ARTICLE
HTTP://DX.DOI.ORG/10.5504/BBEQ.2013.0063 E&BS
EDUCATION & BASIC SCIENCE

FUNCTION OF THE *CYC*-LIKE GENES DURING FLORAL DEVELOPMENT IN SOYBEAN

Ronghua Hao, Baotian Zhao, Haichao Wei, Wen Cheng, Qun Shao, Xianzhong Feng, Yanxiu Zhao
Shandong Normal University, College of Life Science, Jinan, Shandong, P.R. China
Correspondence to: Yanxiu Zhao
E-mail: zhaoyx@sdnu.edu.cn

ABSTRACT

Floral zygomorphy is proved to have evolved many times independently from actinomorphy, exhibiting two kinds of asymmetry, dorsoventral (DV) and organ internal (IN) asymmetry, in flowering plants. Multiple underlying regulators are involved in establishing the complex flowering mechanism during plant development. To understand how genes control the floral dorsoventral asymmetry in soybean, we isolated and analyzed the role of two different copies of CYC-like genes in Glycine max. It was demonstrated that CYC-like genes, GmTCP1 and GmTCP2, encoded the TCP transcription factors that were specifically expressed in the dorsal region of floral meristem. However, the two copies were proved to be functionally divergent during petal development, and by altering the expression of CYC-like genes in transgenic soybean, similar to CYC and DICH, it was found that CYC-like genes had dorsalizing activity. The expression pattern of transgenic plants indicated that the GmTCP2 gene could specifically regulate downstream target genes such as RAD-like and DIV-like genes in the dorsal, lateral and the ventral petals. The relative expression of CUC1 and CUC2 varied in the three different kinds of petals: it was high in the dorsal petals, but low in the ventral petals. At the same time, the expression level of GmTCP1 and GmTCP2 was equally very low. In addition, the study of transgenic Arabidopsis showed that GmTCP2 could play a significant role in cell division during the whole development of plants.

Biotechnol. & Biotechnol. Eq. 2013, **27**(6), 4332-4340

Keywords: soybean, dorsoventral symmetry, *CYC* homologue, TCP transcription factor, *GmTCPs* genes, *GmRADs* genes, *GmDIVs* genes

Introduction

Floral symmetry is a classical characteristic of floral diversity. Two types of floral symmetry in angiosperms are actinomorphy and zygomorphy. During the evolution of angiosperms, floral zygomorphy has developed as a key adaptation to specialized methods of pollination (5). Among the major clades of angiosperms, floral zygomorphy shows great morphological diversity in *Scrophulariaceae*, which includes a major model organism in developmental biology, *Antirrhinum majus* (9, 10). Previous intensive studies on the model plant *Antirrhinum majus* have demonstrated that there are four cardinal genes in the development of dorsoventral asymmetry: *CYCLOIDEA* (*CYC*), *DICHOTOMA* (*DICH*), *RADIALIS* (*RAD*) and *DIVARICATA* (*DIV*) (4, 8, 13, 20, 21). *CYC* and *DICH* genes, whose products belong to the TCP transcription factor family, are involved in the dorsal identity during flower development. Both *RAD* and *DIV* genes code for members of the MYB transcription factor family, with the *RAD* gene promoting dorsal identity, while the *DIV* gene promotes ventral identity. The *CYC* and *DICH* genes are expressed in the dorsal floral primordium, and then activate the expression of the *RAD* gene. The expression of the *DIV* gene is inhibited by *RAD* gene expression in the dorsal and lateral petals. Therefore, dorsal identity is expressed to a different degree in the adaxial domain of dorsal and lateral petals, forming three morphologically different petals.

CYC-like genes have been found to play a significant role in controlling floral dorsoventral (adaxial–abaxial) asymmetry in *Lotus japonicas*, suggesting that the *CYC* homologue independently evolved as a determinant of petal identities along the dorsoventral axis in two distant lineages of flowering plants (11). Two *CYC*-like genes in pea, *LST1* and *K*, were proved to encode TCP transcription factors and were shown to have divergent functions in dorsoventral asymmetry, suggesting a common molecular origin for the mechanisms controlling floral zygomorphy in legume (29).

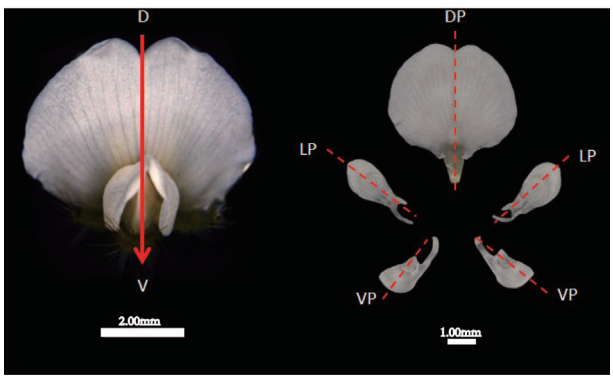

Fig. 1. Zygomorphic flowers and petals of *Glycine max*. Lines with the red arrow show the direction of floral dorsoventral axis. D, V, direction of the floral dorsoventral axis. Red broken lines indicate internal symmetry of different petals; DP, dorsal petal; LP, lateral petal; VP, ventral petal. (Scale bars, 1.00 mm.)

