H), (I), (L), and (M) are scanning electron micrographs. (A) NbGLO1-NbGLO2-VIGS flower, with front sepals and petals removed. Corolla fully transformed into calyx and stamens into carpels. (B) Abaxial sepaloid second-whorl organ epidermis. (C) Adaxial sepaloid second-whorl epidermis. (D) Transformed carpels fused into a central multilocular gynoecium with a lobed stigma; small leaf-like organs (arrow) developed on top of the ovary and at the base of the style. (E) and (F) These third-whorl leaf-like organs developed many trichomes (E), and are characterized by an abaxial epidermis with crenellated cells (F). (G) NbDEF-NbTM6-VIGS flower illustrating a complete transformation of petals into sepals and stamens into carpels. (H) Epidermal cell surface of wild-type ovary. (I) Epidermal cell surface of transformed stamen. (J) Carpeloid stamens can fully or partially fuse to the central gynoecium, and for each fusion event, a third-whorl leaf-like organ (arrow) develops. (K) Close observation shows that two small leaf-like organs develop for every carpelloid stamen (arrows). (L) to (N) Epidermal cells of these organs are puzzle shaped (outline) and are similar to sepals ([L] and [M]) and curl toward the abaxial side ([N]: left abaxial, right adaxial side).
Tomato SIGLO1-RNAi and SIGLO2-RNAi Are Weakly Affected in Stamen Development

To characterize GLO lineage gene function in tomato, we analyzed phenotypes of RNA interference (RNAi) lines in which each GLO gene was silenced separately (Figure 5). We obtained 13 lines in which GLO1 was silenced and five lines in which GLO2 was silenced.

Wild-type tomato flowers of variety Micro-Tom develop five petals alternating with five sepals; the third whorl consists of a staminal cone in which long anthers are joined by lateral hairs (Figure 5A). Petals develop trichomes on the abaxial side, while the adaxial surface develops rounded cells except for the petal vein, where the cells are more elongated (Figures 5B and 5C). The sepal abaxial epidermal cell surface develops irregular cells with characteristic multicellular trichomes and many stomata (Figure 5D), while the adaxial surface cells are even more irregular (Figure 5E). In SIGLO1-RNAi plants, stamens lacked interweaving hairs and did not close (Figure 5F), while petals appeared as wild-type (Figures 5C, 5G, and 5H). We also observed more fully transformed carpelloid stamens (Figure 5I). These organs matured into a sterile carpel attached to the base of a fruit (Figure 5J). We observed essentially the same phenotypes in GLO2-silenced plants (Figures 5K to 5O). Aberrant phenotypes were limited to the stamen whorl (Figure 5K), and petals were unaffected (Figures 5L and 5M). More often, anthers were only weakly affected, and the tip or base of the stamen cone was separated (Figures 5N and 5O).

Double SIGLO1-SIGLO2-RNAi and tap3 Plants Show Complete Homeotic Conversions of Petals and Stamens and Develop Additional Carpel-Like Organs

When both GLO1 and GLO2 were silenced using a concatenated RNAi construct, stamens were fully converted into carpels and petals into sepals (Figures 6A to 6G). Interestingly, we observed a similar phenomenon in these transgenic tomato plants as we saw in the equivalent N. benthamiana transgensics: stamens that are transformed into carpels most often fuse with the central gynoecium (Figure 6D). This fusion is complete at the tissue level as illustrated by scanning electron microscopy of the adjoining tissues (Figure 6E). Scanning electron microscopy was used also to confirm the complete conversion of petals into sepals in these

Figure 4. Floral Phenotypes Produced by VIGS Silencing of Paralogous Genes Using Concatenated Constructs.

