Changes in Expression Pattern of the *Teosinte Brached1*-like Genes in the Zingiberales Provide a Mechanism for Evolutionary Shifts in Symmetry Across the Order

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Symmetry is an important aspect of floral form, from both an ecological and developmental standpoint (Endress, 1999; Giurfa et al., 1999). Flowers are most often described as having many planes of symmetry (actinomorphic) or one plane of symmetry (zygomorphic). Rarely, they may have no observable plane of symmetry (asymmetric) (Endress, 1999). While the earliest flowers were likely to have been actinomorphic, zygomorphy has evolved repeatedly in the angiosperms and is a characteristic of many large and ecologically diverse clades such as Orchidaceae and Lamiales (Stebbins, 1970; Endress, 1994). Shifts to zygomorphy are thought to contribute to floral diversification and speciation through promoting pollinator specificity and subsequently increasing fitness (reviewed in Giurfa et al., 1999). Some experimental evidence for this hypothesis has been found in *Erysimum mediohispanicum* (Brassicaceae) (Gomez et al., 2006), and targeted sister group comparisons across all flowering plants confirm that zygomorphic lineages tend to be more species-rich than closely related actinomorphic lineages (Sargent, 2004).

**Symmetry in the Zingiberales**—Although zygomorphy is dominant in the Zingiberales, all floral whorls across the order have had evolutionary shifts in symmetry, making this an ideal group for examining the evolution of this trait. In addition, an interesting phenomenon occurs in *Heliconia* (Heliconiaceae): the single plane of floral symmetry is not congruent with the median plane of the flower, resulting in a condition known as oblique zygomorphy (Fig. 1) (Eichler, 1878; Kirchoff et al., 2009). It has been suggested that such a shift in the axis of floral symmetry may be essential for correct floral orientation and effective pollination (Ronse De Craene et al., 2000; Kirchoff et al., 2009). Shifts in calyx, corolla, and androecium symmetry have been independently mapped onto the phylogeny in previous studies (Rudall and Bateman, 2004; Bartlett and Specht, 2010) and are discussed below (Fig. 1).

The calycines of representatives from six of the eight families in the Zingiberales (excluding Cannaceae and Marantaceae) are zygomorphic to varying degrees during development (Fig. 1A). In all of these taxa, the abaxial sepal (or anterior sepals in *Heliconia*; Kirchoff et al., 2009) is initiated last and is delayed in...
Corolla zygomorphy is reconstructed as ancestral in the Zingiberales (Bartlett and Specht, 2010) (Fig. 1A). In Cannaceae, Costaceae, Lowiaceae, Musaceae, and Strelitziaceae, corolla zygomorphy is the result of differentiation of the adaxial petal. This is also true in some Marantaceae, but in many taxa of this family, the corolla is actinomorphic (Fig. 1G) (Stevenson and Stevenson, 2004; Ley and Classen-Bockhoff, 2009). The elaborated adaxial petal of Lowiaceae is often described as a labelllum and is reported to be of importance in effective dung-beetle pollination (Kirchoff and Kunze, 1995; Sakai and Inoue, 1999). Perianth zygomorphy is expressed to greater and lesser degrees within the “ginger families” (the clade containing Costaceae, Zingiberaeae, Cannaceae, and Marantaceae), although in these families the perianth is often less important in floral display than the staminode-containing androecial whorls (Smith, 1972; Kay and Schemske, 2003).

The androecium is also reconstructed as ancestrally zygomorphic in the Zingiberales (Rudall and Bateman, 2004) (Fig. 1A). In Lowiaceae, Musaceae, and Strelitziaceae, there are five fertile stamens and the inner-whorl adaxial stamen is either completely absent or is replaced with a staminode (in some Musa spp.) (Kress, 1990b). Ravenala madagascariensis (Strelitziaceae) represents an exception, possessing an actinomorphic androecium with six fertile stamens (Kress et al., 1984). Rarely, six fertile stamens may be present in Musa flowers (Simmonds, 1966). Petaloid staminodes are reconstructed to have evolved on the branch leading to Heliconia plus the ginger families (Fig. 1C), and the androecial whorl in Heliconia is zygomorphic due to the presence of a posterior, outer-whorl, petaloid staminode (Rudall and Bateman, 2004; Kirchoff et al., 2009; Bartlett and Specht, 2010) (Note: Due to oblique zygomorphy, the petaloid staminode in Heliconia is not strictly adaxial; therefore, we use the term posterior to describe its position in the flower (Fig. 1) (Kirchoff et al., 2009)). In Costaceae and Zingiberaeae, five androecial members develop as petaloid staminodes that fuse in various combinations to form the staminodial labellum (Kirchoff, 1988a, b; Kress, 1990b), with the fertile stamen number reduced to one adaxial outer-whorl stamen that defines the single plane of symmetry (Fig. 1E). In Cannaceae and Marantaceae, the androecium is strongly asymmetric (Fig. 1F). There is a single half-stamen, while the remaining androecial members develop as petaloid staminodes (Kirchoff, 1983; Rudall and Bateman, 2004).

The ovary is actinomorphic for much of development in the “banana families” (the grade containing Heliconiaceae, Lowiaceae, Musaceae, and Strelitziaceae), Costaceae, and Zingiberaceae (Fahn and Benouaiche, 1979; Kunze, 1986; Kirchoff, 1988b, 1992; Newman and Kirchoff, 1992; Kirchoff and Kunze, 1995). In Costaceae, Lowiaceae, and Zingiberaceae, the stigma takes on a particular, not strictly actinomorphic form (Pedersen and Johansen, 2004; Box and Rudall, 2006; Specht, 2006). In Cannaceae and Marantaceae, the gynoecium, like the androecium, is asymmetric at maturity (Kirchoff, 1983; Rudall and Bateman, 2004) (Fig. 1F).

**CYC/TB1-like candidate genes**—The CYCOIDEA/TEOSINTE BRANCHED1 (CYC/TB1)-like genes are class II TCP transcription factors, unique in their possession of the ECE (glutamate-cysteine-glutamate) domain (Cubas et al., 1999; Howarth and Donoghue 2005). The ECE domain, a domain of unknown function that has been repeatedly found in CYC/TB1-like genes, has been used to circumscribe the CYC/TB1 subfamily within class II TCP genes (Howarth and Donoghue, 2005,
possesses three flower types, disc, tran, and ray flowers, which is thought to be convergent (Stebbins, 1970; Luo et al., 1996; Citerne et al., 2003; Feng et al., 2006; Busch and Zachgo, 2007). The most complete functional data comes from the asterid Antirrhimum majus (Plantaginaceae) and the rosid Pismum sativum (Fabaceae). In Antirrhimum, the class II TCP genes CYC and DICHOTOMA (DICH) are the products of a gene duplication event that predates the diversification of the tribe Antirrhineae (Hileman and Baum, 2003). In concert with the MYB domain transcription factor RADIALIS (RAD), CYC and DICH specify adaxial floral identity (Luo et al., 1996; Corley et al., 2005). A second MYB domain transcription factor, DIVARICATA (DIV), confers abaxial floral identity (Almeida et al., 1997). This distinction between adaxial and abaxial floral identity enables the development of zygomorphy (Corley et al., 2005).