Flower symmetry is of special interest in understanding angiosperm evolution (16). As flower bilateral symmetry is drawing more and more attention in different species, *CYC*-like genes have been shown to be involved in the development

TABLE 1

Details about the gene fragments cloned and the primers used for PCR amplification

Gene	Size (bp)	Insertion	Primer sequence	Restriction enzyme sites
GmTCP1	1300	Sense	OL0530: 5'-CCGctcgagAGATAGATAAGATACCATCTTCTTG-3'	*Xho*I
			OL0638: 5'-CGgatccTCCATGACCATGATATTCC-3'	*Bam*HI
GmTCP1	1307	Antisense	OL0530: 5'-CCGctcgagAGATAGATAAGATACCATCTTCTTG-3'	*Xho*I
			OL0531: 5'-CCctcgagAGATGCCTCCATGACCATG-3'	*Xho*I
GmTCP1	527	RT-PCR	OL0842: 5'-TCTACCATCCACCAAGACCTACACC-3'	
			OL0843: 5'-AAGCCCTGTAGATAGATTCATGCTG-3'	
GmTCP1	130	qRT-PCR	OL1140: 5'-TCACAAAGTCCAAGAAGGCAATCAAGG-3'	
			OL1141: 5'-CCTTGTTGTTGGTGTAGGTCTTGGTGG-3'	
GmTCP2	1184	Sense/ Antisense	OL0670: 5'-gaattcGTCTAGGGTTTCTGTCCATTGGAAATT-3'	*Eco*RI
			OLO671: 5'-gaattcCAATCATCGGATAATACTGAAAGCTAAC-3'	*Eco*RI
GmTCP2	622	RT-PCR	OL0844: 5'-AAGTCCAAGAAGGCAATTAAGGAGC-3'	
			OL0845 5'-TGAAGCCCTGTAGACAGATTCATGC-3'	
GmTCP2	190	qRT-PCR	OL1018: 5'-ACACCTTTCTTCATGATCCACTTGC-3'	
			OL1019: 5'-ATCTTGCTGTGCCTGTCTTTCTTAG-3'	
GmRAD	92	qRT-PCR	OL1308: 5'-TGGCTCATGGAGTGTTAAGGACAA-3'	
			OL1309: 5'-GAGCAACATTGTACCAACGGTCAG-3'	
GmRAD	156	qRT-PCR	OL1217: 5'-CCTGCCGTTGAGGAAGTGAAGAG-3'	
			OL1218: 5'-CCTTCTTCTATACCTGTTTTCGATTTC-3'	
GmDIV1	179	qRT-PCR	OL1332: 5'-GCCTTGGACTGAAGACGAACATAAAT-3'	
			OL1333: 5'-TAGCCCTCCTCTTGTCTTTGCCTC-3'	
GmDIV3	173	qRT-PCR	OL1215: 5'-GGTGGCACAAAGTGGCTGAGATG-3'	
			OL1216: 5'-GCCAGGAGTGTTCACCCAATCTAAG-3'	
GmDIV4	109	qRT-PCR	OL1334: 5'-CTATGGTGATGGCTTCAAAGGACTC-3'	
			OL1335: 5'-CAACTTGTGTTCTTCTTCAGTCCAT-3'	
GmDIV5	86	qRT-PCR	OL1306: 5'-CAGGCAACTATCAGGAGGCAAGG-3'	
			OL1307: 5'-AAGTTCTGGTGGTTTCTGTGAGATT-3'	
GmCUC	138	qRT-PCR	OL1842: 5'-GGTTTCAGGTTTCACCCCACTGATG-3'	
			OL1843:5'-CACATCTGGAAGTTCCCAAGGCTC-3'	
GmCUC	154	qRT-PCR	OL1844:5'-GGCTTCAGATTCCACCCCACAGATG-3'	
			OL1845: 5'-TCTCACCCATCTTTGCTTTCTCAGG-3'	
Actin 11	142	qRT-PCR	OL1016: 5'-CGGTGGTTCTATCTTGGCATC-3'	
			OL1017: 5'-GTCTTTCGCTTCAATAACCCTA-3'	
AtTCP1	110	qRT-PCR	OL0561: 5'-GGTACGGTGAAGAAGAAGTGG-3'	
			OL0562: 5'-TCCTCTAGCTTTGGCTCCTAG-3'	
Actin 2	130	qRT-PCR	OL0563: 5'-AGGTATCGCTGACCGTATGA-3'	
			OL0564: 5'-GCTGAGGGAAGCAAGAATG-3'	

TABLE 2

List of components in the media used for the modified soybean cotyledonary-node transformation method