Phenotypes of NbTM6-NbGLO1-VIGS ([A] to [C]), NbTM6-NbGLO2-VIGS ([D] to [F]), NbDEF-NbGLO1-VIGS ([G] to [J]), and NbDEF-NbGLO2-VIGS ([K] to [N]) flowers. Bars ~26 μm in (I) and (J) and 23 μm in (M); n = 49. (A) to (H) are stereomicroscopy photographs, and (I), (J), (M), and (N) are scanning electron micrographs. (A) to (C) In NbTM6-NbGLO1-VIGS flowers, the corolla tube petal-to-sepal transformation is most pronounced along the midveins (A), filaments of weakly transformed stamens are not adnate to the corolla (B), and ovules abort acropetally (C). (D) and (E) NbTM6-NbGLO2-VIGS corolla shows similar petal-to-sepal transformation along the midvein (D) and strongly transformed stamens (E), while petal lobes are weakly affected. (F) Placenta with ovules aborting acropetally. (G) NbDEF-NbGLO1-VIGS flowers that show complete transformation of petals into sepals and stamens into carpels. The latter often fuse to the central gynoecium. (H) to (J) For every carpeloid-carpel fusion event, leaf-like organs develop (H), with a sepaloid abaxial (I) and adaxial (J) epidermis. (K) NbDEF-NbGLO2-VIGS flowers develop sepaloid second-whorl organs and carpelloid third-whorl organs that can partially or completely fuse with the central gynoecium. (L) to (N) For every complete fusion of third-whorl-transformed stamens to the central gynoecium (L), small leaf-like organs develop with a sepaloid abaxial (M) and adaxial (N) epidermis.
lines (Figures 6B and 6C). The transformed stamens mature into ripe fruit structures, although they never produce seeds (Figures 6F and 6G). Furthermore, for each transformed third-whorl organ that fuses to the central whorl, a small organ develops with no obvious resemblance to a wild-type tomato floral organ (Figure 6H). This extra organ is located in the position of the transformed stamen that fuses with the central carpels and could thus be interpreted as being a third-whorl organ. Alternatively, these organs could correspond to second-whorl organs as they arise at the margin of second-whorl petals transformed into sepals and alternate in position with the second-whorl organs (Figure 6I). Scanning electron microscopy showed that the tip of this organ consists of stigmatic papillae, and after close inspection, we noticed that the organ can partially mature into a tiny fruit-like carpel (Figures 6J to 6N). The fact that these organs can fuse with either the second or fourth whorl suggests that the capacity to fuse is organ specific, rather than whorl specific.

Because the tomato RNAi lines in which both GLO2 and GLO1 were silenced together phenocopy the previously described tap3 euAP3 lineage mutant (de Martino et al., 2006), we reinvestigated flowers of the tap3 mutant to see if they also developed these small ectopic organs. In tap3 mutant flowers, we also observed similar small carpelloid organs in the position of transformed and fused carpelloid stamens. These organs fuse to the central gynoecium or alternate and fuse with the second-whorl organs. Scanning electron microscopy further identified these organs as carpelloid with a stigma and style (Figures 6P to 6Q), and these organs even have the capacity to ripen (Figures 6R and 6S).

**Early Expression of B-Class Genes in N. benthamiana and Tomato**

To determine where Solanaceae B-class genes are expressed early in flower development, we performed in situ hybridizations. In *N. benthamiana, DEF* is strongly expressed in the region of the floral apex from which the petal and stamen primordia will develop (Figure 7A). This expression is maintained when corolla and stamen primordia separate (Figure 7B). The expression pattern of TM6 is markedly different from that of DEF: expression was weakly observable throughout the floral apex in the early stages of flower development, although expression was strong in the placental tissue and developing ovules at later stages of development (Figures 7C to 7E). Both *N. benthamiana* GLO paralogs have similar expression patterns, which resemble that of DEF. After sepal primordia have developed, a ring-shaped region of the floral apex strongly expresses GLO1 and GLO2 (Figures 7F and 7H). This expression is maintained at later developmental stages (Figures 7G and 7I).

For tomato, the expression patterns of GLO2, TM6, and TAP3 have been previously reported by de Martino et al. (2006). To complement this information, we examined the expression patterns of GLO1 and GLO2 by in situ hybridization at various stages of flower development. GLO1 expression was detected in the central region of the floral apex, similar to that of DEF (Figures 7I to 7L). GLO2 expression was also detected in the central region of the floral apex, but with a more diffuse pattern than GLO1 (Figures 7M to 7O). These results indicate that GLO1 and GLO2 are expressed in similar regions of the floral apex, but with different expression patterns. This suggests that these genes may have different roles in floral development.

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(L) and (M) Petals seem unaffected, as illustrated by scanning electron micrographs of the abaxial epidermis (L) and adaxial epidermis (M). (N) and (O) Weakly affected stamens separate at the tip (N) or base (O) of the stamen cone.
pattern of the GLO1 gene in flowers. GLO1 had a very similar expression pattern to that of GLO2 (Figures 7J and 7K). In early developmental stages, GLO1 is expressed in incipient petal and stamen primordia, while in later stages, its expression was restricted to tapetal tissue and the lateral edges of the growing petals.

Protein–Protein Interaction Specificity Differs Quantitatively between Species

Based on work in Arabidopsis and A. majus, it has been shown that B-class proteins form obligate heterodimers that increase protein stability, facilitate nuclear localization, and are necessary for DNA binding (Tröbner et al., 1992; McGonigle et al., 1996; Riechmann et al., 1996). To determine if there are differences in protein–protein interactions of the B-class proteins in Solanaceae, we examined the abilities of the N. benthamiana B-class proteins to dimerize in all possible combinations using yeast two-hybrid assays. Both GLO1 and GLO2 interacted strongly with DEF, while NM6 showed stronger interactions with GLO2 than with GLO1 (Table 2). Furthermore, TM6 and DEF failed to interact, and we did not observe an above-background interaction between GLO1 and GLO2. In addition, we did not detect homodimerization capabilities for any of the four tested proteins. These observations indicate that the GLO and DEF lineage gene products preferentially interact with each other and that there is some degree of partner preference.