CYC and DICH have different effects on different whorls of the zygomorphic Antirrhimum flower. They have been shown to promote growth of the adaxial petals while restricting growth of the adaxial sepal and the adaxial stamen, which in wild-type flowers aborts to become a staminode. The double cyd/dich mutant displays an actinomorphic, abaxialized phenotype, with six, rather than five, sepals and petals, as well as six fully formed stamens. Internal petal asymmetry is also lost in the double mutant (Luo et al., 1996). Thus, these genes play a role in controlling organ number as well as size, two key aspects of floral zygomorphy. In Pismum, floral symmetry is controlled by two CYC-like genes (PaCYC2 and PaCYC3) and an uncharacterized single locus, SYPI. PaCYC2 and PaCYC3 control adaxial–abaxial symmetry at the level of the entire flower: the double mutant displays an abaxialized corolla, but petals still display internal asymmetry. Internal organ asymmetry is controlled by the third locus, SYPI. The triple mutant is radially symmetric with all petals possessing an abaxialized identity (Wang et al., 2008).

In Asteraceae, CYC-like genes seem to play a role in specifying floral identity across the inflorescence. Gerbera hybrida possesses three flower types, disc, trans, and ray flowers, which differ in a number of characteristics including symmetry. In G. hybrida, one CYC-like gene, GhCYC2, is expressed only in ray flower primordia once the disk and ray flowers begin to differ morphologically. GhCYC2 overexpresser lines have disk flowers with more ray-like characteristics: they possess a ligular structure that resembles the zygomorphic corolla of ray and trans flowers, stamen development is disrupted, and in one line all the disk flower petals were fused. GhCYC2 over-expression also results in changed petal length in all three flower types (Broholm et al., 2008). In Senecio, introgression of the RAY locus, which contains a CYC-like gene, from S. squalidus into S. vulgaris resulted in the development of a morphological polymorphism within the S. vulgaris population. Senecio vulgaris inflorescences usually consist entirely of actinomorphic flowers, but those S. squalidus RAY locus have inflorescences with some zygomorphic ray florets on the periphery of the floral head (Kim et al., 2008). These results suggest that in Asteraceae, CYC-like genes are contributing, along with multiple other factors, to disc vs. ray floral identity.

In the monocots, most studies have focused on TB1 and its orthologs in the grasses. TB1 represses lateral branching in Zea mays (maize) (Doebely et al., 1997; Hubbard et al., 2002). Similar roles for TB1 orthologs have been demonstrated in Sorgum and Oryza (Takeda et al., 2003; Kebrom et al., 2006). In addition, there is evidence that a CYC/TB1-like gene from Oryza sativa (rice), RETARDED PALEA 1 (REPI), contributes to the specification of zygomorphy in the first whorl of the rice flower (Yuan et al., 2009).

Because of their demonstrated role driving the development of zygomorphy in model systems, recent studies have focused on the CYC/TB1-like genes as potential candidates for evolutionary changes in zygomorphy in a number of lineages. Numerous studies in the core eudicots associate CYC-like gene duplications and expression patterns with floral symmetry. Asymmetric CYC-like gene expression was found to be correlated with corolla zygomorphy in Chirita hetrotricha (Gesneriaceae), Malpighiaceae, Iberis amara (Brassicaceae), Veronica montana, and Gratiola officinalis (Plantaginaceae) (Busch and Zachgo, 2007; Gao et al., 2008; Preston et al., 2009; Song et al., 2009; Zhang et al., 2010). The absence of expression or symmetric CYC-like gene expression was associated with derived corolla actinomorphy in Plantago lanceolata (Plantaginaceae), Cadia purpurea (Fabaceae), and Bournea leiophylla (Gesneriaceae) (Citerne et al., 2006; Zhou et al., 2008; Reardon et al., 2009). In Papaveraeae, the only basal eudicot family where TCP genes have been studied thus far, correlations have been found between CYC-like gene expression and corolla symmetry. CYC-like gene expression was observed only in the outer petals of the disymmetric Lamprocapnos spectabilis and the zygomorphic Capnosoides sempervirens. Additionally, this CYC-like gene expression was often found to be asymmetric in C. sempervirens (Damerval et al., 2007).

In the eudicots, there is an emerging pattern of numerous gains and losses of CYC-like genes in different lineages (Citerne et al., 2003; Fukuda et al., 2003; Gubitz et al., 2003; Hileman and Baum, 2003; Reeves and Olmstead, 2003; Howarth and Donoghue, 2005, 2006; Kolsch and Gleissberg, 2006). In the Dipsacales, these changes in CYC-like copy number are correlated with changes in floral form (Howarth and Donoghue, 2005). This pattern of gene evolution suggests an ideal candidate gene family for the study of morphological evolution by gene duplication and diversification (Ohno, 1970; Lynch and Force, 2000).

Furthermore, the CYC/TB1-like genes seem to have a particular role in stamen abortion associated with zygomorphy (Hileman and Cubas, 2009; Preston and Hileman, 2009). The CYC-like genes are implicated in causing stamen abortion in many taxa, most notably in Antirrhimum majus, where CYC and DICH, one of CYC’s paralogs, restrict growth of the abaxial stamen, which aborts to become a staminode (Luo et al., 1996). Apart from its expression in axillary meristems and branches, TB1 in Z. mays is strongly expressed in the stamens of female florets: floral organs destined to abort. TB1 expression is much weaker in the fertile stamens of male florets (Hubbard et al., 2002). Overexpression of a CYC2 homolog in Gerbera hybrida (Asteraceae) disrupts stamen development in both disc and ray florets. Stamens in the transgenic line 35S::GhCYC2 were dis-colored and unable to release pollen (Broholm et al., 2008). In Mohavea conferti flora (Plantaginaceae), a close relative of Antirrhinum, the two lateral stamens and the single adaxial stamen develop as infertile staminodes. CYC and DICH expression is expanded into the region occupied by the lateral staminodes, possibly accounting for their abortion (Hileman et al., 2003). In Opithandra (Gesneriaceae), expression of two CYC2-like genes is correlated with adaxial and abaxial stamen abortion and is negatively correlated with expression of OpdycyclinD3, a positive regulator of cell division (Gaudin et al., 2000; Song et al., 2009). OpdCYC1 is initially expressed in petals, fertile stamens,
and staminodes, and later becomes localized to the staminodes (Song et al., 2009). In Veronica montana and Gratiola officinalis (Plantaginaceae), CYC-like gene expression is correlated with adaxial (but not abaxial or lateral) stamen abortion, suggesting a second mechanism for controlling abaxial stamen abortion in this family (Preston et al., 2009). Because stamen abortion is a process of key importance when considering zygomorphy of the androecial whorl in the Zingiberales, we hypothesize that the CYC/TB1-like genes have been recruited in the Zingiberales to generate zygomorphy.

In this paper, we report on our investigation into the evolution of the CYC/TB1-like gene family in the context of floral symmetry within the Zingiberales. We retrieved CYC/TB1-like genes from 29 taxa spanning the Zingiberales. The nucleotide sequences were analyzed in a phylogenetic context, revealing evidence of a CYC/TB1-like (henceforth referred to as TBL1 or TBL) gene duplication that occurred prior to the divergence of the commelinid monocots, and at least one Zingiberales-specific TBL gene duplication.

We focused our analyses of gene expression on two Zingiberales taxa with divergent patterns of floral symmetry: Costus spicatus and Heliconia stricta (Fig. 1). On the basis of described patterns of symmetry in these flowers, we expected asymmetric CYC/TB1 expression early in development in the abaxial/anterior sepal of Costus and Heliconia. We further expected this expression to persist in the zygomorphic calyx of Heliconia, but not in Costus where the calyx is actinomorphic at maturity. We also expected CYC/TB1 expression in the petaloid staminode of Heliconia and in the petaloid labellum (fused petaloid staminodes) of Costus. We found that the expression of these genes is associated with evolutionary shifts in floral symmetry.