Components	GM (germination medium)	CCM (co-cultivation medium)	SIM (shoot induction medium)	SEM (shoot elongation medium)	RM (rooting medium)
MS salts	-	-	-	1×	-
B5 salts	1×	1/10×	1×	-	1/2×
MS iron stock	1×	1/10×	1×	1×	1×
B5 vitamins	1×	1×	1×	1×	-
Mes	3 mmol×L^{-1}	20 mmol×L^{-1}	3 mmol×L^{-1}	3 mmol×L^{-1}	3 mmol×L^{-1}
Sucrose (w/v)	3 %	3 %	3 %	3 %	3 %
Agar (w/v)	0.8 %	0.5 %	0.8 %	0.8 %	0.8 %
pH	5.8	5.4	5.6	5.6	5.6
6-Benzyl-aminopurine	-	1.67 mg×L^{-1}	1.67 mg×L^{-1}	-	-
Gibberellic acid	-	0.25 mg×L^{-1}	-	0.5 mg×L^{-1}	-
Acetosyringone	-	0.2 mmol×L^{-1}	-	-	-
L-Cysteine	-	8.8 mmol×L^{-1}	-	-	-
Sodium thiosulfate	-	1.0 mmol×L^{-1}	-	-	-
DTT	-	1.0 mmol×L^{-1}	-	-	-
Hygromycin B	-	-	0.5 mg×L^{-1}	0 mg×L^{-1}, 5 mg×L^{-1}, 10 mg×L^{-1}	-
Cefotaxime	-	-	300 mg×L^{-1}	300 mg×L^{-1}	-
Carbenicillin	-	-	300 mg×L^{-1}	300 mg×L^{-1}	-
Asparagine	-	-	-	50 mg×L^{-1}	-
Pyroglutamic acid	-	-	-	100 mg×L^{-1}	-
Indole-3-acetic acid	-	-	-	0.1 mg×L^{-1}	-
Zeatin-riboside	-	-	-	1 mg×L^{-1}	-
Silver nitrate	-	-	-	10 mg×L^{-1}	-
Indole-3-butyric acid	-	-	-	-	1 mg×L^{-1}

of zygomorphy in different species (22, 27, 28). In legumes, numerous *CYC*-like genes have been isolated and found to have undergone repeated duplication events, implying that they might have divergent functions (1, 11, 12). However, it remains unknown whether such TCP-box genes could be involved in the regulation of floral asymmetry in *Glycine max* belonging to the *Faboideae* subfamily of the *Leguminosae* family, which is an ancient polyploidy with a dorsoventral axis of the flower comprising five petals of three types: a single bilaterally symmetrical petal in the dorsal position, two asymmetric lateral petals and two asymmetric ventral petals (**Fig. 1**). To address this question, in this study we focused on the divergent function of different *CYC*-like gene copies in *Glycine max*. The data indicated that the *CYC*-like gene *GmTCP2* had dorsalizing activity in the control of floral zygomorphy development.

Materials and Methods

Phylogenetic analysis

The phylogenetic tree of the TCP proteins was constructed with the neighbor-joining method, using MEGA 5.0. Branches with bootstrap support (1 000 replicates) of ≥50 % are indicated for each branch (3).

Isolation of RNA and RT-PCR

Total RNA was isolated from tissues with Trizol, according to the manufacturer's procedure (Invitrogen Life Technologies). The RNA was treated with RNase-free DNase I (NEB) at 37 °C for 30 min. RT-PCR was performed with the PrimeScript™ RT-PCR Kit (TAKARA), according to the manufacturer's instructions.

Gene cloning

Fragments of *CYC*-like genes were amplified from *Glycine max* with appropriate primers, as shown in **Table 1**. PCR amplifications were carried out by using Taq and reagents (Trans Start Fast Pfu DNA Polymerase) in a 50 µL mix containing 200 µmol·L^{-1} of dNTP, 0.4 µmol·L^{-1} of each primer, 1 unit of Pfu and appropriate amount of cDNA. PCR was performed with gene-specific primers for 39 cycles.

Plasmid constructs

The vector pCAMBIA1301M was used as a basal plasmid in the construction of the sense and antisense transformation in this study. The vector pCAMBIA1301M was modified from

pCAMBIA1301 by adding a CaMV *35S* promoter and a *Nos* terminator of the pRT101 plasmid in the site of *Hin*dIII. The sense and antisense DNA fragments were amplified using the specific primers and then were inserted into appropriate sites of pCAMBIA1301M (shown in **Table 1**).

Plant material and transformation

Glycine max ecotype Gao feng 1 was used in this study. Soybean was transformed using the *Agrobacterium* mediated transformation procedure in the cotyledonary-node method, as described by Olhoft et al. (25). *Agrobacterium tumefaciens* strain EHA105 was used for transformation. The cotyledonary-node explants were inoculated in the liquid CCM/ *Agrobacterium tumefaciens* suspension and incubated for 30 min with shaking at 60 $r \cdot min^{-1}$. Next, explants (adaxial side down) were placed on solid CCM (shown in **Table 2**) in Petri dishes (100 mm×15 mm) with sterile 70 mm Whatman #1 filter paper. The Petri dishes were incubated at 25 °C for 5 days in the dark. Explants were then briefly washed in liquid SIM (shown in **Table 2**) to remove excess *Agrobacterium tumefaciens* and transferred onto solid SIM without hygromycin B, with the hypocotyl and cotyledonary node placed within the medium to stimulate shoot induction for the first 14 days. Then, they were incubated in an incubator at 22 °C and an 18/6 h photoperiod at 150 $\mu mol \times m^{-2} \times s^{-1}$. After this incubation, the explants were transferred to fresh SIM containing 5 $mg \times L^{-1}$ hygromycin B for another 14 days, and then the shoots were transferred to fresh SEM (shown in **Table 2**) containing 10 $mg \times L^{-1}$ hygromycin B, every 14 days for selection. The elongated shoots (3 cm to 5 cm) were excised and basal portions were dipped into solidified RM (shown in **Table 2**). Rooted T0 plants were grown in the incubator to maturity at 25 °C and a 16/8 h photoperiod at 150 $\mu mol \times m^{-2} \times s^{-1}$ and 80 % humidity.

