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Significance of consensus CYC-binding sites found in the promoters of both *ChCYC* and *ChRAD* genes in *Chirita heterotricha* (Gesneriaceae)

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Abstract *CYC*-like genes are widely conserved in controlling floral dorsoventral asymmetry (zygomorphy) through persistent expression in corresponding domains in core eudicots. To understand how *CYC*-like gene expression is maintained during flower development, we selected *Chirita heterotricha* as a material and isolated the promoter sequences of the *ChCYC1C* and *ChCYC1D* genes, homologs of *CYC*, by inverse polymerase chain reaction. Further promoter analyses led to the identification of a putative *cis*-regulatory element in each promoter matching the consensus DNA binding site for *Antirrhinum* CYC protein: GGCCCCTC at −165 for *ChCYC1C*, and GGCCCCCC at −163 for *ChCYC1D*. This indicates that both the *ChCYC1C* and *ChCYC1D* genes have probably evolved autoregulatory loops to sustain their expression in developing flowers. We also isolated the coding and promoter sequences of the *ChRAD* gene, a homolog of *Antirrhinum RAD*. Promoter analysis showed that the *ChRAD* gene promoter also contained a putative CYC-binding site (GGCCCAC at −134). Therefore, *ChRAD* is likely a direct target of the *ChCYC1* genes, which is similar to *Antirrhinum RAD*. These results imply that the establishment of floral zygomorphy in *Chirita* may have been achieved by the evolution of an autoregulatory loop for *CYC*-like genes, which was probably accompanied by simultaneous co-option of the *RAD*-like gene into their regulatory network.

Key words autoregulatory loop, *Chirita heterotricha*, CYC-binding sites, *CYCLOIDEA*, *RADIALIS*.

Promoters, functional *cis*-regulatory sequences located in non-coding DNA regions at varying distances from transcription initiation sites, provide the ultimate information, controlling where, when, and at what level a gene is expressed through interactions with *trans*-acting factors (Wittkopp et al., 2004; Kim et al., 2006).

The TCP transcription factor family, comprising TEOSINTE BRANCHED1 (TB1) from maize (*Zea mays*; Doebley et al., 1995, 1997), CYCLOIDEA (CYC) from snapdragon (*Antirrhinum majus*; Luo et al., 1996), and PROLIFERATING CELL FACTORS 1 and 2 (PCF1 and PCF2, respectively) from rice (*Oryza sativa*; Kosugi & Ohashi, 1997), is plant specific and characteristic of a so-called TCP domain, a 59-amino acid basic helix–loop–helix (bHLH) motif involved in DNA binding and protein–protein interactions (Cubas et al., 1999; Kosugi & Ohashi, 2002). Based on sequence features in the TCP domain, the TCP family can be divided into two subfamilies, namely PCF (Class I or TCP-P) and CYC/TB1 (Class II or TCP-C; Cubas et al., 1999; Kosugi & Ohashi, 2002; Navaud et al., 2007). In rice, two classes of PCF proteins, namely I and II, recognize different but partially overlapping consensus DNA binding sites: GGNCCCAC for Class I, and GTGGNCCC for Class II (Kosugi & Ohashi, 2002). Although *Antirrhinum* CYC belongs to the Class II subfamily based on its TCP domain, its consensus DNA binding site (GGNCCCNC) resembles that of Class I TCP proteins (Costa et al., 2005).

Increasing evidence shows that *CYC*-like genes have important roles in establishing floral dorsoventral asymmetry (zygomorphy) in angiosperms, especially in core eudicots. The expression of the *CYC*-like genes in the second and third whorls of floral organs usually represses stamen development while promoting or retarding petal growth depending on the trait concerned (Luo et al., 1996, 1999; Hileman et al., 2003; Costa et al., 2005; Citerne et al., 2006; Feng et al., 2006; Busch & Zachgo, 2007; Gao et al., 2008; Wang et al., 2008; Song et al., 2009). In *Antirrhinum*, *CYC*, together with its paralog *DICHOTOMA* (*DICH*), patterns floral zygomorphy by promoting dorsal petal growth while repressing dorsal stamen development to become a staminode (Luo et al., 1996, 1999). In *Chirita* and *Opithandra* (Gesneriaceae), retardation of the dorsal petal and abortion of both dorsal and lateral or ventral stamens are due to the expanded expression domains of *CYC*-like genes from dorsal to the corresponding regions, as in *Mohavea*, a close relative of *Antirrhinum* (Hileman et al., 2003; Gao et al., 2008; Song et al., 2009). In legume flowers, the dorsal-specific expression of the *LegCYC* gene

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establishes dorsal petal identity (Citerne et al., 2006; Feng et al., 2006; Wang et al., 2008). The *CYC* ortholog *TCP1* is expressed early in the floral dorsal meristem of *Arabidopsis*, but the mature flowers are actinomorphic due to the lack of a later effect of *TCP1* (Cubas et al., 2001). In zygomorphic *Iberis* (Brassicaceae), closely related to *Arabidopsis*, the strong dorsal-specific expression of *IaTCP1* after the early floral stage markedly reduces dorsal petal size (Busch & Zachgo, 2007). Downregulation of *BlCYC1* gives rise to a derived actinomorphy in *Bournea* (Gesneriaceae) flowers (Zhou et al., 2008; Busch & Zachgo, 2009). As outlined above, the duration of *CYC*-like gene expression is strongly related to whether the expression of these genes has an actual morphological effect on floral symmetry. It is suggested that early expression of *CYC*-like genes may not be essential for the establishment of floral zygomorphy, whereas the acquisition of late or persistent expression is likely to be important for generating morphological zygomorphy in flowers (Cubas, 2004; Preston & Hileman, 2009; Song et al., 2009). However, it remains unclear how the asymmetric expression of *CYC*-like genes is maintained until the late stages in floral organs: does this involve autoregulatory loops or does it depend on continuous activation by upstream transcription factors?