**MATERIALS AND METHODS**

**Amplification of CYC/TB1 homologs—TBL1-like (TBL) genes were amplified from Zingiberales taxa (Table 1) using primers situated in the conserved SP, TC, and R regions (Lukens and Doebely, 2001; Howarth and Donoghue, 2005). We achieved the most success amplifying TBL genes with nondegenerate forward primers situated in the TCP domain. Once TCP genes were recovered, clade-specific primers were designed. Multiple primer pair combinations were used on all taxa investigated and are listed in Table 2. PCR reactions had a final volume of 20 µL and contained 0.5 µmol each of the forward and reverse primers, 0.4 U iProof DNA polymerase (BioRad, Hercules, California, USA), 4 µmol dNTPs, and 2 µg BSA. Final MgCl2 concentration was 2.5 mM/L. The full 20-µL PCR volumes were separated in 1.2% agarose gels, and bands of the appropriate size were gel extracted and cloned using the CloneJet cloning kit (Fermentas, Ontario, Canada). Inserts were sequenced using vector-specific primers and BigDye v3.1 on an ABI 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA). The number of colonies sequenced for each taxon ranged from 20 to 100. All sequences were deposited in GenBank (accession numbers HM775093–HM775147).

Multiple sequence alignment and phylogenetic analyses—To explore the full complement of TBL1-like genes present in grass genomes, we retrieved TBL1-like sequences from the genome sequences of Brachypodium distachyon, Sorghum bicolor, Oryza sativa (rice), and Zea mays (maize) (all Poaceae) using the COGE genome browser (Lyons and Freeling, 2008). The genomes were searched for significant BLAST hits using TBL1, Os08g33530, and Os09g24480 (REPT1) as query sequences in three separate searches. A similar procedure was used to search the Vitis vinifera (Vitaceae), Arabidopsis thaliana (Brassicaceae), Carica papaya (Caricaceae), and Populus trichocarpa (Salicaceae) genomes using CYC1, CYC2, and CYC3 from Antirrhinum majus.

These genes were included with the retrieved Zingiberales sequences to generate an alignment of CYC/TB1-like genes. A second alignment of a broader swathe of class II TCP transcription factors included the described CYC/TB1 sequences, CINNINATA, and its closest homologs from O. sativa and A. thaliana.

| Table 1. Taxon sampling for phylogenetic analysis of TBL1-like genes in the Zingiberales |
|--------------------------|------------------|----------------------|
| Taxon                     | Locationa          | Accession            |
| Acrocarae                 |                   |                      |
| Acorus calamus L.         | UCGB              | 94.1392              |
| Cannacea                  |                   |                      |
| Costus sp. L.             | UC               | mb0602               |
| Costaceae                 |                   |                      |
| Costus amazonicus (Loes.) J.F. Machr. | NMNH   | M0936               |
| Costus dubius (Afzel.) K. Schum. | UCBG  | 89.0918             |
| Costus guanaiensis Rusby  | NMNH             | L80.0707             |
| Costus spicatus Swartz    | NMNH             | 2002-127             |
| Monocostus uniflorus (Poep. ex Petersen) | NMNH | 1994-725             |
| Maas                      |                   |                      |
| Heliconiaceae             |                   |                      |
| Heliconia chartacea Lane ex Barreiros | HLA | L96-5689          |
| Heliconia pendula Wawra   | McBryde           | 711003-003           |
| Heliconia stricta Huber   | NMNH             | 1994-637             |
| Lowiaaceae                |                   |                      |
| Orechindantha maxillarioides (Ridl.) K. Schum. | McBryde | 970091          |
| Marantaceae               |                   |                      |
| Calathea insignis Petersen | UCBG | 90.1612             |
| Calathea ornata (Linden) Korn. | UCBG | 90.1624             |
| Maranta leuconoeura E. Morren | UCBG | 51.0711             |
| Musaceae                  |                   |                      |
| Musa basjoo Siebold      | UCBG             | 89.0873              |
| Musa sp. L.               | UC               | mb0602               |
| Srelitziaceae             |                   |                      |
| Srelitizia nicolai Regel & Korn. | UC | mb0601             |
| Srelitizia reginae Aiton  | UC               | mb0607               |
| Zingiberaceae             |                   |                      |
| Alpinia vitata W. Bull    | RBGE             | 19961132             |
| Barbidegia nitida Hook. F. | NMNH | 1996-282             |
| Curcuma longiflora Salisb. | NMNH | 2000-056b           |
| Curcuma rubraebracteata Korn., | NMNH | 1998-172           |
| M. Sabu & Prasanthk.     |                   |                      |
| Elettariopsis unifolia (Gagnep.) | RBGE | 19901449           |
| Elettaria cardamomum Maton | HLA   | L67-1100            |
| Globba laeta K. Larsen    | HLA              | L92-0182             |
| Plagiotachys albiflora Ridl. | NMNH | KS1745              |
| Plagiotachys mucida Holtum | NMNH | KS1761              |
| Pleuranthodium helligii (K. Schum.) | HLA | L-99-0492           |
| Riedelia lanata (Scheff.) K. Schum. ex Valeton | NMNH | 16327            |
| Zingiber officinalis Roscoe | UC     | MB0876              |
| Zingiber ostiessi Valeton  | NMNH             | 94-770               |

* Location of live accessions or herbarium sheets: Lyon Arboretum, Oahu, Hawaii, USA (HLA); McBryde Botanical Garden, Kauai, Hawaii, USA; University of California Botanical Garden (UCBG); University of California Berkeley Herbarium (UC), Smithsonian Greenhouses (NMNH).
Table 2. Primers used to amplify TBL genes from the Zingiberales

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence: 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td>CYC-F1a*</td>
<td>AAA GAY CGV GAC AGC AA</td>
</tr>
<tr>
<td>CYC-F1 (T/G)a</td>
<td>AAA GAT CGG CAC AGC AA</td>
</tr>
<tr>
<td>ZinTBL-F1a</td>
<td>AAA GAT CGG CAC AGC AAG AT</td>
</tr>
<tr>
<td>ZinTBL-F1b</td>
<td>TCC CAT CAG TAA AGC ACA TGT TCC TCC</td>
</tr>
<tr>
<td>ZinTBL-F2</td>
<td>AAR GAY CGG CAC AGC AA</td>
</tr>
</tbody>
</table>

* From Howarth and Donoghue (2005)
* Modified from Lukens and Doebely (2001)

2003). For each data set, two analyses were run in parallel until convergence (standard deviation of split frequencies ≤ 0.01). Likelihood scores were examined using the program Tracer (Rambaut and Drummond, 2007), and the first 10% of trees were discarded as burnin. Maximum likelihood searches and ML bootstrap analyses (1000 reps class II TCP, 531 reps CYC/TBI) were performed on both data sets using the program GARLI v0.96 (Zwickl, 2006) with the same model parameters.