The Columbia-0 ecotype of *Arabidopsis* was used as the wild type. The resulting plasmid constructs were mobilized to *Agrobacterium tumefaciens* strain GV3101 and used for transformation of 5-week-old *Arabidopsis* plants by the floral-dip method (2). After being grown in the greenhouse, their seeds were collected and screened in MS (23) medium supplemented with 20 $mg \times L^{-1}$ hygromycin B for 10 days. Plants were then grown in a greenhouse on soil at 22 °C and a 16/8 h photoperiod at 150 $\mu mol \times m^{-2} \times s^{-1}$ and 80 % humidity.

Real-time PCR analysis

The expression of genes was analyzed by real-time quantitative RT-PCR, using SYBR® *premix Ex Taq*™ (TAKARA) in the GeneAmp 5700 sequence Detection System (Applied Biosystems, http://www.appliedbiosystems.com) according to the manufacturer's instructions. The relative expression level was calculated from three replicates using the $2^{-\Delta\Delta Ct}$ method after normalization to the *Actin11* control in soybean and the *Actin2* control in *Arabidopsis* (shown in **Table 1**).

Scanning electron microscopy

Scanning electron microscopy (Hitachi S-570) was performed as described (14).

Leaf surface area measurement

The area of petals was measured from digital images of dissected organs with the Image J program.

Results and Discussion

Phylogenetic analysis of CYC-like proteins in *Glycine max*

To determine the copy number of CYC-like proteins in soybean, 58 protein sequences with TCP and R domain were searched at: http://www.phytozome.net/soybean. According to the level of sequence similarities, the *CYC*-like proteins can be divided into three branches, the CYC/TB1 subgroup, PCF subgroup, and a relatively independent unit. Obviously, the numerous soybean sequences here were the best candidates for CYC homologous proteins. The event of duplication gave rise to two copies of CYC-like proteins in *Glycine max*. The GmTCP1 and GmTCP2 proteins are two copies of CYC-like proteins that were grouped in a close clade corresponding to the CYC and DICH proteins (**Fig. 2**), providing evidence that both GmTCP1 and GmTCP2 proteins may play an analogous role in the control of dorsal petal identity. Here we report the functional characterization of GmTCP1 and GmTCP2 proteins.

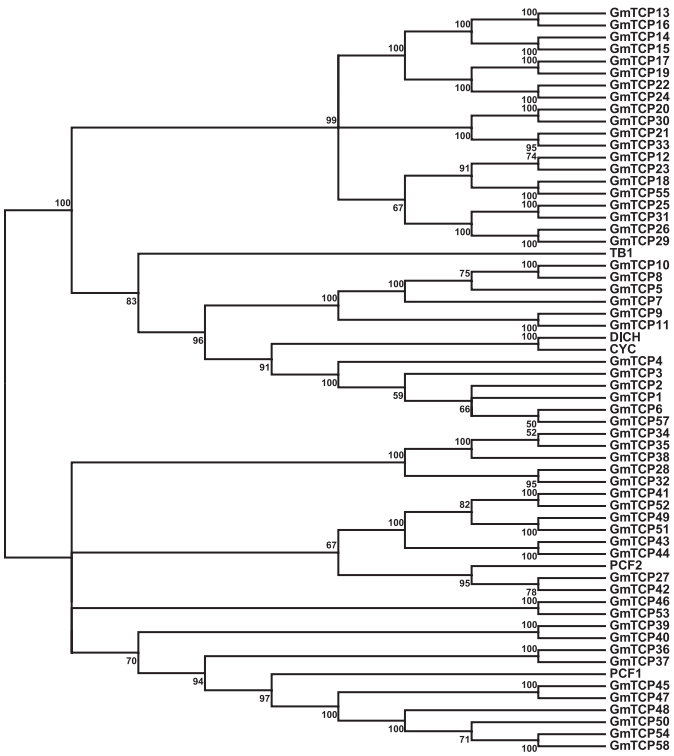

Fig. 2. Phylogram analysis of TCP proteins. *GmTCP1-GmTCP58*, *Glycine max*, CYC, Antirrhinum majus, TB1, Zea mays ; PCF1, PCF2, Oryza sativa. The phylogenetic tree was constructed with the neighbor-joining method using MEGA 5.0. Branches with bootstrap support (1,000 replicates) 50% are indicated for each branch.

CYC homologous proteins contain a basic-Helix-Loop-Helix (bHLH) domain

Analysis was made on the structure of CYC homologous proteins in soybean, the GmTCP1 and GmTCP2 proteins. According to our prediction, the two copies preserved a

conserved bHLH domain similar to CYC, TB1, PCF1 and PCF2 proteins (**Fig. 3A**). Therefore, we supposed that the GmTCP1 and GmTCP2 proteins could belong to the gene family of TCP transcription factors characterized by a bHLH domain.