In *Antirrhinum*, *CYC* activity in controlling floral dorsal identity is mediated in part by a *RADIALIS* (*RAD*) gene, a direct target of *CYC* because its promoter and intron contain several putative CYC-binding sites that have been shown to be bound by recombinant CYC protein in *in vitro* electrophoresis mobility shift assays (Corley et al., 2005; Costa et al., 2005). The *RAD* gene can be activated by the *CYC* gene in transgenic *Arabidopsis* plants, but endogenous *Arabidopsis RAD*-like genes cannot be activated due to the lack of consensus CYC-binding sites in their promoters (Costa et al., 2005). In *Bournea* (Gesneriaceae), the expression domain of *BlRAD* overlaps with that of the *BlCYC1* gene, implying that *BlRAD* may be activated by *BlCYC1* (Zhou et al., 2008). However, it remains uncertain whether *RAD*-like genes are direct targets of *CYC*-like genes outside the model plant *Antirrhinum*.

As the basal-most family probably representing the ancient and unelaborated forms of floral dorsoventral asymmetry within Lamiales s.l., Gesneriaceae is an interesting group with respect to the evolution of floral asymmetry (Endress, 1998; Cubas, 2002, 2004). Even though considerable expression data show that the persistent expression of *CYC*-like genes is correlated with the formation of floral zygomorphy in Gesneriaceae (Wang et al., 2006; Du & Wang, 2008; Gao et al., 2008; Zhou et al., 2008; Song et al., 2009), the underlying molecular mechanism remains unknown. *Chirita heterotricha* is an ideal candidate in which to address this question owing to its typical zygomorphic flowers and available expression data for the *ChCYC1C* and *ChCYC1D* genes (Gao et al., 2008). Therefore, in the present study we selected *C. heterotricha* as the material and isolated the promoter sequences of the *ChCYC1C* and *ChCYC1D* genes by inverse polymerase chain reaction (IPCR). In addition, we isolated and analyzed the coding and promoter sequences of the *ChRAD* gene, a putative direct target of *ChCYC* genes. The aim of the present study was to provide basic information as to why *CYC*-like gene expression is maintained in the corresponding floral domains in plant with morphological zygomorphic flowers, and to investigate whether *RAD*-like genes are co-opted into the regulatory network of *CYC*-like genes in the basal group of Lamiales s.l. The results would provide further information on, and promote progress in exploring, the floral symmetry gene network and its evolutionary mechanisms outside model genetic organisms.

1 Material and methods

1.1 Plant materials

Plants of *Chirita heterotricha* were grown in the greenhouse of the Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing, China. For total RNA extraction, young flower buds (<1 cm in length) were collected, frozen immediately in liquid nitrogen, and stored at −80°C for future use. Fresh leaves were collected for genomic DNA extraction.

1.2 Isolation of the coding region of the *ChRAD* gene

Total RNA was extracted from young flower buds of *C. heterotricha* using an SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). To amplify the coding region of the *ChRAD* gene, a pair of primers was designed according to the *BlRAD* gene (Zhou et al., 2008) as follows: forward 5′-TCCATGTCTAGTCGTTCGAG-3′ and reverse 5′-TCACCTCTG CTCATCGTTCTTC-3′. The PCR product was cloned into the pGEM-T easy vector (Promega) and sequenced.

1.3 Sequence and phylogenetic analyses of the *ChRAD* gene

In the present study, DNAMAN software (Lynnon Biosoft, Los Angeles, CA, USA) was used to

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translate the *ChRAD* gene. The amino acid sequence of the *ChRAD* gene was first compared with that of the *BlRAD* gene from *Bournea* and the *RAD* gene from *Antirrhinum* using Clustal X (Thompson et al., 1997) and then adjusted manually with BioEdit (Hall, 1999).

Neighbor-Joining (NJ) analyses were performed using the full-length amino acid sequences of *ChRAD*, *BlRAD* from *Bournea*, *RAD* and four *RAD*-like genes (*AmRL1*, *AmRL3*, *AmRL4*, and *AmRL5*) from *Antirrhinum*, and six *RAD*-like genes from *Arabidopsis* (*AtRL1*, *AtRL2*, *AtRL3*, *AtRL4*, *AtRL5*, and *AtRL6*) to ascertain the phylogenetic position of the *ChRAD* gene in the *RAD*-like MYB gene family. The *Antirrhinum DIV* and *Bournea BlDIV1* genes were used as outgroups. All genes used in the phylogenetic analyses are listed in Table 1. Amino acid sequences were first aligned using Clustal X (Thompson et al., 1997) and further adjusted manually with BioEdit (Hall, 1999). MEGA4 software (Sudhir Kumar, Tempe, AZ, USA) was used to construct NJ trees, with the bootstrap values calculated for 1000 replicates.