More pronounced observations of partner specificity have been noted for interactions among B-class proteins in tomato...
Tomato TAP3 only interacts with Sl GLO1, while TM6 only interacts with Sl GLO2 (Leseberg et al., 2008), and Petunia TM6 interacts only with GLO2, while DEF interacts with both GLO1 and GLO2 (Vandenbussche et al., 2004). This suggests that the degree of protein interaction differs between species. However, we did not identify differences in early expression between the *N. benthamiana* GLO orthologs, nor did we identify differences in early expression between tomato GLO1 and GLO2, and the early expression of Petunia GLO paralogs is also very similar, suggesting that functional divergence of GLO paralogs is not likely to be related to divergence in expression but rather to differences in protein–protein interaction specificity. If the latter is true, we may expect that the protein sequences evolve adaptively to maintain interaction specificity.

**Positive Selection of Sequence Sites Likely Contributed to the Functional Differentiation of GLO Lineages**

To further test this hypothesis, we investigated selective constraints on GLO amino acid sequence sites using the topology depicted in Figure 1 and Supplemental Data Set 1 online (Table 3). Sites in the euasterid GLO proteins evolve on average under purifying selection, as the one ratio model yielded an average dN/dS = 0.1421. However, selective constraints strongly vary among sites along the sequence (M0 versus M3, 2dL = 612.932, df = 4, P < 0.01). This is partly because some sequence sites are evolving under positive selection (M1a versus M2a, 2dL = 7.784, df = 2, P < 0.05; M7 versus M8, 2dL = 13.529, df = 2, P < 0.01). Bayes prediction of sites under positive selection estimates that in the MADS and I-domains, most sites are evolving under purifying selection. In these domains, <1% of sites evolve under relaxed purifying selection, and no sites are detected that evolve under positive selection. The K domain has 16% of sites for which purifying selection is relaxed, while all other sites evolve under purifying selection. Finally, in the C-terminal domain, selection on approximately half of the sites is either purifying or relaxed, and a single site close to the C-terminal PISTILLATA motif is significantly detected to evolve under positive selection.

To better understand how the Solanales GLO sequences acquired specific functions after duplication, we highlighted the

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**Figure 7. Early Expression of Class-B Genes as Observed Using in Situ Hybridization.**

Expression as determined by in situ hybridization of Nb DEF ([A] and [B]), Nb TM6 ([C] to [E]), Nb GLO1 ([F] and [G]), and Nb GLO2 ([H] and [I]) in wild-type *N. benthamiana* and Sl GLO1 in wild-type *S. lycopersicum* ([J] and [K]). s, sepal; p, petal; st, stamen; p/st, fused primordium; g, gynoecium; pl, placenta; ov, ovule.

([A] and [B]) DEF is expressed in joint petal-stamen primordial ([A]), and this expression is maintained when the petal primordia develop ([B]).

([C] to [E]) TM6 expression is weak in petal-stamen primordia ([C]) but strong in placenta tissue ([D]) and ovules ([E]).

([F] and [G]) GLO2 expression is strong in joint petal-stamen primordia ([F]) and maintained in petals and stamens ([G]).

([H] and [I]) GLO1 is expressed in joint petal-stamen primordia ([H]), and this expression is maintained in the developing corolla and stamens ([I]).

([J] and [K]) SIGLO1 is strongly expressed in incipient petal and stamen primordia ([J]) and becomes more confined to the corolla margins and the anther wall in later development ([K]).

[See online article for color version of this figure.]
sites that are conserved within their gene lineage but are different between lineages in a sequence alignment of GLO proteins (see Supplemental Figure 2 online). These sites are densely grouped within the K-domain, the domain encoded by MIKC-type genes that is involved in protein–protein interaction (Yang and Jack, 2004; Kaufmann et al., 2005), suggesting that differences in interaction specificity between GLO lineage gene products are derived from mutations in the K-domain.

Maintenance of B-Gene Expression Is under Different Transcriptional Control in *N. benthamiana* and Tomato

Regulatory evolution of transcription factors has been proposed as an important evolutionary mechanism driving morphological change (Doebley and Lukens, 1998), yet we still do not have a clear view of how such changes occur among closely related species. To understand the evolution of gene regulation of the B-class genes in Solanaceae, we performed a quantitative analysis of gene expression in the loss-of-function lines we generated for analysis of gene expression in the loss-of-function lines we

Table 2. Protein–Protein Interaction Specificity as Found Using Yeast Two-Hybrid Assays