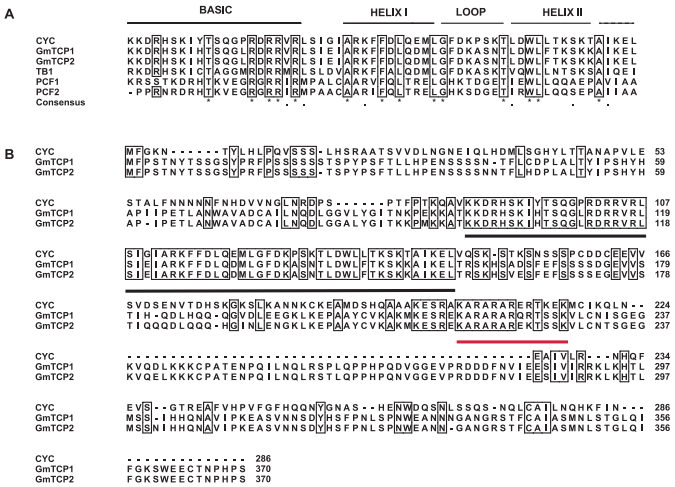

Fig. 3. Alignment of the amino acid sequences of GmTCP1 and GmTCP2. Alignment of the amino acid sequences of GmTCP1, GmTCP2, CYC, TB1, PCF1 and PCF2. Only the bHLH architecture in the TCP domain is shown. Identical amino acids are in black boxes. Alignment of amino acid sequences of GmTCP1, GmTCP2 and CYC. The TCP domain is underlined with black bold straight lines and the R domain is underlined with red bold straight lines.

To further confirm this, the GmTCP1 and GmTCP2 protein sequences were analyzed. The result showed that they both coded a conserved TCP and R domain similar to CYC (**Fig. 3B**), and apparently, were affiliated to the TCP transcription factors. Also, we found that the similarity of sequences for GmTCP1 and GmTCP2 proteins amounted to 94 %, which meant that they were two copies descending from a common ancestor.

Evidence from CYC homologous proteins in *Glycine max* strongly suggested that the CYC-like proteins could be easily aligned and they were homologous to the floral symmetry genes in *Antirrhinum majus*. The GmTCP1 and GmTCP2 proteins preserving the highly conserved TCP and R domains, were isolated and verified to belong to the TCP family of transcription factors. This suggested that the conserved TCP domain was necessary for the dorsal activity of CYC-like proteins.

Expression pattern of *GmTCP1* and *GmTCP2* genes

The TCP domain genes are universally expressed in rapidly growing primordial meristem in plants, which suggests that many members of the TCP family may affect cell division. To investigate the expression patterns of *CYC* homologues in soybean, RT-PCR analysis was carried out. The result showed that *GmTCP1* and *GmTCP2* were only specifically transcribed in the flowers, but were not detectable in the roots, stems, leaves, pods and seeds (**Fig. 4A, B**).

We further characterized the expression pattern by qRT-PCR in the dorsal, lateral and ventral petal flowers. The two copies were proved to share a uniform expression pattern, mostly transcribed in the dorsal petals in the early period of flower development (**Fig. 5**). Taken together, the expression pattern suggested that the two copies were actively expressed in flowers and involved in the establishment of the dorsoventral axis.

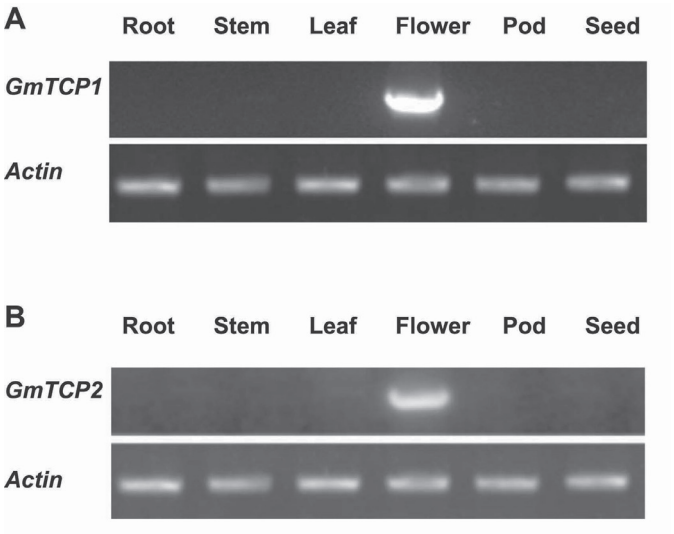

Fig. 4. RT-PCR showing the expression pattern of *GmTCP1* and *GmTCP2* in soybean. (A) Expression pattern of GmTCP1. (B) Expression pattern of *GmTCP2*. Total RNA was separately extracted from root, stem, leaf, flower, pod and seed of soybean after 50 days of sowing. The RT-PCR product of *Actin11* was loaded as an internal control.