1.4 Isolation of genomic regions upstream of the *ChCYC1* and *ChRAD* genes

Genomic DNA was prepared from fresh leaves using a modified cetyl trimethyl ammonium bromide (CTAB) method described by Doyle & Doyle (1987). After searching for non-cutting enzymes in the known coding regions of *ChCYC1* and *ChRAD* with Oligo 6.0 software (Molecular Biology Insights, West Cascade, Washington, USA), *Eco*RI and *Hin*dIII were selected to digest genomic DNA to isolate the genomic region upstream of each gene. Briefly, approximately 500 ng pure genomic DNA (80–100 ng/μL) was digested with excessive enzymes (TaKaRa, Dalian, China) in a final volume of 25 μL by incubation at 37°C for 3–5 h. After being examined on a 1.2% agarose gel, the digested DNA was self-ligated using T4 DNA ligase (TaKaRa) in a volume of 5 μL by incubation at 16°C for 6–8 h. The resulting circle DNA was then used as a template for IPCR.

The IPCR was performed using Ex Taq polymerase (TaKaRa) and primer pairs directed outwards from the genes as follows: *ChCYC1C* and *ChCYC1D*, 5′-TGACAAGCCAAGTAAAACAC-3′ (forward) and 5′-GAAGAGACTGTGAAACCTGAG-3′ (reverse); and *ChRAD*, 5′-CCTAATTACAGGACCACTCGG-3′ (forward) and 5′-CACGTTGTCCCAACGATC-3′ (reverse). The following thermocycling conditions were used: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 50–55°C for 45 s, and 72°C for 2 min and 30 s, with a final extension at 72°C for 10 min. A 1-μL aliquot of PCR products from the first round of the PCR was then used as a template for the second round of PCR with a pair of nested primers further out: *ChCYC1C* and *ChCYC1D*, 5′-AGGCAAGAGCAAGGGCTAGG-3′ (forward) and 5′-GACTGTGAAACCTGAGGAGGATG-3′ (reverse); and *ChRAD*, 5′-GTACGTTGGCTTTGGGTGAAG-3′ (forward) and 5′-TCGCTGTCCAATTTCTCGAAC-3′ (reverse). The PCR conditions were initial denaturation at 94°C for 3 min, followed by 15–25 cycles of 94°C for 30 s, 50–55°C for 30 s, and 72°C for 2 min and 30 s, with a final extension at 72°C for 10 min. The amplified products were resolved on 1.2% agarose gels and the putative fragments were cut from the gels and purified before being cloned into the pGEM-T easy vector (Promega) and sequenced.

The resulting upstream genomic regions were further confirmed by general PCR using gene-specific primers: *ChCYC1C*, 5′-CATGAGTTGTTCACTGGCATACC-3′ (forward) and 5′-GAG CTCTTGCCAAACATTGTTG-3′ (reverse); *ChCYC1D*, 5′-GCTAGGCTTACCATTACCAACC-3′ (forward) and

Table 1 Accession numbers for genes used in the phylogeny analysis of the *ChRAD* gene in the present study

Taxon	GenBank. Accession no.	Gene name	Reference
Antirrhinum majus L.	AY954971	*RAD*	Corley et al. (2005)
	AJ791699	*AmRL1*	Baxter et al. (2007)
	DQ375227	*AmRL3*	Baxter et al. (2007)
	DQ375228	*AmRL4*	Baxter et al. (2007)
	AJ793240	*AmRL5*	Baxter et al. (2007)
Arabidopsis thaliana	At4g39250	*AtRL1*	Baxter et al. (2007)
	At2g21650	*AtRL2*	Baxter et al. (2007)
	At4g36570	*AtRL3*	Baxter et al. (2007)
	DQ395345	*AtRL4*	Baxter et al. (2007)
	At1g19510	*AtRL5*	Baxter et al. (2007)
	At1g75250	*AtRL6*	Baxter et al. (2007)
Bournea leiophylla	EF207557	*BlRAD*	Zhou et al. (2008)
	EF211118	*BlDIV1*	Zhou et al. (2008)
Chirita heterotricha Merr.	NA	*ChRAD*	Present study

NA, not available.

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5′-GCAGAAGTAGACCCACGAGAT-3′ (reverse); and *ChRAD*, 5′-AATGAGTAACCTCG TGCACCC-3′ (forward) and 5′-TCGTCTTCCACGTTTCCCTC-3′ (reverse). The PCR products were purified and sequenced.

1.5 Promoter analyses

To predict the promoter regions and the transcription start sites, genomic regions upstream of the *ChCYC1* and *ChRAD* genes were submitted to an online TSSP (Plants Pol II promoter region and start of transcription) tool (using RegSite Plant DB (Softberry Inc.); http://linux1.softberry.com/berry.phtml?topic=plantprom&group=data&subgroup=plantprom, accessed 13 March 2010). We also used the TSSP tool to predict the transcription factor binding sites (RegSite). DNAMAN software (Lynnon Biosoft) was used to search for putative CYC-binding sites in the promoters of these genes.