Analysis of protein sequences—Sequences were translated into protein using the program MacClade (Maddison and Maddison, 1989). The program MEME (Bailey et al., 2009) was used to search for conserved motifs in the CYC/TBI like genes. All identified motifs were individually assessed and verified. The Protein Data Bank (Berman et al., 2000) was searched with verified motifs using the programs FIMO and MAST (Bailey et al., 2009). The TCP domains of the Zingiberales TBL genes were analyzed for nonconservative amino acid replacements using Grantham’s amino acid distances (Grantham, 1974) and Yang et al.’s’ (Yang et al., 2000) categories. If an amino acid replacement was considered nonconservative using both methods, it was scored as such. Protein structure prediction for the ZinTBL genes was performed using the Phyre server (Kelley and Sternberg, 2009). To facilitate comparison between TBL genes in different clades, we converted alignments of the ECE regions into sequence logos using the program WebLogo (Crooks et al., 2004).

Tests for selection on the TCP domain of the TBL genes—the ratio of nonsynonymous nucleotide substitutions to synonymous substitutions (ω = dNd/s) is often used as a measure of selection acting on protein-coding sequences. An ω value of less than one implies that the protein under investigation is under negative (purifying) selection, ω equal to one implies neutral evolution, and ω greater than one implies positive selection acting on a protein (Kimura, 1977; Miyata and Yasunaga, 1980; Yang, 2002). Using the programs Datamonkey (Kosakovský Pond and Frost, 2005b) and PAML v4.2 (Yang, 2007), we employed various tests to identify selection acting on the TBL genes. The Datamonkey web server allows the user to estimate ω at each site in an alignment using three different likelihood-based methods: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), and random effects likelihood (REL) (Kosakovský Pond and Frost, 2005a,b). We estimated ω at individual codons using SLAC and FEL analyses (Kosakovský Pond and Frost, 2005a).

Tests for variation in selection regime across the TBL phylogeny were performed using PAML v4.2a (Yang, 2007). The codeml program within PAML estimates the likelihood of the data being analyzed given various codon substitution models, each with differing values of ω. These likelihoods are then compared using the likelihood ratio test (Yang, 1998, 2007). Using the programs Tracer (Rambaut and Drummond, 2007), and the first 10% of trees were discarded as burnin. Maximum likelihood searches and ML bootstrap analyses (1000 reps class II TCP, 531 reps CYC/TBI) were performed on both data sets using the program GARLI v0.96 (Zwickl, 2006) with the same model parameters.

RESULTS

Phylogenetic analyses—We conducted phylogenetic analyses to determine the evolutionary history of TBL genes in the monocotyledons. Maximum likelihood and Bayesian phylogenetic analysis of both the complete class II TCP transcription factor data set and the CYC/TBI-like data set resolved trees with very similar topologies. Analysis of the full TCP class II data set (Fig. 2) resolved two main gene lineages: a weakly supported CYC/TBI clade (posterior probability [pp] = 0.55, ML bootstrap support [BS] = 55%), and a well-supported PCF/CIN clade (pp = 1.00, BS = 99%). Tree topology was in agreement with previous analyses and confirms that the gene duplication events that led to the CYC1, CYC2, and CYC3 gene lineages were eudicot-specific (Howarth and Donoghue, 2006). With the exception of two PCF-like genes from Plagiostachys albiflora and P. mucida (Zingiberaceae), all genes that we recovered from the Zingiberales formed as described previously (Bartlett et al., 2008). Considering the extremely low levels of nucleotide divergence within the ZinTBL1a and ZinTBL2 clades, we used a single probe specific to each homolog in both C. spicatus and H. stricta. The probes were designed to exclude the conserved TCP domain.

RNA in situ hybridization—The expression of ZinTBL1a and ZinTBL2 was assessed in C. spicatus and H. stricta. RNA in situ hybridizations were performed as described previously (Bartlett et al., 2008). Considering the extremely low levels of nucleotide divergence within the ZinTBL1a and ZinTBL2 clades, we used a single probe specific to each homolog in both C. spicatus and H. stricta. The probes were designed to exclude the conserved TCP domain.
gene duplication event that occurred after the divergence of Acorus from the remaining monocots, but predated the divergence of the commelinid monocots. The TBL1 clade includes Zea mays TB1 and its orthologs from other grasses, while the TBL2 clade includes REP1 (Yuan et al., 2009), Os08g24480 and their orthologs from other grasses. Our analyses reconstructed a Zingiberales-specific gene duplication in the TBL1 gene lineage, leading to two clades: ZinTBL1a and ZinTBL1b. Homologs in both of these gene lineages were retrieved from all eight families in the Zingiberales with the exception of Cannaceae (only ZinTBL1b was retrieved from Cannia). ZinTBL1b and ZinTBL1a are in a moderately well-supported sister relationship with the TBL1 clade from Poaceae, which includes TB1 from Zea mays.

The moderately supported TBL2 clade (pp = 0.93, BS = 63%) comprised two more well-supported sister lineages containing genes from either Zingiberaceae (ZinTBL2 clade) or Poaceae (PoaTBL2 clade). There is some evidence for TBL2 duplications in both the Poaceae and the Zingiberales lineages. Two ZinTBL2 copies have been recovered from Zingiberaceae and Marantaceae (Fig. 3). One ZinTBL2 homolog shows extremely low sequence divergence, similar to what is seen in ZinTBL1a. The second putative copy shows a higher level of sequence divergence. Each of the grasses included in the analysis are represented by two genes in the TBL2 clade, except for Z. mays, which is represented by five TBL2 genes. Thus, we have found evidence for several Zingiberales-specific TBL gene duplication events and for an independent gene duplication event that predated the diversification of the commelinid monocots.

**ZinTBL protein evolution**—We screened the Zingiberales TBL genes for both previously defined and as yet unrecognized protein domains. As expected, the TCP domains of ZinTBL1a, ZinTBL1b, and ZinTBL2 were predicted to form a basic helix-loop-helix, distinct from the canonical domain of the bHLH transcription factors (Cubas et al., 1999). No structural homologs were found, and no further predictions of ZinTBL structure could be made. As is common in TCP transcription factors (Cubas et al., 1999), there have been many nonconservative amino acid substitutions in both the basic and the helix-turn-helix domains of the protein (Fig. 4).

A putative ECE domain was found by visual inspection in all the Zingiberales TBL genes and in the Acorus calamus TBL sequence. MEME also identified the ECE region of ZinTBL1a, ZinTBL1b, and ZinTBL2 as a domain, despite the fact that the ZinTBL1a ECE domain appears to have diverged considerably (Fig. 4C). The PoaTBL2 genes from Oryza sativa have previously been described as lacking both ECE and R domains.
(Howarth and Donoghue, 2006; Yuan et al., 2009). However, upon close inspection of PoaTBL2 genes, what could be highly divergent ECE domains could be identified. ECE domain architecture differed between PoaTBL2 genes, and two subdivisions could be identified, PoaTBL2a and PoaTBL2b (Fig. 4C).