<table>
<thead>
<tr>
<th>AD/BD</th>
<th>E-BD</th>
<th>NbGLO1-BD</th>
<th>NbGLO2-BD</th>
<th>NbTM6-BD</th>
<th>NbDEF-BD</th>
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</thead>
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<td>E-AD</td>
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<td>–</td>
</tr>
<tr>
<td>NbDEF-AD</td>
<td>–</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

DEF, GLO1, and GLO2 show expression patterns in dissected mature floral organs of *N. benthamiana* (Figures 8A to 8D) that are consistent with our in situ hybridization experiments. For these three genes, expression is nearly absent from first-whorl sepals but is strong in second- and third-whorl organs. By contrast, TM6 expression is much weaker in mature *N. benthamiana* petals and stamens but is strongly expressed in the ovary. In GLO1 loss-of-function flowers, expression of GLO2 and TM6 is reduced in the second- and third-whorl organs, and expression of DEF is reduced in third-whorl organs, suggesting that GLO1 positively regulates all three other B-class genes. In GLO2 loss-of-function plants, GLO1 and DEF expression is significantly reduced in second- and third-whorl organs, while TM6 expression is strongly reduced in the fourth whorl. Loss of TM6 function shows a pronounced effect on DEF expression in third-whorl organs, and expression of GLO1 in second-whorl organs is somewhat reduced. Together, these data demonstrate extensive and whorl-specific cross-regulation between B-class genes in *N. benthamiana*.

We also investigated the expression of the tomato B-class genes in mature floral organs in the wild-type and various loss-of-function situations (Figures 8E to 8H). Again, expression of GLO2, GLO1, and TAP3 is strongest in wild-type second- and third-whorl organs. Knockdown of GLO1 results in reduced second-whorl GLO2 expression, and knockdown of GLO2 causes a reduction in GLO1 expression, suggesting cross-activation of GLO2 and GLO1 in the second-whorl organs. TAP3 expression is higher in second- and third-whorl organs of SIGLO1-RNAi plants than the wild type, while TAP3 expression was reduced in response to GLO2 knockdown. This suggests that apart from responses that can be explained by direct regulation, there appear to be additional compensatory effects on gene expression. In the tap3 mutant, expression of GLO2 and GLO1 is completely absent in second- and third-whorl organs. The strong phenotype and pronounced effects of TAP3 on the expression of other B-class genes suggest that TAP3 has a primary role in establishing the regulatory pathways necessary for petal and stamen development.

MicroRNA169 Has a Different Expression Pattern in Flowers of Tomato and Tobacco

Because of the qualitative difference between the phenotypes of double GLO1-GLO2 loss of function in tobacco and tomato, we investigated whether a different expression pattern of miR169 could explain the differences between these closely related species. The BLIND locus in *Petunia* encodes miR169 and was found to act as a repressor of C-function in petals (Tsuchimoto et al., 2000; Cartolano et al., 2007). While the third-whorl organs in *NbGLO1-NbGLO2* double VIGS plants produce third-whorl petaloid sepals, the double SIGLO1-SIGLO2-RNAi lines in tomato produce third-whorl carpelloid organs. We followed expression of mature miR169 in wild-type organs of *N. benthamiana* and *S. lycopersicum* quantitatively by stem-loop RT-PCR (Chen et al., 2005). Interestingly, this putative repressor of C-function has a qualitatively different expression pattern in each species. In tomato, miR169 is strongly expressed in the ovary but mostly absent from the stamens (Figure 9A), whereas the inverse is true for tobacco, where expression in stamens is roughly sevenfold the expression level in the ovary (Figure 9B). Because miR169 functions as a repressor of C-function, this could explain why third-whorl transformations as seen in the double knockdown plants are different in both species: miR169 represses C-function in petals (Tsuchimoto et al., 2005). Interestingly, this putative repressor of C-function has a qualitatively different expression pattern in each species. In tomato, miR169 is strongly expressed in the ovary but mostly absent from the stamens (Figure 9A), whereas the inverse is true for tobacco, where expression in stamens is roughly sevenfold the expression level in the ovary (Figure 9B).

Table 3. Likelihood of Models Used for Likelihood Ratio Tests to Test for Adaptive Sequence Evolution

<table>
<thead>
<tr>
<th>Codon Model</th>
<th>Likelihood</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>M1a</td>
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<tr>
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</tr>
<tr>
<td>M8</td>
<td>-8831.098777</td>
</tr>
</tbody>
</table>
DISCUSSION

Gene duplication has long been recognized as an important driver in the evolution of variation that can lead to adaptive change (Ohno, 1970). Considerable information is now available on sequence diversification of gene duplicates through phylogenetic and molecular evolution studies, and a theoretical framework for various evolutionary fates is being refined (Conant and Wolfe, 2008; Innan and Kondrashov, 2010). Yet, studies integrating functional assays with comparative data are rare and may contribute to the understanding of gene fates in evolution and how these in turn contribute to phenotypic innovation and biodiversity.