2 Results

2.1 Identification of the *ChRAD* gene

We isolated a *RAD*-like gene from *Chirita heterotricha* that contained a 276-bp open reading frame that potentially encodes a 92-amino acid protein. We designated this gene *ChRAD* based on its high sequence similarity with the *BlRAD* and *AmRAD* genes in the BLAST analyses and the results of phylogenetic analyses (Fig. 1). Phylogenetic analyses based on the NJ method showed that ChRAD protein is sister to BlRAD from *Bournea* (100% bootstrap) and they are further clustered with *Antirrhinum* RAD protein with high support (99% bootstrap; Fig. 1). In addition, of six RAD-like proteins from *Arabidopsis*, AtRL1 and AtRL2 form a clade with high support (96% bootstrap) that is further sister to the monophyletic clade including ChRAD, BlRAD, and *Antirrhinum* RAD proteins. All the results indicate that the *ChRAD* gene we isolated is closely related to the *Antirrhinum RAD* gene.

Subsequently, the amino acid sequence of the *ChRAD* gene was compared with that of the *BlRAD* and *AmRAD* genes using Clustal X and BioEdit software. As shown in Fig. 2, ChRAD has a single MYB domain that is characterized by three conserved tryptophan residues spaced 18–20 residues apart. In addition, similar to BlRAD and AmRAD, the third tryptophan residue of the ChRAD protein is replaced by tyrosine.

2.2 Sequence analyses of *ChCYC1* and *ChRAD* upstream genomic regions

Excessive *Eco*RI, an enzyme with no recognition site in the known coding regions of *ChCYC1* genes,

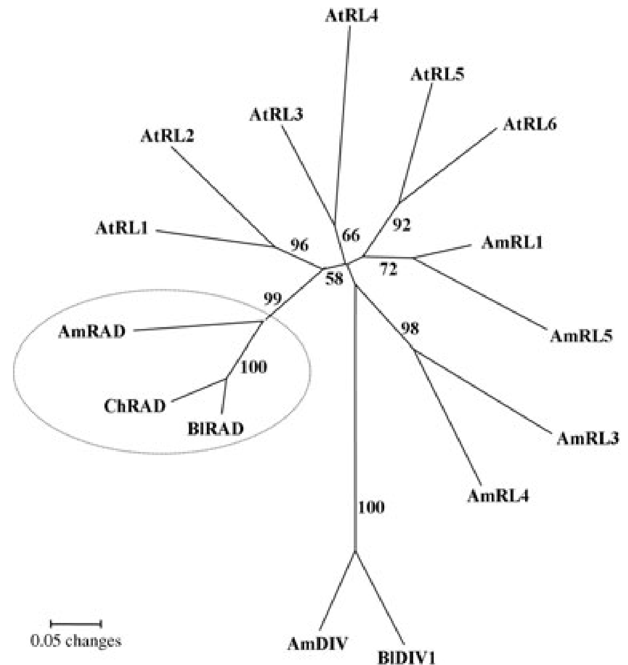

Fig. 1. Neighbor-joining (NJ) tree showing the relationships among ChRAD, BlRAD, RAD and RAD-like proteins from *Antirrhinum* and *Arabidopsis*. The sequences used in the tree include RAD and four RAD-like proteins (AmRL1 and AmRL3–AmRL5) from *Antirrhinum*, six RAD-like proteins (AtRL1–AtRL6) from *Arabidopsis*, and BlRAD from *Bournea*. Bootstrap values with support >50% are shown.

was used to digest genomic DNA. After two rounds of IPCR reactions, 1286- and 1683-bp upstream genomic regions were isolated for the *ChCYC1C* and *ChCYC1D* genes, respectively. Subsequent general PCR using gene-specific primers led to the validation of 1015- and 1454-bp upstream genomic sequences for the respective genes (Fig. 3). Sequence alignment revealed 82.11% sequence similarity between the *ChCYC1C* and *ChCYC1D* upstream regions, which is slightly lower than that between the respective coding sequences (86.59%; data not shown).

We also performed IPCR to isolate the genomic region upstream of the *ChRAD* gene. Excessive *Hin*dIII, a non-cutting enzyme in the *ChRAD* coding region, was used to digest genomic DNA. After IPCR amplification and general PCR validation, we isolated an 892-bp genomic sequence upstream of the *ChRAD* gene (Fig. 3).