Apart from the previously described SP (Lukens and Doebley, 2001), TCP (Cubas et al., 1999), ECE (Howarth and Donoghue, 2005), and R domains (Cubas et al., 1999), MEME identified five further motifs (Fig. 4; Appendix S3 in online Supplemental Data). The SP domain was found and characterized in TBL-like genes from the grasses and is rich in serine and proline residues (Lukens and Doebley, 2001). Motif 2 was found in all Zingiberales TBL genes and in Poaceae TBL1. MEME did not identify motif 2 in either of the Acorus calamus TBL genes, although examining the sequences enabled us to identify a potential (highly diverged) motif 2 in A. calamus TBL1a and TBL1b. The PoaTBL2 genes shared motif 4, and motif 5 was found in PoaTBL1 and in PoaTBL2a. Motif 1 was found in ZinTBL1b and ZinTBL1a, while the two ZinTBL2 clades of genes shared motif 3. None of these additional domains were found in the eudicot CYC proteins. Neither FIMO nor MAST genes shared motif 3. None of these additional domains were identified in Costus spicatus and Heliconia stricta — To investigate the TBL genes’ roles in floral development, we examined the expression of the ZinTBL genes in two taxa with divergent floral symmetries: Costus spicatus and Heliconia stricta. Preliminary RT-PCR experiments testing for expression of TBL genes in C. spicatus inflorescences containing flowers at early to late developmental stages showed expression of ZinTBL1a and ZinTBL2 in C. spicatus flowers, but not ZinTBL1b (data not shown). We therefore investigated the expression patterns of the C. spicatus and H. stricta orthologs of ZinTBL1a and ZinTBL2 in developing flowers using RNA in situ hybridization.

The inflorescence of C. spicatus is bracteate (Fig. 5A). As in C. scaber (Kirchoff, 1988b), a reduced cincinnus of a single flower occurs in the axil of each primary bract. The flowers of C. spicatus are strongly zygomorphic and consist of three fused sepals, a floral tube formed by the proximal fusion of the androecium and the corolla, and a trilocular inferior ovary. The petals are free distally, and the adaxial petal is larger than the lateral petals. There are two trimerous androecial whorls. A single adaxial, interior-whorl stamen is fertile. The remaining androecial members develop as petaloid staminodes and fuse to form the staminodial labellum. CsTBL1a expression was detected in the early floral meristem and the primary bract of C. spicatus (Fig. 5B). Later in development, once floral organs were clearly discernable, CsTBL1a expression became restricted to the abaxial side of the flower and the region of the bract closest to the abaxial side of the flower (Fig. 5C–D). Expression of CsTBL1a in older flowers was detected in the abaxial side of the floral tube, the labellum, the gynoeicum, and the anther thecae. The C. spicatus ortholog of ZinTBL2, CsTBL2, was expressed in the fertile thecae of the single adaxial, fertile stamen (Fig. 5E). No expression of CsTBL2 was observed in the sepal of C. spicatus. After examining numerous sections, we did not observe CsTBL2 expression in the labellum, but the absence of CsTBL2 expression in the labellum remains to be confirmed. No signal was observed in either of the CsTBL sense controls (Fig. 5F, G).

The Heliconia inflorescence consists of showy bracts that enclose cincinni of multiple flowers. The trimerous calyx and trimerous corolla are similar and fuse postgenitally to form the floral tube. The posterior sepal is larger than the other perianth members and separates from the floral tube at anthesis (Kress, 1990a; Kirchoff et al., 2009). As in all Zingiberales, there are two trimerous androecial whorls. There are five fertile stamens, while the exterior-whorl posterior androecial member develops as a petaloid staminode. The ovary is trilocular and inferior.
**H. stricta**, *HsTBL1a* expression was detected in the gynoeicum and the posterior staminode. Weak expression was also detected in the petals (Fig. 5H). Expression of the *H. stricta* ortholog of *ZinTBL2*, *HsTBL2*, was found throughout the anterior sepals and in the sepal margins (Fig. 5I). No signal was observed in either of the *HsTBL* sense controls (Figs. 5J, K).

In summary, *ZinTBL1a* expression in *H. stricta* was found primarily in the staminodes and the gynoeicum. In *C. spicatus*, *ZinTBL1a* was observed in the labellum (petaloid staminodes) and the gynoeicum. *ZinTBL1a* expression was also observed in the fertile stamen and the abaxial side of the floral tube. *ZinTBL2* expression was found in the adaxial fertile stamen of *C. spicatus* and the anterior sepals of *H. stricta*.

**DISCUSSION**

**TB1-like genes have diversified in the commelinid monocots and in the Zingiberales**—Our data show evidence of *TB1* gene duplications that predate the divergence of Zingiberales and Poales (Poaceae) and are thus possibly shared by all commelinid monocots. The duplications that led to the three clades of *CYC* genes (*CYC1*, *CYC2*, and *CYC3*) found in core eudicots are hypothesized to have occurred prior to the origin and diversification of the core eudicots (Howarth and Donoghue, 2006). The duplications in the *TBL* gene lineage that we have uncovered in monocots mirror these *CYC* duplications in the core eudicot lineage. Because of the current limited sampling within monocots, however, we still do not know precisely when these duplications occurred. They may have occurred just prior to the diversification of the commelinid monocots, or they may appear earlier in monocot history but after the divergence of the lineage leading to *Acorus*.

Based on our results, there appears to be evidence for similar gene duplication histories between *CYC*-like and *TB1*-like lineages: early duplications generated core gene lineages (i.e., *TB1* and *TBL2*: *CYC1*, *CYC2*, and *CYC3*) that further diversified via subsequent lineage-specific gene duplications. There is evidence for gene diversification in both the *ZinTBL1* and the *ZinTBL2* lineages, similar to the reported *CYC2* diversification in many eudicot lineages (Fukuda et al., 2003; Howarth and Donoghue, 2005; Kolsch and Gleissberg, 2006; Damerval et al., 2007). Unlike what has been discovered in the Dipsacales (Howarth and Donoghue, 2005) and Plantaginaceae (Reardon et al., 2009), however, no correlations can be made between *ZinTBL* copy number and floral symmetry pattern.

We show evidence that at least one Zingiberales-specific gene duplication occurred in the *TBL1* gene lineage, leading to the clades *ZinTBL1a* and *ZinTBL1b*. The *ZinTBL1a* clade was well supported (pp = 1.00, BS = 98%), the *ZinTBL1b* clade less so (pp = 1.00, BS < 50). Homologs from both of these gene lineages were recovered from members of all eight families in the order Zingiberales with the exception of *ZinTBL1a* from Cannaceae, suggesting that the gene duplication that led to these two clades occurred prior to the diversification of the order, approximately 100–120 million years ago (Ma) (Kress and Specht, 2006). Sequence divergence in the *ZinTBL1a* clade is extremely low. Except for the sequence from *Orchidanthaxialloridoideae* (Lowiaceae), the amplified region spanning the TCP, ECE, and R regions of the protein is identical across the order. Although contamination is always a concern with PCR-based methods, we are confident that these results represent a biological reality. Identical *TBL1a* sequences were retrieved from numerous taxa with different sets of primers.

These low levels of sequence divergence are in stark contrast to *PoaTBL1b* where sequence divergence is quite high. The nucleotide and protein sequences of the genes in the *ZinTBL1b* clade cannot be unambiguously aligned over much of their length, and internal resolution in the *ZinTBL1b* clade is weakly supported for the most part. These results suggest that the *ZinTBL1b* clade may represent more than one *TB1* homolog. Alternatively, this clade may represent a single gene that has undergone significant diversification in the Zingiberales, indicating a potentially important role in developmental diversification. Internal clade topology is broadly congruent with taxonomic phylogeny, consistent with this hypothesis. Although the much greater variation seen in *ZinTBL1b* may be suggestive of this paralog being nonfunctional, this level of variation is not unprecedented for *CYC/TB1*-like genes. There is evidence for rapid molecular evolution of *CYC*-like genes in many eudicot lineages (Fukuda et al., 2003; Gubitz et al., 2003; Citerne, 2005). Molecular evolution may be occurring at a similarly rapid rate in this clade of *TBL* genes in the Zingiberales.