To comprehensively assess the divergence in functions of duplicate MADS box genes in the Solanaceae, we have characterized orthologs of B-class genes in N. benthamiana and S. lycopersicum and compared these results to previously published analyses performed in Petunia (Vandenbussche et al., 2004; Rijpkema et al., 2006). Our phylogenetic analyses and previous studies indicate that the duplication in the GLO lineage of MADS box genes occurred at least before the radiation of the core lamiids and possibly before that of the euasterids as suggested by the analyses of Viaene et al. (2009). The presence of both duplicate lineages in Solanales allowed us to examine the diversification in function of orthologous genes using a combination of stable and transient transgenic loss-of-function approaches.
Comparisons of Loss of Function of Orthologs Indicates Divergence in Function

The phenotypic effects of loss of function of individual orthologs are quite distinct for each Solanaceae species examined, suggesting that there is considerable plasticity in the roles of each of these genes. For instance, loss of GLO1 lineage gene function in *N. benthamiana* results in considerable defects in both second- and third-whorl development. Similar second- and third-whorl phenotypic effects have been reported for the loss of Ph GLO1 function in *Petunia* (Vandenbussche et al., 2004). However, our analyses indicate that in tomato, GLO1 loss of function has no obvious effect on petal development. Furthermore, GLO1 function is required in both Petunia (Vandenbussche et al., 2004) and *N. benthamiana* (this work) flowers for corolla fusions, a feature that is not observed in tomato flowers. Similarly, GLO2 orthologs show different loss-of-function phenotypes in *Petunia*, *Nicotiana*, and tomato (Vandenbussche et al., 2004; this work).

While loss-of-function phenotypes of euAP3 homologs in *N. benthamiana*, *Petunia*, and tomato are similar (Liu et al., 2004; Vandenbussche et al., 2004; de Martino et al., 2006; Rijpkema et al., 2006; this work), loss-of-function phenotypes of TM6 orthologs are qualitatively distinct. Loss of *Nicotiana* TM6 results in weak defects in the petals, stamens, and ovules (this work). Loss of function of tomato TM6 results in defects predominantly in the stamens (de Martino et al., 2006), a phenotype that is similar to that of loss of *Petunia* TM6 function (Rijpkema et al., 2006). Together, these observations underscore the divergence in orthologous gene functions among closely related species.

Additionally, the degree of redundancy within the DEF gene lineage differs among Solanaceae species. This appears to reflect differences in the patterns of expression of euAP3 and TM6 gene orthologs in each species. In *Petunia*, DEF is strongly expressed in petal and stamen primordia, while TM6 is only weakly expressed in petals and strong expression can be observed in stamens (Rijpkema et al., 2006). Consistent with this pattern, loss of Ph DEF function results in defects only in petals, reflecting the redundant compensatory function of Ph TM6 in stamens (Rijpkema et al., 2006). However, in both tomato and *Nicotiana*, TM6 does not compensate for loss of the euAP3 paralog, since both tap3 mutant and NbDEF-VIGS plants show complete conversion of petals into sepals and stamens into carpels (Liu et al., 2004; de Martino et al., 2006; this work). TM6 function is completely redundant in *Petunia*, since loss of TM6 does not produce any obvious phenotype (Rijpkema et al., 2006). This is not the case for either tomato or *Nicotiana* for which mild homeotic phenotypes are observed in TM6 loss-of-function plants.

Together, these results suggest that there has been a shift in the role of TM6 in tomato and *Nicotiana* compared with *Petunia*. This is consistent with the most likely relationship of these three Solanaceae subfamilies, suggested to be [Petunioideae + Solanoideae + Nicotianoideae] (Olmstead et al., 1999; Wu et al., 2006; Olmstead and Bohs, 2007). In turn, this suggests that the functions we have ascribed to tomato and *Nicotiana* B-class genes can be interpreted as being derived from a possibly ancestral condition retained in *Petunia*. Alternatively, *Petunia* may represent an independent derivation of a distinct TM6 function from an ancestral condition present in species branching off earlier in the evolution of Solanales.

Conservation of B-Function Despite Differences in the Roles of Individual Components

Either the combined knockdown of GLO1/GLO2 or the combined knockdown of euAP3/TM6 lineage genes results in the complete homeotic conversion of organ identity, indicating that despite diversification of members within a lineage as shown by the single knockdown phenotypes, the combined function of both gene lineages is identical between species. This can be explained by the fact that products of both gene lineages are likely involved in establishing protein complexes regulating similar processes.