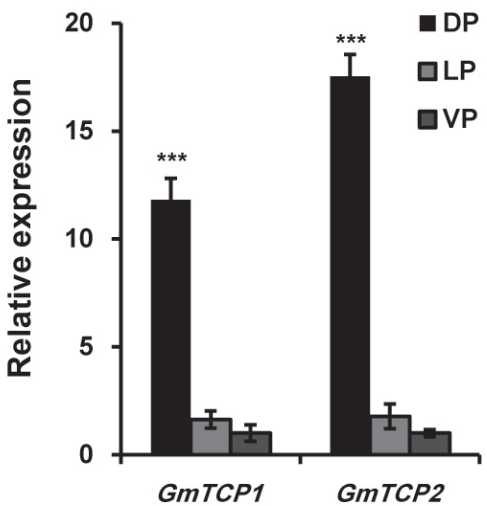

Fig. 5. QRT-PCR showing the expression pattern of *GmTCP1* and *GmTCP2* in soybean flowers. DP, dorsal petal; LP, lateral petal; VP, ventral petal (t-tests: * P <0.05;** P <0.01; *** P<0.001).Total RNA was separately extracted from dorsal petal, lateral petal and ventral petal of soybean flowers after 50 days of sowing. The qRT-PCR product of *Actin11* was used as a normalizer.

Previous studies on the model plant *Antirrhinum majus* demonstrated that the *CYC gene* is expressed at a very early stage in the dorsal regions of floral meristems (21). However, the study of actinomorphic flowers in *Bergia texana* (Elatinaceae) indicated that the *CYC*-like genes are expressed across all floral organs (32). Therefore, it could be considered that the expression pattern of *CYC* genes shows special

divergence with further evolution. In soybean, the *GmTCP1* and *GmTCP*2 genes were mainly expressed in the dorsal flower primordial region but less in the lateral and the ventral region. Significantly, the *CYC*-like genes were associated with the control of dorsoventral symmetry and cell proliferation. The expression patterns of the two *CYC*-like gene copies indicated that the TCP transcription factors act subtly to control the morphogenesis of flowers.

Effects of altered expression of *GmTCP2* in transgenic *Glycine max*

To explore the molecular scheme that dominates plant growth and development, we separately constructed sense and antisense vectors of *GmTCP1* and G*mTCP2* for plant transformation (**Fig. 6**). The results from the qRT-PCR analysis showed that the *GmTCP2* transcript level was increased in the *35S::GmTCP2* lines, confirming the validity of transformation (**Fig. 8**). However, none of the *35S::GmTCP1* plants showed any specific alteration in the soybean (data not shown). On the other hand, all of the *35S::GmTCP2* lines were morphologically distinguishable from the wild type plant, with a specific effect in the lateral petals, which developed symmetrical shapes similar to those of dorsal petals (**Fig. 7B**). In the adaxial epidermal layer of lateral petals dorsal-like conical cells were observed. Even the ventral petals were more symmetrical and the adaxial epidermal layer contained lateral-like dome cells, whereas the dorsal petal maintained the wild-type shape and cell type (**Fig. 7F–H**). These results illustrated that overexpression of *GmTCP2* could exert a dorsalizing effect and play a critical role in the establishment of dorsoventral asymmetry in *Glycine max*.

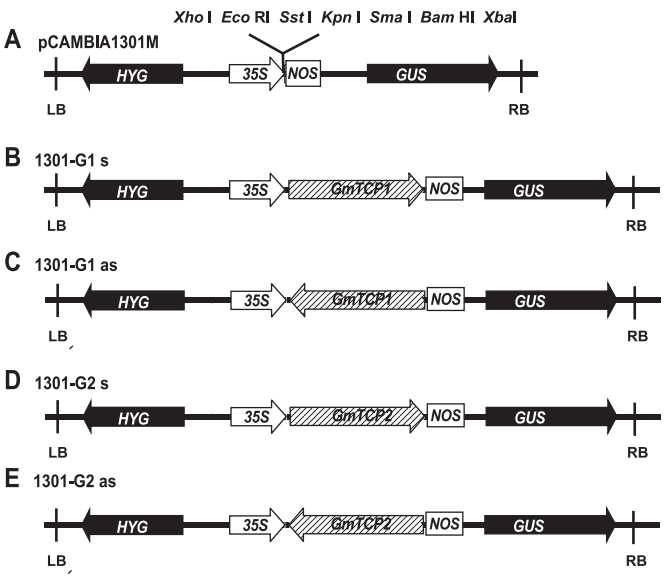

Fig. 6. Construction of different transgenes. (A) pCAMBIA1301M was a modified binary vector by adding a CaMV *35S* promoter, a T7 terminator. The restriction enzyme sites for the insertions of different *GmCYC* genes were indicated. (B–E) Schematic diagram of different transgenes, 1301-G1 s, 1301-G1 as, 1301- G2 s, and 1301-G2 as. Each of these constructs contains a sense or antisense sequence of *GmCYC* genes driven by the CaMV*35S* promoter and hygromycin-resistant gene as selectable marker gene.

To explore the pathway involved in the establishment of dorsoventral asymmetry, six homologs were optimized based on data previously obtained by us (30): *GmRAD3*(*Gm03g28050.1*), *GmRAD5*(*Gm19g30810.1*), *GmDIV1*(*Gm02g39000.1*), *GmDIV3*(*Gm 14g37050.1*), *GmDIV4*(*Gm11g22960.1*), and *GmDIV5*(*Gm18g07250.1*).