There is little evidence for extensive gene duplication of either the *TBL1* or the *TBL2* gene lineages in grasses. *TBL1* is single copy in *Orzya*, *Sorghum*, and *Brachypodium*. In maize, there are two *TBL1* loci, *TBL1* and *TBL2*, which probably originated from a segmental allotetraploidy event that generated the maize genome ~12 Ma (Gaut and Doebley, 1997; Swigonová et al., 2004). Apart from maize, *TBL1* has been found to be single copy in a large number of grasses (Lukens and Doebley, 2001). Our phylogenetic analyses confirm this finding. The single *TBL1* gene in the grasses is in contrast to the inferred homologs of *TB1* in the Zingiberales, *ZinTBL1a* and *ZinTBL1b*, which have both been maintained in Zingiberales genomes following a duplication event in the *TBL1* gene lineage. It is also in stark contrast to *CYC*-like genes in the eudicots, which appear to have undergone several rounds of duplication and gene retention in numerous lineages (Citerne et al., 2003; Gubitz et al., 2003; Reeves and Olmstead, 2003; Ree et al., 2004; Howarth and Donoghue, 2005, 2006; Kolsch and Gleissberg, 2006; Damerval et al., 2007).

**TBL protein evolution**—Protein structure in the *TBL* gene family suggests an interesting history of gain and loss of particular motifs. The ECE region is present in *ZinTBL2*, as well as in the *Acorus TB1* gene family. A short stretch of the R domain beyond the primers is also present in all of the Zingiberales and *Acorus* TBL sequences. These results imply that *PoaTBL2* lost the R domain and that the ECE domain has either diverged considerably or has been lost from these genes. A novel motif not found in *TBL2*, domain 4, was uncovered in *PoaTBL2*. 
Motif 5 was found only in *PoaTBL1* and *PoaTBL2a*. This motif is outside the region amplified by our primers and may be present in the *ZinTBL* sequences. The presence and absence of domains within specific gene lineages lends support to our phylogenetic hypothesis of gene family evolution: *ZinTBL1a* and *ZinTBL1b* are in a weakly supported sister relationship, but the presence of motif 1 in both of these gene lineages allows us to be more confident in their close relationship.

The majority of the amino acid residues in the TCP domain of the *TBL* genes are under negative selection. This is not surprising given that the TCP domain is the most conserved domain of the protein, involved in DNA binding. No positive selection was found to be acting on the full coding sequence of grass *TB1* orthologs (Lukens and Doebley, 2001). In contrast, positive selection was found to be acting on three residues in the TCP domain and one residue of the R domain of *CYC*-like genes of *Senecio vulgaris* and *Helianthus annuus* (Asteraceae) (Chapman et al., 2008). In Asteraceae, *CYC*-like genes have been shown to play a role in specifying floral identity across the inflorescence, where ray florets are zygomorphic and disc florets actinomorphic (Broholm et al., 2008; Chapman et al., 2008; Kim et al., 2008).

Although negative selection is dominant, selection pressure differs significantly across branches in the TBL phylogeny. Hypothesis 8, where separate $\omega$ values were estimated for each of the labeled branches, is significantly more likely than a scenario in which there were three different values of $\omega$ for the branches leading to *TBL1*, *TBL2*, and *Acorus TBL*. This result implies that there are distinct forces acting on each of the TBL gene lineages, suggesting functional diversification of the TBL genes in the monocots.

There have been shifts in selection regime following gene duplication in the *ZinTBL1* gene lineage. In the more sensitive *Motif 5* was found only in *PoaTBL1* and *PoaTBL2a*. This motif is outside the region amplified by our primers and may be present in the *ZinTBL* sequences. The presence and absence of domains within specific gene lineages lends support to our phylogenetic hypothesis of gene family evolution: *ZinTBL1a* and *ZinTBL1b* are in a weakly supported sister relationship, but the presence of motif 1 in both of these gene lineages allows us to be more confident in their close relationship.

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Table 3. Log likelihood values and parameter estimates under models of variable $\omega$ among sites and along branches and sites.

<table>
<thead>
<tr>
<th>Model</th>
<th>$L^*$</th>
<th>Parameter estimates</th>
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<tbody>
<tr>
<td>Sites</td>
<td></td>
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<tr>
<td>M0 (one ratio)</td>
<td>$-2077.98$</td>
<td>$\omega = 0.075$</td>
</tr>
<tr>
<td>M1a (nearly neutral, $\omega_1 = 1$)</td>
<td>$-2057.37$</td>
<td>$p_0 = 0.827$, $p_1 = 0.173$, $\omega_0 = 0.056$</td>
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<tr>
<td>M2a (positive selection)</td>
<td>$-2057.37$</td>
<td>$p_0 = 0.827$, $p_1 = 0.155$, $\omega_0 = 0.056$, $\omega_1 = 1$</td>
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<tr>
<td>M7 (beta)</td>
<td>$-1999.67$</td>
<td>$p = 0.3481$, $q = 3.88713$</td>
</tr>
<tr>
<td>M8 (beta &amp; $\omega &gt; 1$)</td>
<td>$-1999.67$</td>
<td>$p_0 = 0.999$, $p = 0.348$, $q = 3.887$, $\omega = 2.672$</td>
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<tr>
<td>Branch-sites</td>
<td>$-2053.24$</td>
<td>$p_0 = 0.831$, $p_1 = 0.148$, $p_2 = 0.182$, $p_3 = 0.003$</td>
</tr>
<tr>
<td>ZinTBL1a foreground</td>
<td>$-2055.98$</td>
<td>$\omega_0 = 0.056$, $\omega_2 = 0.056$, $\omega_2b = 1$</td>
</tr>
<tr>
<td>A ($\omega_{for} &gt; 1$)</td>
<td>$-2055.98$</td>
<td>$\omega_0 = 0.058$, $\omega_1 = 1$, $\omega_{2a b} = 28.128$</td>
</tr>
<tr>
<td>A1 ($\omega_{for} = 1$)</td>
<td>$-2055.98$</td>
<td>$\omega_{2a b} = 0.058$, $\omega_{2b b} = 1$</td>
</tr>
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*Models significantly more likely than the corresponding null model are in boldface.

a $28 = 5.4826$, d.f =1, $P = 0.0192$. Not significant after Bonferroni correction for multiple comparisons, $\alpha_{0.05} = 0.0045$. 

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**Fig. 4.** TBL protein evolution in the monocots. (A) Protein motifs detected by MEME are shown in their approximate positions in TBL proteins from *Acorus*, Zingiberales, and Poaceae. Domains that we could identify, but were not detected by MEME, are shown with dashed outlines. (B) TCP domain structure prediction. Nonconservative amino acid replacements are boxed. The open box indicates the *ZinTBL1a* residue identified as under positive selection in the PAML BEB analysis. (C) Aligned sequence logos of the ECE domain. Amino acids are colored according to hydrophobicity.
branch-site test for selection, positive selection was found to be acting on the branch leading to ZinTBL1a, on the asparagine residue at position 22 of the amplified TCP domain. This residue is predicted to be the first residue in the first helix of the HLH domain. In bHLH transcription factors of the MyoD type, the HLH domain is thought to be involved in mediating protein dimerization (Murre et al., 1989). This nonconservative amino acid replacement on the branch leading to ZinTBL1a may have resulted in new protein–protein interactions and new TBL gene function. Sequence divergence within the ZinTBL1a clade, however, is extremely low. These results suggest a scenario where there was positive selection acting on ZinTBL1a after the ZinTBL1a/lb gene duplication, resulting in a new protein function that was maintained through extreme purifying selection acting on the ZinTBL1a gene.