2.3 Promoter predictions for the *ChCYC1* and *ChRAD* genes

To predict promoters of the *ChCYC1* and *ChRAD* genes, their upstream genomic regions were submitted to an online TSSP tool. As shown in Fig. 3, the 5′-untranslated regions of the *ChCYC1C*, *ChCYC1D*, and

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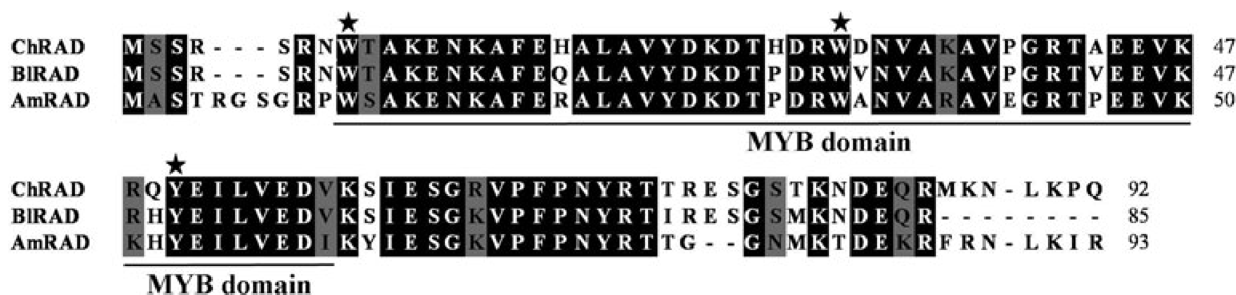

Fig. 2. Alignment of the amino acid sequences of *ChRAD*, *BlRAD*, and *AmRAD* genes. The stars indicate three conserved tryptophan residues spaced 18–20 residues apart (the third tryptophan residue is replaced by tyrosine).

ChRAD genes are 784-, 804-, and 260-bp in size, respectively. To test the results of promoter prediction, conventional reverse transcription–PCR was performed using several sets of elaborately designed primers. After PCR amplification and DNA sequencing, the results of the promoter predictions were further corroborated (data not shown). As a result, we obtained 231-, 650-, and 632-bp promoter sequences for the *ChCYC1C*, *ChCYC1D*, and *ChRAD* genes, respectively. The length of all three sequences corresponds with the usual length of promoters of plant genes (usually 200 bp). RegSite prediction indicated that the promoters of *ChCYC1C* and *ChCYC1D* contained a considerable number of transcription factor binding sites, most of which were shared by them (data not shown), indicating that they are probably regulated by a common set of transcription factors.

2.4 Identification of consensus CYC-binding sites

To understand why *ChCYC1C* and *ChCYC1D* are persistently expressed in developing *C. heterotricha* flowers, we used DNAMAN software to analyze their promoters by searching for "GGNCCC", the core sequence for the consensus CYC-binding site (GGNCCNC). As a result, a putative *cis*-regulatory element matching the consensus CYC-binding site was identified in each promoter: GGCCCCTC at position −165 for *ChCYC1C*, and GGCCCCCC at position −163 for *ChCYC1D* (Fig. 3) (the transcription initiation site is appointed as position +1). The presence of putative CYC-binding sites in the *CYC*-like gene promoters indicates that these genes may be regulated by their own products.

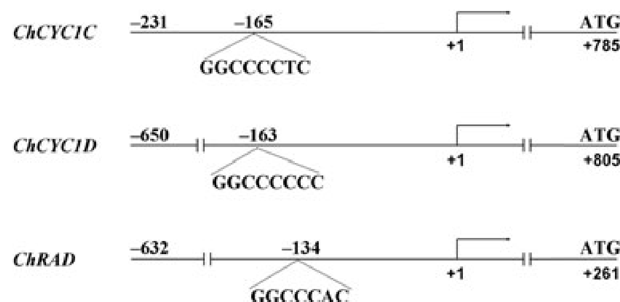

Fig. 3. Upstream genomic regions of *ChCYC1C*, *ChCYC1D*, and *ChRAD* genes showing the putative CYC-binding sites identified. Nucleotide positions are numbered relative to the transcription initiation site (appointed as position +1).

In addition, we analyzed the promoter sequence of the *ChRAD* gene using DNAMAN software. The *ChRAD* promoter also contains a putative CYC-binding site: GGCCCAC at position −134 (Fig. 3), indicating that the *ChRAD* gene is likely a direct target of *ChCYC1* genes, which is similar to the *Antirrhinum RAD* gene.

3 Discussion

3.1 Consensus CYC-binding sites relating to persistent expression of *ChCYC1* and regulation of *ChRAD* by *ChCYC1* in developing *Chirita heterotricha* flowers

Like the model plant *Antirrhinum majus*, *C. heterotricha* produces typical zygomorphic flowers. In *A. majus*, the *CYC* gene plays a key role in the establishment of zygomorphic flowers because of its dorsal identity function (i.e. controlling the fate of dorsal floral organs in the second and third whorls; Luo et al., 1996, 1999). In *C. heterotricha*, the *CYC* orthologs *ChCYC1C* and *ChCYC1D* are expressed not only in the dorsal petals and stamen, but also in the two lateral stamens (Gao et al., 2008). Cubas (2004) suggests that the maintenance of *CYC* expression after early floral development is likely to be important for generating the morphological zygomorphy in snapdragon flowers. Accordingly, the persistent expression of *ChCYC1C* and *ChCYC1D* in both the dorsal and lateral regions is tightly correlated with the formation of the bilateral corolla and the abortion of both dorsal and lateral stamens (Gao et al., 2008). Costa et al. (2005) suggest that the maintenance of *CYC* expression may be due to *CYC* containing