Based on the differential ability of individual DEF and GLO lineage gene products to form protein complexes, we suspect that several distinct B-class gene product protein complexes are formed in vivo. This may also be reflected by the pattern of molecular evolution of the K-domain within Solanales B-class genes. This domain is important in protein–protein interaction specificity and shows several sites that are conserved within individual GLO lineage genes but differ between lineages. Extrapolating from the different affinities of the GLO lineage gene products for their interaction partners in different species, the relative abundance of these complexes may also be different between species (Vandenbussche et al., 2004; de Martino et al., 2006; Leseberg et al., 2008; this study). As these complexes can be considered to regulate different subsets of target genes, target genes may be parsed differently between different protein complexes in different species. Furthermore, there appear to be compensatory shifts in overall B-class gene expression levels, which is consistent with the expectations of the gene balance hypothesis (Birchler and Veitia, 2007). Because knockdown of all B-class gene function results in homeotic transformations of both second- and third-whorl organs in *Nicotiana*, tomato, and *Petunia*, the complete suite of downstream targets is likely to be conserved across Solanaceae species.

Diversification in the Mechanism of Whorl Identity Specification

Probably the most dramatic example of functional divergence we have observed are qualitatively distinct outcomes of eliminating B-function from the flower altogether. In *Nicotiana*, strong loss of B-function results in the formation of ectopic sepal-like third-whorl organs, while in tomato, these organs develop as carpeloid structures. The differences in the expression of miR169 could explain these differences in third-whorl organ identity. In this model, miR169 would repress C-function in third-whorl organs of *Nicotiana* but not repress C-function in third-whorl organs of tomato because it is not expressed in this region. As a consequence, retention of C-function in the third whorl of tomato B-class loss-of-function plants would confer carpeloid characteristics. These differences in *Nicotiana* and tomato loss of B-function phenotypes could reflect species-specific differences in the postulated threshold at which miR169 exerts its effects on the regulation of C-function (Cartolano et al., 2007).
Several models have been used to explain the functional fates of genes after duplication. These models are not necessarily distinct and capture different aspects of the selective pressures and mechanisms acting on duplicate gene lineages (Innan and Kondrashov, 2010). Cases describing gene fates based on both sequence and functional analyses are rare, but the combination of models and detailed functional studies can refine the interpretation of cases and the theoretical framework of models (e.g., Force et al., 1999; Des Marais and Rausher, 2008).

Although we cannot unequivocally determine the timing of the GLO lineage duplication event, our data suggest an origin prior to the diversification of the core lamiids as a minimal estimate. This duplication could have occurred concomitant with the whole-genome duplication that is inferred to have occurred early in the radiation of the core eudicots (Blanc and Wolfe, 2004), which also corresponds to the presumed occurrence of the DEF lineage duplication at the base of the core eudicots (Kramer et al., 1998). However, an early core eudicot dating for the GLO gene duplication does imply that considerable gene loss events also occurred as these gene lineages evolved. Assuming a parallel duplication origin for both GLO and DEF lineage genes, we can postulate that this event allowed for subsequent diversification through a variety of changes. Because the GLO and DEF lineage gene products form obligate heterodimers, this may have constrained the mechanisms by which all four gene lineages evolved. The DEF lineages appear to have evolved in three ways: through changes in expression domains, changes in protein–protein interaction with GLO lineage gene products, and changes in the C-terminal domain. In addition, the euAP3 lineage may have undergone neofunctionalization as evidenced from the presence of positively selected sites and the origin of a novel C-terminal domain as a consequence of a frameshift mutation (Vandenbussche et al., 2004; Kramer et al., 2006; Hernández-Hernández et al., 2007). The GLO lineage, however, seems to have subfunctionalized mainly through changes in protein–protein interaction specificity as the two lineages have similar expression patterns in the species studied but have specialized interaction preferences. Positive selection would have remained or become active long after the duplicate lineages originated. Such selected sites may either be evolving adaptively or represent compensatory changes to maintain protein function. As such, they need not reside in the domain responsible for protein–protein interaction, since compensatory changes could contribute to overall stability or function of the protein (Camps et al., 2007). Alternatively, a more recent dating of the GLO lineage duplication would imply that the evolution of the DEF and GLO lineages may reflect a temporal order to this process, with the DEF lineage genes being subfunctionalized first and then driving the subfunctionalization of the GLO lineage gene duplicates through a dosage balance mechanism.

Because B-class genes encode transcription factors and regulate downstream target genes, they perform multiple functions. It is clear that the specificity of DNA binding is sensitive to many biochemical variables, and it can be considered that adaptive conflict is continuously present and reinforces the maintenance of multiple functions prior to gene duplication (Hughes, 1994). In such a situation, gene duplication would allow for each duplicate to escape these constraints and specialize such that both ancestral and novel functions can evolve. This “escape from adaptive conflict” model (Hughes, 1994; Des Marais and Rausher, 2008) seems somewhat less likely at first because one may expect sites in the MADS domain to have undergone positive selection. However, DNA binding specificity determinants are not limited to the protein-DNA binding interface but are also strongly influenced by protein–protein interactions (e.g., Egea-Cortínez et al., 1999). It may be that the processes captured by this model may help to understand how the subfunctions of the B-class genes have continued to evolve in Solanaceae. Together, our data for B-class genes reveal the complexity with which duplicate lineages evolve. In general, subfunctionalization, which sometimes involves loss of specific functions (e.g., loss of specific protein interaction capabilities) and sometimes involves the fixation of apparently deleterious mutations that then undergo adaptive compensation (e.g., as reflected in the positive selection on specific sites observed in the GLO lineages), appears to best describe the evolution of the duplicate B-class genes in the Solanaceae.