**ZinTBL1a expression changes along with changes in androecium zygomorphy—**ZinTBL1a expression is observed in the aborted, petaloid staminodes of both C. spicatus and *H. stricta*. *CstBL1a* expression is observed in the abaxially placed (anterior) staminodal labelum of *Costus spicatus*, while *HstBL1a* expression is observed in the posterior staminode of *H. stricta*. The observed changes in expression domain may indicate that ZinTBL1a expression results in abaxial stamen abortion in *Costus* and posterior stamen abortion in *Heliconia*, thus causing the shift in androecium zygomorphy. A strict correlation cannot be made, however, because ZinTBL1a expression is also observed in the fertile, adaxial stamen of *C. spicatus*. This expression in both the fertile and infertile stamens does not preclude the involvement of the TBL genes in stamen abortion, but their expression will have to be investigated in a broader group of Zingiberales taxa with differential staminode placement.

*CstBL1a* is also expressed in the early floral meristem and primary bract of *C. spicatus*. Similarly, *TBl1* is expressed in the axillary meristems and husk leaves of *Zea mays* (Hubbard et al., 2002). Later in development, *CstBL1a* expression becomes localized to the abaxial side of the flower and bract, suggesting that *CstBL1a*, similar to *CYC* in *Antirrhinum*., has a broader role than causing stamen abortion and may be defining abaxial floral identity. Both *CstBL1a* and *HsTBL1a* are expressed in the gynoecium of *Costus* and *Heliconia*. This expression domain has not yet been described for TBL genes, although *GocCYC2* and *GoCYC1* were both expressed at a low level in the gynoecium of *Gratiola officinalis* (Plantaginaceae) (Preston et al., 2009). The *Heliconia* and *Costus ZinTBL1a* homologs are expressed throughout the gynoecium, which notably is the only actinomorphic floral whorl in both *Heliconia* and *Costus*. Similarly, symmetrical *CYC2*-like gene expression is observed in the actinomorphic flowers of *Bhesa paniculata* (Centropalaceae), a close relative of the Malphigiaceae where asymmetric *CYC*-like gene expression is associated with zygomorphy in the perianth (Zhang et al., 2010). In *Codiaeum purpureum* (Fabaceae), a symmetric *CYC*-like gene expression domain in the perianth is associated with a shift to floral actinomorphy (Citerne et al., 2006). This actinomorphic expression of *ZinTBL1a* in the gynoecia of both *C. spicatus* and *H. stricta* may be instrumental in developing actinomorphy in this floral whorl, or it may be indicative of an as yet undescribed role of TBL genes in gynoecium development.

The complex *ZinTBL1a* expression patterns we have observed, in multiple floral whorls, are not unprecedented. *CYC* is expressed in the sepalas, petals, and stamens of *A. majus* and has different effects on different whorls of the *Antirrhinum* flower; it appears to promote growth of the adaxial petals and restrict growth of the adaxial sepal and the adaxial stamen, which aborts to become the staminode (Luo et al., 1996). In Fabaceae, much of the investigation into zygomorphy and *CYC*-like genes has focused on the corolla, although all four floral whorls are zygomorphic to varying degrees. There is some evidence that *CYC*-like genes are controlling zygomorphy of legume flowers outside the corolla. In *Lotus japonicus* *CYC*-like expression is evident in the adaxial side of the developing calyx (Feng et al., 2006). When two *CYC*-like genes, *K-1* and *LST1-1*, are mutated in *Pisum sativum*, the calyx appears to have a more actinomorphic form with reduced abaxial lobes (fig. 1d in Wang et al., 2008). As our expression patterns indicate, *TBL* gene action is likely just as complex in the flowers of the Zingiberales.

**ZinTBL2 is expressed in the anterior sepalas of Heliconia and the posterior stamen of Costus**—Expression of the *ZinTBL2* homolog *HstBL2* is strongest in the anterior sepalas of *Heliconia*. At maturity, *Heliconia* flowers look noticeably zygomorphic because the anterior sepals are considerably smaller than the posterior sepal from inception and the posterior sepal separates from the other sepals at anthesis (Kirchoff et al., 2009). *HstBL2* may restrict the growth of the anterior sepalas, causing zygomorphy of the sepal whorl throughout development. *ZinTBL2* is a putative homolog of *REP1* from *Orzya* sativa. *REP1* is expressed in the adaxially placed palea early in development.
later it is expressed in the stamen thecae and the vascular bundles of the palea and lemma. The rep1 mutant shows a partial loss of palea identity: the palea is delayed in development and has an expanded marginal tissue domain. These results suggest that REP1 is contributing to the specification of adaxial identity in the first whorl of the developing rice flower (Yuan et al., 2009). Similarly, HsTBL2 may also specify anterior sepal identity in Heliconia flowers.

CsTBL2 is expressed in the adaxial anther thecae of the Costus spicatus flower. Apart from weak TB1 expression in the stamens of maize male florets, similar expression of IaTCP1 is observed in the fertile stamens of Iberis amara (Busch and Zachgo, 2007). REP1 is also expressed in the fertile stamens of Oryza, but no stamen defects were observed in the rep1 mutant (Yuan et al., 2009). The absence of a rep1 stamen phenotype may be due to redundancy with Os08g24480 (sister to REP1 in our analysis), both genes being the product of a potentially grass-specific TBL1b gene duplication event. What role CYC/TBL homologs might play in fertile stamens has yet to be determined. What is intriguing is the expression of ZinTBL2 abaxially

Fig. 5. ZinTBL1a and ZinTBL2 expression in Heliconia stricta and Costus spicatus. The adaxial side of Costus flowers and the posterior side of Heliconia flowers is uppermost in all micrographs. The C. spicatus inflorescence shown in (A) was photographed, sectioned, and probed for CsTBL1a and CsTBL2 expression. The region of the inflorescence shown in section in (B) is marked with the red box. (B) CsTBL1a expression was detected early on the developing primary bract. Later in development, (C, D) CsTBL1a expression was detected in the gynoecium, the labellum (fused petaloid staminodes), and the abaxial side of the floral tube and bract. (E) CsTBL2 expression was detected in the thecae of the fertile stamen. There was no significant signal development with sense probe of (F) CsTBL1a or (G) CsTBL2. In H. stricta, (H) HsTBL1a expression was detected in the petaloid staminode and gynoecium and (I) HsTBL2 expression was detected in the anterior sepals and the sepal margins. No significant signal development was observed with sense probe of (J) HsTBL1a or (K) HsTBL2. Abbreviations: a, fertile stamen; br, bract; ca, calyx tube; fm, floral meristem; ft, floral tube; gy, gynoecium; im, inflorescence meristem; lab, staminodial labellum; p, petal; s, sepal; st, petaloid staminode. All scale bars represent 200 µm.
in *H. stricta* and adaxially in *C. spicatus*: ZinTBL2 is expressed in the anterior (abaxial) sepals of developing *Heliconia* flowers, but in the adaxial fertile stamen of developing *Costus* flowers (Fig. 6). It is possible that ZinTBL2 contributes to positional identity in both *Heliconia* and *Costus*, but specifies abaxial floral identity in *Heliconia* and adaxial floral identity in *Costus*. Understanding this pattern across the Zingiberales will be important to assessing the possible evolution of abaxial vs. adaxial identity specification within the order.