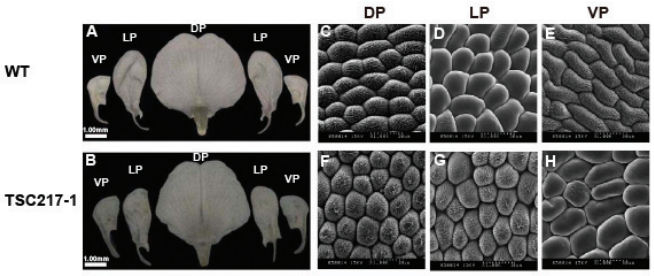

Fig. 7. Flowers of the wild type (*Glycine max*) and the *35S::GmTCP2* plant. (A) Wild type flower; (B) A typical flower of the 35S::GmTCP2 plant (TSC217-1); (C-H) Scanning electron microscopy of the representative epidermal cells from different petals in the wild type and transgenic plant. The paraxial region of cells are objected for scanning. DP, dorsal petal; LP, lateral petal; VP, ventral petal. (Scale bars in Fig 7A-B, 1.00mm; C-H, 30 μm.)

In order to study the effect of *GmTCP2* and downstream target genes in the *35S::GmTCP2* lines (TSC217-1), a series of qRT-PCRs were carried out. Up-regulated *GmTCP2* expression was detected in flowers at stages when all flower organs had been initiated. The transcription levels of the *GmRAD3* and *GmRAD5* genes were also increased when the expression level of *GmTCP2* was high in flowers, but the transcription levels of the *GmDIV1*, *GmDIV3*, *GmDIV4* and *GmDIV5* genes showed an opposite expression pattern to that of the *RAD*-like genes, with a reduction in the same transgenic lines (**Fig. 8**). These results indicated that overexpression of *GmTCP2* led to an increase of *GmRADs* expression and a reduction of *GmDIVs* expression in flowers.

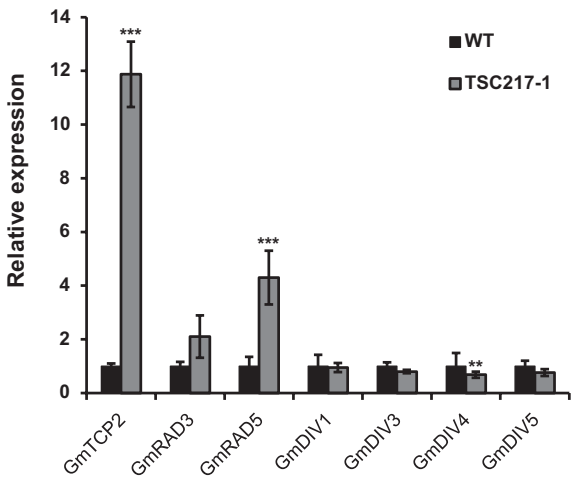

Fig. 8. The relative expression of genes in the *35S::GmTCP2* plant (TSC217-1). Asterisk (*) denotes a significant difference between the wild type and transgenic plant respectively (t-tests: * P <0.05;** P <0.01; *** P <0.001). Total RNA was extracted from soybean flowers after 50 days of sowing. The qRT-PCR product of *Actin11* was used as a normalizer.