From a functional point of view, B-class MADS box gene products form a complex, but the individual roles of such genes are clearly different among different Solanaceae species. However, loss of function of the entire complex, through multiple mutations that disrupt the entire complex (or complexes), produces equivalent homeotic transformations, suggesting that selective pressure for a consistent protein complex function is maintained, despite plasticity of individual components. This kind of change presumably reflects compensatory changes in the complex components, resulting in an overall maintenance of complex function. Furthermore, our results indicate that considerable variation has occurred in the genetic mechanisms by which organ identity is specified, even among closely related Solanaceae species. These differences include differential parsing of B-class gene functions, different degrees of redundant gene activity, as well as differences in the likely roles of microRNAs involved in regulating organ identity specification. Despite these differences, though, floral organ identities across eudicots are strongly conserved, pointing to an overall evolutionary robustness in maintaining floral architecture through compensatory variation.

**METHODS**

**Phylogenetic Analysis**

We manually modified the aligned sequence matrix of Viana et al. (2009) in MacClade 4.05 (Maddison and Maddison, 2002) by adding cloned sequences of Hedera helix (Hh GLO), Heptacodium miconoides (Hm GLO), Scabiosa sp (Ssp GLO), Osmanthus sp (Os GLO1), Torenia fournieri (Tf GLO1), Borago sp (Bsp GLO), and Nicotiana benthamiana (Nb GLO1 and Nb GLO2). The primer sequences are presented in Supplemental Table 1 online.

We performed parsimony analysis using Paup 4b10 with 1000 bootstrap replicates with 10 random addition replicates and TBR branch swapping. Maximum likelihood analysis to estimate the single most likely topology was performed using PhyML and the GTR+I+G model (Guindon et al., 2010).
and Gascuel, 2003). This model was selected using Modeltest 3.7 (Posada and Crandall, 1998) according to the Akaike information criterion. Model parameters were likely estimated along with the phylogeny. Bootstrap analysis was chosen as a measure of branch support, and for each pseudoreplicate data set, the maximum likelihood tree was estimated using the GTR+I+G model with likelihood optimized model parameters. Finally, MrBayes (Ronquist and Huelsenbeck, 2003) was used to approximate the posterior probability distribution over tree space, again using the same substitution model (GTR+I+G). Also for Bayesian analyses, parameter estimations were likelihood optimized. Two analyses were run in parallel for 10 million generations, and convergence of the markov chains was followed using standard deviation of split frequencies. Because of the relatively simple tree space searched, apparent convergence was reached relatively early in the analysis, and exclusion of the first 50% of the sampled trees from the posterior distribution was judged ample sufficient.

Adaptive Evolution Tests

To test for signatures of adaptive evolution, the PAML program codeML (Yang, 2007) was used. This analysis is based on the idea that non-synonymous substitutions, dN, are expected to occur roughly with the same frequency as synonymous substitutions, dS, when a sequence is evolving without selective constraints (measured as omega = dN/dS). By default, PAML excludes from the data set all sites with gaps or ambiguities. To test for signatures of adaptive evolution, the PAML program codeML was run in parallel for 10 million generations, and convergence of the chains was followed using standard deviation of split frequencies. To formally test for adaptive evolution among sites, from the analysis; therefore, we opted to include missing data in the analyses, however, the data set contained some C-terminal partial sequences. To test for signatures of adaptive evolution, the PAML program codeML was run in parallel for 10 million generations, and convergence of the chains was followed using standard deviation of split frequencies. Because of the relatively simple tree space searched, apparent convergence was reached relatively early in the analysis, and exclusion of the first 50% of the sampled trees from the posterior distribution was judged ample sufficient.