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a promoter sequence matching the consensus CYC-binding site, upon which an autoregulatory loop may have arisen in *A. majus*. To investigate why *ChCYC1* genes are persistently expressed in developing *Chirita* flowers, we performed IPCR to isolate genomic regions upstream of the *ChCYC1C* and *ChCYC1D* genes. More than 1 kb of an ATG upstream sequence was isolated for each gene (Fig. 3), and subsequent promoter predictions indicate that the entire promoter sequence (usually 200 bp in size) has been isolated for each gene, which is also supported by the existence of a mass of transcription factor binding sites (data not shown). Further promoter analyses showed that the promoter of each gene contains a putative *cis*-regulatory element matching the consensus DNA binding site for the *Antirrhinum* CYC protein (Costa et al., 2005). All these facts indicate that both *ChCYC1C* and *ChCYC1D* may have evolved autoregulatory loops to maintain their expression in developing flowers to form zygomorphic flowers in *C. heterotricha* (Gao et al., 2008). Although Costa et al. (2005) mentioned that the *Antirrhinum CYC* promoter probably contains a CYC-binding site, as yet there is no direct evidence supporting this claim. Therefore, the present study provides the first and direct evidence for the existence of a consensus CYC-binding site in the promoters for each of the genes, suggesting that an autoregulatory loop evolved in *CYC*-like genes, corresponding to the formation of actual morphological zygomorphy.

It has been suggested that *CYC* activity in establishing floral dorsal identity is mediated by cell cycle-related genes and the MYB gene *RAD*, specifically that *CYC* directly or indirectly suppresses *AmCYCLIN D3b* activity in the stamen whorl while activating *RAD* expression in the petal whorl (Luo et al., 1996; Gaudin et al., 2000; Corley et al., 2005). Further research has shown that the *RAD* promoter and intron contain several consensus CYC-binding sites that can be bound by recombinant CYC protein in *in vitro* gel shift assays, indicating that *RAD* is a direct target of *CYC* in *A. majus* (Costa et al., 2005). Even though expression data have demonstrated that *CYC*-like genes may also activate *RAD*-like genes outside the model organism *A. majus* (Zhou et al., 2008; Preston & Hileman, 2009), no direct evidence of this has been provided to date. Therefore, the finding in the present study that the *ChRAD* promoter contains the consensus CYC-binding site is suggestive of the *RAD*-like gene being likely a direct target of *CYC*-like genes in other Lameales plants.

3.2 Significance of the consensus CYC-binding site found in both *ChCYC1* and *ChRAD* promoters

Although transient expression of *CYC*-like genes in the dorsal regions of floral meristems usually has no morphological effect on floral symmetry (Cubas et al., 2001; Zhou et al., 2008), the persistent expression of the genes in the corresponding domains of whorl 2 and 3 floral organs have clear morphological effects in species with zygomorphic flowers (Luo et al., 1996, 1999; Hileman et al., 2003; Citerne et al., 2006; Feng et al., 2006; Busch & Zachgo, 2007; Gao et al., 2008; Wang et al., 2008; Song et al., 2009). Taking into consideration previous genetic and expression data together with the results of the present study, we postulate an interpretation that may provide a reasonable explanation for the establishment of morphological zygomorphy relying on the persistent expression of *CYC*-like genes as follows (Fig. 4): at the early stage of flower development, *CYC*-like genes are activated by upstream transcription factors (Fig. 4, Step a) and are expressed at basal levels (Fig. 4, Step b); the basal level expression of *CYC*-like genes is maintained and/or strengthened (Fig. 4, Step e) by autoregulation (Fig. 4, Step d) to yield to more products; once the products reach a certain threshold, downstream genes are activated and their expression controls floral dorsoventral asymmetry (Fig. 4, Step f). In *Arabidopsis* (Fig. 4: A), the lack of a consensus CYC-binding site in the *TCP1* promoter is correlated to its early and transient expression in the dorsal floral meristems, a pre-pattern of ancestral *CYC* gene expression (Cubas et al., 2001; Costa et al., 2005). In contrast, the maintenance of *CYC* and *ChCYC1* expression in corresponding domains of floral organs in zygomorphic *Antirrhinum* and *Chirita* flowers depends on the acquisition of an autoregulatory loop during evolution (Fig. 4: B, C). In addition, *Arabidopsis RAD*-like genes lack consensus CYC-binding sites in their promoters, whereas *Antirrhinum RAD* and *Chirita ChRAD* promoters contain such sites, indicating that they are likely direct targets of *CYC* and *ChCYC1*, respectively.

It is believed that the first flower was zygomorphic in Lamiales s.l. (Donoghue et al., 1998; Endress, 1998, 1999). The plants of Lamiales s.l. have further evolved from early and moderate zygomorphy towards advanced and strong zygomorphy in different clades (Donoghue et al., 1998; Endress, 1998). The family Gesneriaceae, as the basal-most group in this order, is sister to the remainder of Lamiales s.l. (Cubas, 2004; Wortley et al., 2005). This family is characteristic of diverse forms of zygomorphy related to the floral organ differentiation early in the order (Li & Wang, 2004; Zhou et al., 2008; Song et al., 2009). Therefore, the findings of the present study indicate that floral zygomorphy has been established at the evolutionary beginning of the Lamiales s.l., achieved by the evolution of an autoregulatory loop for *CYC*-like genes, which was probably accompanied by the simultaneous co-option of