In addition to sequence changes, the regulation of *TBL* genes has likely diversified throughout the course of plant diversification. The coding sequence of the amplified region of both *ZinTBL1a* and *ZinTBL2* is identical in *Heliconia* and *Costus*, yet they have very different expression patterns during flower development in these taxa (Figs. 5, 6), providing some evidence for evolution of development through cis-regulatory evolution (Stern, 2000; Carroll, 2008; but see Hoekstra and Coyne, 2007). *Zea mays* *TB1* is a major maize domestication gene (Doebely et al., 1997). The differences between the phenotypic effects of maize *TB1* and teosinte *TB1* are thought not to be due to differences in coding sequence, but rather due to differences in the regulation of expression (Hubbard et al., 2002; Clark et al., 2006). *TB1* expression is observed in axillary meristems of maize in which it represses tiller outgrowth. *TB1* is not expressed in axillary meristems of the wild progenitor of maize, teosinte (Hubbard et al., 2002), which has extensive axillary branching (tillering). The *TB1* gene has pleiotropic effects on plant morphology, including differences in the degree of axillary meristem outgrowth and aspects of inflorescence architecture (Doebely et al., 1995; Clark et al., 2006). Introggression experiments demonstrate that these pleiotropic effects are under the control of distant cis-elements, more than 41 kb upstream of the *TB1* coding region, that act to alter *TB1* transcription (Clark et al., 2006). Similarly, *ZinTBL1a* may be under divergent transcriptional control in *Heliconia* and *Costus*.

**CYC/TB1-like genes control both structural and presentation zygomorphy**—Floral morphology may be viewed in light of Endress’ three interconnected levels of floral organization: Bauplan, construction, and mode (Endress, 1994). Bauplan refers to the basic organization of the flower, the floral diagram, and is the most deeply rooted in phylogeny. Bauplaene are often characteristic of families (e.g., the families of the Zingiberales) and sometimes of orders (e.g., Orchidales). Construction refers to the architecture of flowers: flowers with different Bauplaene may look superficially similar because of similar architectural and functional constraints (e.g., the lip flowers of orchids and Lowiaceae). Mode refers to the most plastic floral traits, such as organ color and size, and may vary within a population or species (Endress, 1994). Individual flowers may be understood in terms of all three levels of organization. A striking example is found in the Marantaceae: Kunze (1984) described the secondary zygomorphy of the asymmetric flowers of *Calathea* (Marantaceae). Because of reorientation of the floral organs at anthesis, the flowers look superficially like the lip flowers typical of Costaceae and Zingiberaceae. The orientation of this staminode as a lip is common in the Marantaceae and has been shown to be of importance in pollination in numerous African members of the family (Ley and Classen-Bockhoff, 2009). The flowers are structurally asymmetric (Bauplan), but changes in orientation (mode) render them superficially zygomorphic in order for successful pollination to occur (construction).

Rudall and Bateman in their 2004 (p. 27) analysis of zygomorphy in monocots made a distinction between structural zygomorphy and “more subtle causes of bilateral symmetry”, what we term presentation zygomorphy. Structural zygomorphy occurs as a result of organ loss, suppression, or elaboration and is thought to be more deeply rooted in phylogeny (Rudall and Bateman, 2004). Presentation zygomorphy involves smaller changes in floral form, such as differential organ coloration or differential organ expansion late in development and can vary within a species (Endress, 2001; Rudall and Bateman, 2004). Structural zygomorphy might be thought of as changes in floral Bauplan, while presentation zygomorphy results from changes in floral mode (Endress, 1994). Within the Zingiberales, there are numerous examples of both structurally and presentationally zygomorphic flowers, and there have been evolutionary shifts in symmetry involving both levels of floral organization (Rudall and Bateman, 2004; Bartlett and Specht, 2010).

What is intriguing is that there is a growing body of evidence that the *CYC/TB1*-like genes control floral zygomorphy at the level of both structural (Bauplan) and presentational (mode) zygomorphy. Changes in Bauplan may be considered macromutation in
nature, while changes in mode are most often considered microevolutionary (Endress, 1994): thus homologous genes may be controlling what are often considered vastly different evolutionary processes.

The calyx, corolla, and androecium of Antirrhinum flowers are all zygomorphic. The calyx is zygomorphic because of reduced growth of the adaxial sepal (presentational zygomorphy), whereas the corolla and androecium are structurally zygomorphic because of organ elaboration and organ suppression, respectively (Vincent and Coen, 2004). Mutant analyses have revealed that CYC and DICH control both the structural zygomorphy of the corolla and androecium of Antirrhinum and the less pronounced presentational zygomorphy of the calyx (Luo et al., 1996). CYC is expressed in the adaxial sepal, and both the single (cyc) and double (cyc, dich) mutants have six, rather than five sepals, indicating that CYC, possibly in concert with DICH, represses adaxial sepal initiation and growth (Luo et al., 1996).

Apart from controlling the development of structural zygomorphy in the corolla of Lotus and Pisum (Fabaceae) (Feng et al., 2006; Wang et al., 2008), CYC-like genes appear to have a role in controlling the presentational zygomorphy of the Lotus and Pisum calyces. In Lotus, expression of CYC2 homologs is found in the adaxial sepals, which are smaller than abaxial sepals at maturity (Tucker, 2003; Feng et al., 2006). Although the authors did not mention it, the zygomorphy of the calyx in Pisum k-1, lst-1 mutants is less pronounced than in the wild-type flowers (fig. 1d in Wang et al., 2008).

Iberis amara has flowers with a presentationally zygomorphic perianth in which the abaxial petals expand more than the adaxial petals. Higher expression of the CYC homolog IaTCP1 in Iberis amara is correlated with decreased adaxial petal growth (Busch and Zachgo, 2007). In a peloric mutant, IaTCP1 is expressed at a much lower level, and this low expression level is correlated with increased, equal growth of adaxial and abaxial petals. Transgenic Arabidopsis plants overexpressing IaTCP1 have reduced petal size, supporting a role for IaTCP1 in controlling presentational corolla zygomorphy in Iberis (Busch and Zachgo, 2007).

In the Zingiberales, the zygomorphy of the calyx of Heliconia might be considered presentation zygomorphy, while the androecium of both Heliconia and Costus is rendered structurally zygomorphic through differential sitemap abortion (Rudall and Bateman, 2004). Our expression data supports the hypothesis that TBL genes contribute to zygomorphy in both of these cases. If this is the case, homologous genes are controlling the development of nonhomologous morphological features. Similarly, the decreased growth of adaxial petals observed in Iberis produces floral zygomorphy analogous to the zygomorphy seen in Antirrhinum, but both are controlled by homologous genes. Thus, CYC/TBL-like genes are deployed in different whorls of the developing flower and during different phases of development to control both structural and presentational zygomorphy, mostly likely via control over underlying processes such as cell proliferation, cell identity, and meristematic activity (Kosugi and Ohashi, 1997, 2002; Tremeouyague et al., 2003; Li et al., 2005; Wang et al., 2008).

In conclusion, we have found evidence of an ancient duplication in the TBL gene lineage that predates the divergence of the commelinid monocots. In addition, there have been TBL gene duplications in the Zingiberales, one of which is associated with significant shifts in selection regime. Expression patterns of two ZintTBL genes are associated with differences in floral symmetry, adding to the growing body of evidence of continued recruitment of CYC/TBL-like genes in the evolution of floral symmetry. Further exploration of TBL gene evolution, expression, and function in the Zingiberales will shed more light on these intriguing phenomena.

LITERATURE CITED


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