In Situ Hybridization

Templates for RNA probe synthesis were amplified from the 3’ end of cDNA sequences using a reverse primer that included a T7 promoter (primer sequences between the two genes in these regions, these constructs with hygromycin resistance. Judging from the strongly different sequences between the two genes in these regions, these constructs were silenced together or individually using RNAi interference. A ±400-bp PCR product targeting the 3’ untranslated and C-terminal coding region was cloned using Gateway technology (Invitrogen) into a vector expressing a hairpin construct (Karimi et al., 2007) with hygromycin resistance. Judging from the strongly different sequences between the two genes in these regions, these constructs would target either SIGLO1 or SIGLO2 specifically. The double RNAi construct was made by concatenating the single PCR fragments in the order SIGLO2-SIGLO1 using blunt-end ligation. Constructs were transformed into LBA4404 Agrobacterium for cotyledon explant infection. For plant transformation, we followed the same procedures as described by Vrebalov et al. (2009). Correlation between transcript knockdown and phenotypic effects was demonstrated using RT-PCR using primer sets outside vector constructs (see Supplemental Table 1 online). For each construct, endogenous transcript levels were measured in four flowers that showed apparent phenotypes (n = 4) and four wild-type flowers. PCR reactions were three times repeated and end products were quantitated by measuring band intensity using the ImageJ software. In these same samples, actin expression was measured using the same procedure. Standard errors of the mean were calculated for the differences in expression between the gene of interest and actin, and relative expression was plotted in bar graphs using DeltaGraph.

Scanning Electron Microscopy

Standard formalin/acetic acid/alcohol fixative was used to fix tissues overnight with subsequent dehydration in an ethanol-water series. After dissection, the material was critical point dried and sputter gold coated for scanning electron microscopy according to a previously published protocol (Irish and Sussex, 1990).

VIGS in N. benthamiana

We followed the procedures described in Dinesh-Kumar et al. (2003). PCR fragments were cloned behind the 2× 35S promoter of TRV2 vectors using primers with BamHI or Xbal restriction sites (primer sequences in Supplemental Table 1 online). Constructs aimed at silencing two genes simultaneously were made by concatenating two sequences either by blunt-end ligation or by incorporating an EcoRI restriction site between the two sequences. Sequence verified constructs were transformed into GV101 Agrobacterium tumefaciens cells. Plants were started in growth chambers and 1 week after Agrobacterium infiltration of the leaves with TRV2 together with TRV1, the plants were transferred to the March Botanical greenhouse facilities of Yale University. Correlation between transcript knockdown and phenotypic effects was demonstrated using RT-PCR using primer sets outside vector constructs (see Supplemental Table 1 online). For each construct, endogenous transcript levels were measured in four flowers that showed apparent phenotypes (n = 4) and four wild-type flowers. PCR reactions were three times repeated and end products were quantitated by measuring band intensity using the ImageJ software. In these same samples, actin expression was measured using the same procedure. Standard errors of the mean were calculated for the differences in expression between the gene of interest and actin, and relative expression was plotted in bar graphs using DeltaGraph.

RNAi in Solanum lycopersicum var Micro-Tom

SIGLO1 and SIGLO2 were silenced together or individually using RNAi interference. A ±400-bp PCR product targeting the 3’ untranslated and C-terminal coding region was cloned using Gateway technology (Invitrogen) into a vector expressing a hairpin construct (Karimi et al., 2007) with hygromycin resistance. Judging from the strongly different sequences between the two genes in these regions, these constructs would target either SIGLO1 or SIGLO2 specifically. The double RNAi construct was made by concatenating the single PCR fragments in the order SIGLO2-SIGLO1 using blunt-end ligation. Constructs were transformed into LBA4404 Agrobacterium for cotyledon explant infection. For plant transformation, we followed the same procedures as described by Vrebalov et al. (2009). Correlation between transcript knockdown and phenotypic effects was demonstrated using the same procedure as for VIGS experiments.

Yeast Two-Hybrid Analysis

Because MADS domain sequences were not available, we tested dimeric interactions of IKC fragments of N. benthamiana TM6, GLO1, GLO2, and DEF. PCR products were ligated into vectors pGBT9 and pGADT424 (Clontech) using EcoRI and Sal restriction sites in primers (see Supplemental Table 1 online). Constructs were cotransformed into yeast strain Y109. As previously described, we used a β-gal liquid assay with ortho-nitrophenyl-β-galactoside as a substrate and detected yellow coloring as a measure for reporter gene activation and B-GAL unit measurement (de Martino et al., 2006).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: GLOBOSA sequences of Hesperocodium miconium, Borago officinalis, Hedera helix, Scabiosa sp, N. benthamiana NbGLO1, and N. benthamiana NbGLO2.
were submitted to GenBank and received accession numbers HQ005413 to HQ005418.

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure 1. Reduction of Transcript Levels as a Consequence of VIGS or RNAi Silencing in N. benthamiana or Tomato.
Supplemental Figure 2. K-Domain Alignment of Solanales and Gentianales GLOBOSA Lineage Proteins.
Supplemental Table 1. Primer Sequences.
Supplemental Data Set 1. Alignment of GLOBOSA Sequences.

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REFERENCES


Hidden Variability of Floral Homeotic B Genes in Solanaceae Provides a Molecular Basis for the Evolution of Novel Functions
Koen Geuten and Vivian Irish

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