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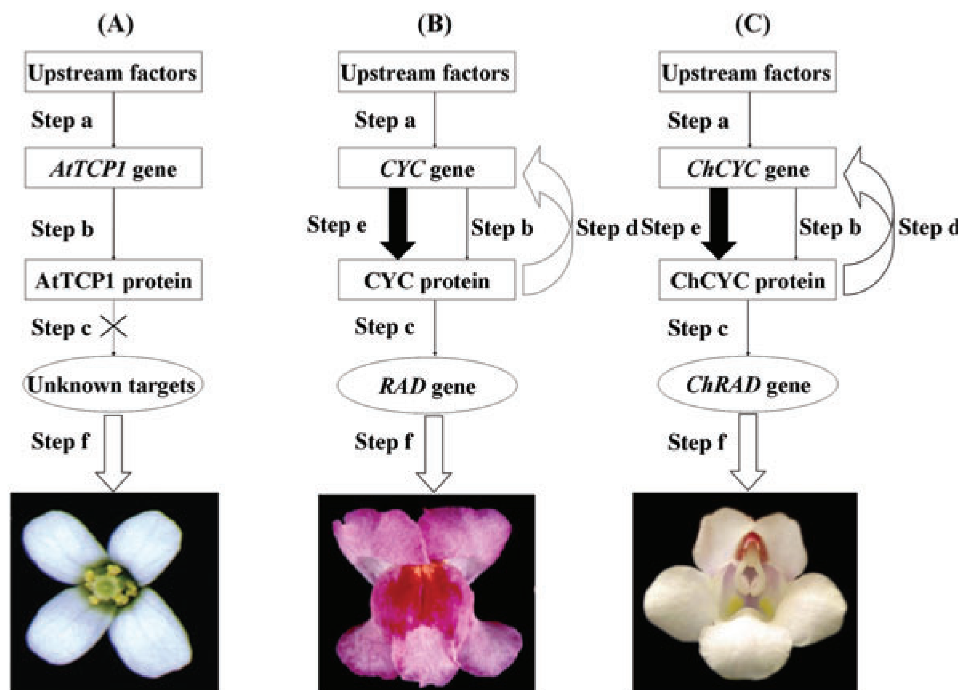

Fig. 4. Scheme showing the role of autoregulatory loops in the control of floral dorsoventral asymmetry in angiosperms. **A,** After floral asymmetry gene are activated by upstream factors (Step a), they are expressed at basal levels (Step b), which may be insufficient to activate downstream genes (Step c). **B, C,** Floral asymmetry genes have evolved autoregulatory loops (Step d; the dashed arrow indicates an unproven autoregulatory loop) to maintain and strengthen basal level expression; thus, sufficient products are produced (Step e) to activate downstream genes to form dorsoventral asymmetric flowers (Step f).

RAD-like genes into the regulatory network of *CYC*-like genes.

Further functional analyses of protein–DNA interactions with respect to the autoregulation of *CYC*-like genes and the regulatory relationships between *CYC*-like genes and upstream or downstream transcription factors are important for the regulatory network of floral symmetry genes to be revealed in addition to the complex combinatorial mechanisms underlying the evolutionary pathways and diversification of zygomorphy in angiosperms, especially in Lamiales s.l.

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References

Baxter CEL, Costa MMR, Coen ES. 2007. Diversification and co-option of *RAD*-like genes in the evolution of floral asymmetry. The Plant Journal 52: 105–113.

Busch A, Zachgo S. 2007. Control of corolla monosymmetry in the Brassicaceae *Iberis amara*. Proceedings of the National Academy of Sciences USA 104: 16 714–16 719.

Busch A, Zachgo S. 2009. Flower symmetry evolution: Towards understanding the abominable mystery of angiosperm radiation. BioEssays 31: 1181–1190.

Citerne HL, Pennington RT, Cronk QCB. 2006. An apparent reversal in floral symmetry in the legume *Cadia* is a homeotic transformation. Proceedings of the National Academy of Sciences USA 103: 12 017–12 020.

Corley SB, Carpenter R, Copsey L, Coen E. 2005. Floral asymmetry involves an interplay between TCP and MYB transcription factors in *Antirrhinum*. Proceedings of the National Academy of Sciences USA 102: 5068–5073.

Costa MMR, Fox S, Hanna AI, Baxter C, Coen E. 2005. Evolution of regulatory interactions controlling floral asymmetry. Development 132: 5093–5101.

Cubas P. 2002. Role of TCP genes in the evolution of key morphological characters in angiosperms. In: Cronk QCB, Bateman RM, Hawkins JA eds. Developmental genetics and plant evolution. London: Taylor & Francis. 247–266.

Cubas P. 2004. Floral zygomorphy, the recurring evolution of a successful trait. BioEssays 26: 1175–1184.

Cubas P, Coen E, Zapater JM. 2001. Ancient asymmetries in the evolution of flowers. Current Biology 11: 1050–1052.

Cubas P, Lauter N, Doebley J, Coen E. 1999. The TCP domain: A motif found in proteins regulating plant growth and development. The Plant Journal 18: 215–222.

Doebley J, Stec A, Gustus C. 1995. *teosinte branched1* and the origin of Maize: Evidence for epistasis and the evolution of dominance. Genetics 141: 333–346.

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Doebley J, Stec A, Hubbard L. 1997. The evolution of apical dominance in maize. Nature 386: 485–488.

Donoghue MJ, Ree RH, Baum DA. 1998. Phylogeny and the evolution of flower symmetry in the Asteridae. Trends in Plant Science 3: 311–317.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11–15.

Du ZY, Wang YZ. 2008. Significance of RT-PCR expression patterns of *CYC*-like genes in *Oreocharis benthamii* (Gesneriaceae). Journal of Systematics and Evolution 46: 23–31.

Endress PK. 1998. *Antirrhinum* and Asteridae: Evolutionary changes of floral symmetry. Symposia of the Society for Experimental Biology 51: 133–140.

Endress PK. 1999. Symmetry in flowers: Diversity and evolution. International Journal of Plant Sciences 160: S3–S23.

Feng X, Zhao Z, Tian Z, Xu S, Luo Y, Cai Z, Wang Y, Yang J, Wang Z, Weng L, Chen J, Zheng L, Guo X, Luo J, Sato S, Tabata S, Ma W, Cao X, Hu X, Sun C, Luo D. 2006. Control of petal shape and floral zygomorphy in *Lotus japonicus*. Proceedings of the National Academy of Sciences USA 103: 4970–4975.

Gao Q, Tao JH, Yan D, Wang YZ, Li ZY. 2008. Expression differentiation of *CYC*-like floral symmetry genes correlated with their protein sequence divergence in *Chirita heterotricha* (Gesneriaceae). Development Genes and Evolution 218: 341–351.

Gaudin V, Lunness PA, Fobert PR, Towers M, Riou-Khamlichi C, Murray JAH, Coen E, Doonan JH. 2000. The expression of *D-Cyclin* genes defines distinct developmental zones in snapdragon apical meristems and is locally regulated by the *cycloidea* cene. Plant Physiology 122: 1137–1148.

Hall TA. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hileman LC, Kramer EM, Baum DA. 2003. Differential regulation of symmetry genes and the evolution of floral morphologies. Proceedings of the National Academy of Sciences USA 100: 12 814–12 819.

Kim DW, Lee SH, Choi S-B, Won S-K, Heo Y-K, Cho M, Park Y-II, Cho H-T. 2006. Functional conservation of a root hair cell-specific *cis*-element in angiosperms with different root hair distribution patterns. The Plant Cell 18: 2958–2970.

Kosugi S, Ohashi Y. 1997. PCF1 and PCF2 specifically bind to *cis* elements in the rice proliferating cell nuclear antigen gene. The Plant Cell 9: 1607–1619.

Kosugi S, Ohashi Y. 2002. DNA binding and dimerization specificity and potential targets for the TCP protein family. The Plant Journal 30: 337–348.

Li ZY, Wang YZ. 2004. Plants of Gesneriaceae in China. Zhengzhou: Henan Science and Technology Publishing House.

Luo D, Carpenter R, Copsey L, Vincent C, Clark J, Coen E. 1999. Control of organ asymmetry in flowers of *Antirrhinum*. Cell 99: 367–376.

Luo D, Carpenter R, Vincent C, Copsey L, Coen E. 1996. Origin of floral asymmetry in *Antirrhinum*. Nature 383: 794–799.

Navaud O, Dabos P, Carnus E, Tremousaygue D, Herve C. 2007. TCP transcription factors predate the emergence of land plants. Journal of Molecular Evolution 65: 23–33.

Preston JC, Hileman LC. 2009. Developmental genetics of floral symmetry evolution. Trends in Plant Science 14: 147–154.

Song CF, Lin QB, Liang RH, Wang YZ. 2009. Expressions of ECE-CYC2 clade genes relating to abortion of both dorsal and ventral stamens in *Opithandra* (Gesneriaceae). BMC Evolutionary Biology 9: 244, doi:10.1186/1471-2148-9-244.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876–4882.

Wang L, Gao Q, Wang YZ, Lin QB. 2006. Isolation and sequence analysis of two *CYC*-like genes, *SiCYC1A* and *SiCYC1B*, from zygomorphic and actinomorphic cultivars of *Saintpaulia ionantha* (Gesneriaceae). Acta Phytotaxonomica Sinica 44: 353–361.

Wang Z, Luo YH, Li X, Wang LP, Xu SL, Yang J, Weng L, Sato S, Tabata S, Ambrose M, Rameau C, Feng XZ, Hu XH, Luo D. 2008. Genetic control of floral zygomorphy in pea (*Pisum sativum* L.). Proceedings of the National Academy of Sciences USA 105: 10 414–10 419.

Wittkopp PJ, Haerum BK, Clark AG. 2004. Evolutionary changes in *cis* and *trans* gene regulation. Nature 430: 85–88.

Wortley AH, Rudall PJ, Harris DJ, Scotland RW. 2005. How much data are needed to resolve a difficult phylogeny: Case study in Lamiales. Systematic Biology 54: 697–709.

Zhou XR, Wang YZ, Smith JF, Chen RJ. 2008. Altered expression patterns of TCP and MYB genes relating to the floral developmental transition from initial zygomorphy to actinomorphy in *Bournea* (Gesneriaceae). New Phytologist 178: 532–543.

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