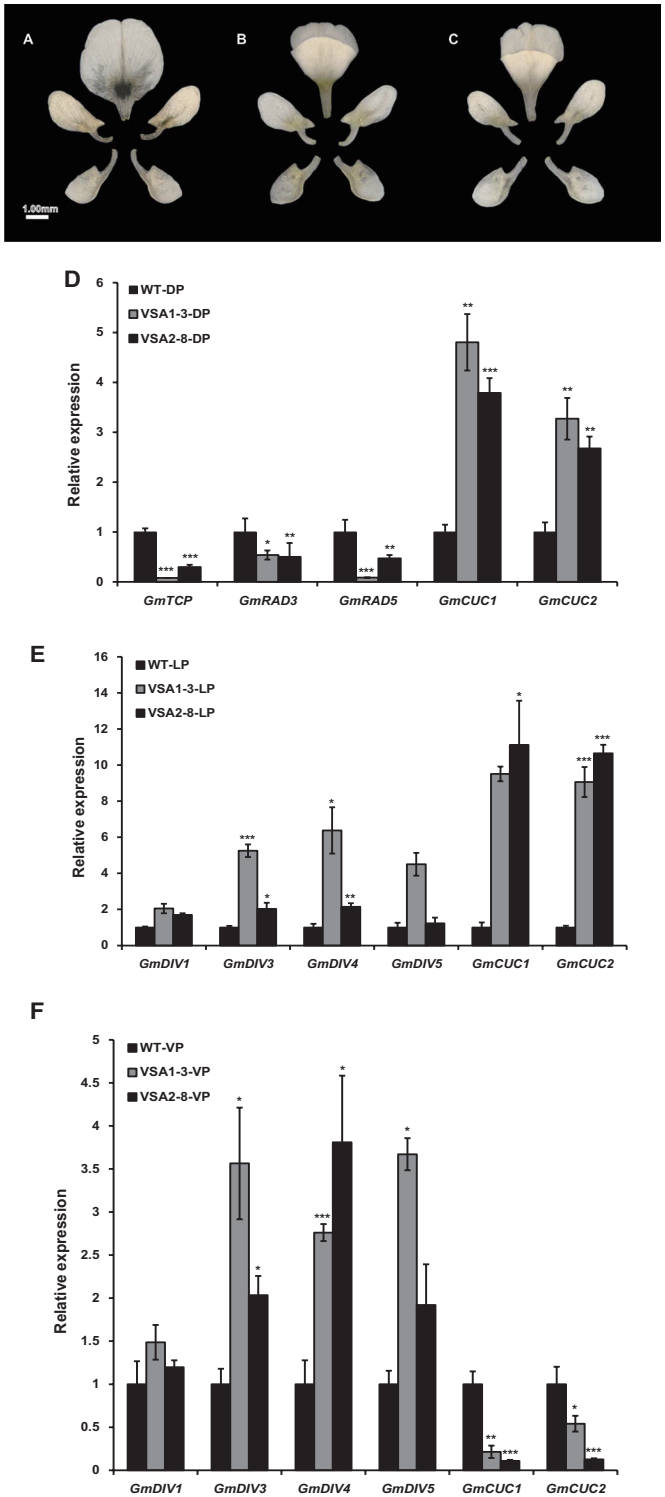

Fig. 9. Phenotype of antisense lines of *GmTCP1*, *GmTCP2* and the differences of relative expression of three type petals in transgenic soybean. (A) Wild type control; (B) Phenotype of antisense lines of GmTCP1 (VSA1-3); (C) Phenotype of antisense lines of *GmTCP2* (VSA2-8); (D-F) The differences of the relative expression of *GmTCP1* and GmTCP2 in the dorsal (D),lateral (E) and the ventral (F) petal of antisense lines are presented.Total RNA was separately extracted from dorsal, lateral and ventral petal of soybean flowers after 50 days of sowing. The qRT-PCR product of Actin11 was used as a normalizer.(Scale bars of flower, 1.00mm) DP, dorsal petal; LP, lateral petal; VP, ventral petal. Asterisk (*) denotes a significant difference.

The antisense lines of *GmTCP1* and *GmTCP2* displayed a similar phenotype, with tubular cup dorsal petals abnormal to various extents, and organ internal (IN) asymmetry completely lost in the dorsal petals, shifting to radially symmetrical petals (**Fig. 9B, C**). In order to study the effect of *GmTCP1*, *GmTCP2*, and the downstream target genes in the antisense lines, a series of qRT-PCRs were carried out. As a result, a reduction in the transcription level of *GmTCP1* and *GmTCP2* was detected in dorsal petals. *GmRAD3* and *GmRAD5* were down-regulated when the expression level of *GmTCP1* and *GmTCP2* were low in dorsal petals (**Fig. 9D**). However, the transcription levels of the *GmDIV1*, *GmDIV3*, *GmDIV4* and *GmDIV5* genes were enhanced in lateral and ventral petals (**Fig. 9E, F**). The relative expression of *CUC1* and *CUC2* varied in the three different kinds of petals. It was high in the dorsal petals, but low in the ventral petals; at the same time, the expression level of *GmTCP1* and *GmTCP2* was equally very low (**Fig. 9D, E**). Previous studies reported that CIN-like proteins control the morphology of lateral shoot organs via negative regulation of expression of NAC domain transcription factors, such as *CUC1*, *CUC2* and *CUC3* in *Arabidopsis* (18). Besides, it is well known that CIN-like CYC and TCP transcription factors negatively regulate the expression of *CUC* genes to inhibit the undifferentiated state of cells (19).

Overexpression of *CIN*-like genes, which is associated with a fused cotyledon phenotype, can lead to suppression of NAC domain transcription factors (26). Also, in *Antirrhinum*, the TIC protein has been proved to interact with a NAC domain protein that regulates organ fusion (31). Thus, NAC proteins are associated with TCP domain proteins, either as downstream targets or as interaction partners. However, the exact mechanism has not been revealed yet. It is worth to mention that altering the expression of the *GmTCP1* gene results in a dorsalization phenotype in antisense lines, but not in sense lines. There is probably a functional divergence for the different *GmTCP1* and *GmTCP2* copies during floral development. This indicates that the different copies contribute differently to the dorsal characteristics. It is possible that *GmTCP2* may play a more vital role in the transcriptional regulatory pathway, whereas *GmTCP1* might be recruited to other transcriptional regulatory pathways in soybean.

The *ECE-CYC2* clade of *TCP* genes show persistently asymmetric expression in the corresponding floral domains, indicating that they are functionally conserved in controlling floral zygomorphy. The altered *GmTCP2* expression in transgenic soybean supports the idea that the coded protein has a dorsalizing activity in the control of morphogenesis of the zygomorphic flowers, suggesting that *GmTCP2* expression is essential for floral development. Both of the *GmTCP1* and *GmTCP2* transformation events resulted in flower shape variation in soybean. As a whole, *GmTCP* genes, *GmRAD* genes and *GmDIV* genes could be considered to be involved in the control of flower bilateral symmetry in soybean. *GmRAD* genes might be the downstream targets of *GmTCP* genes, while *GmRAD*s might antagonize *GmDIV* genes in

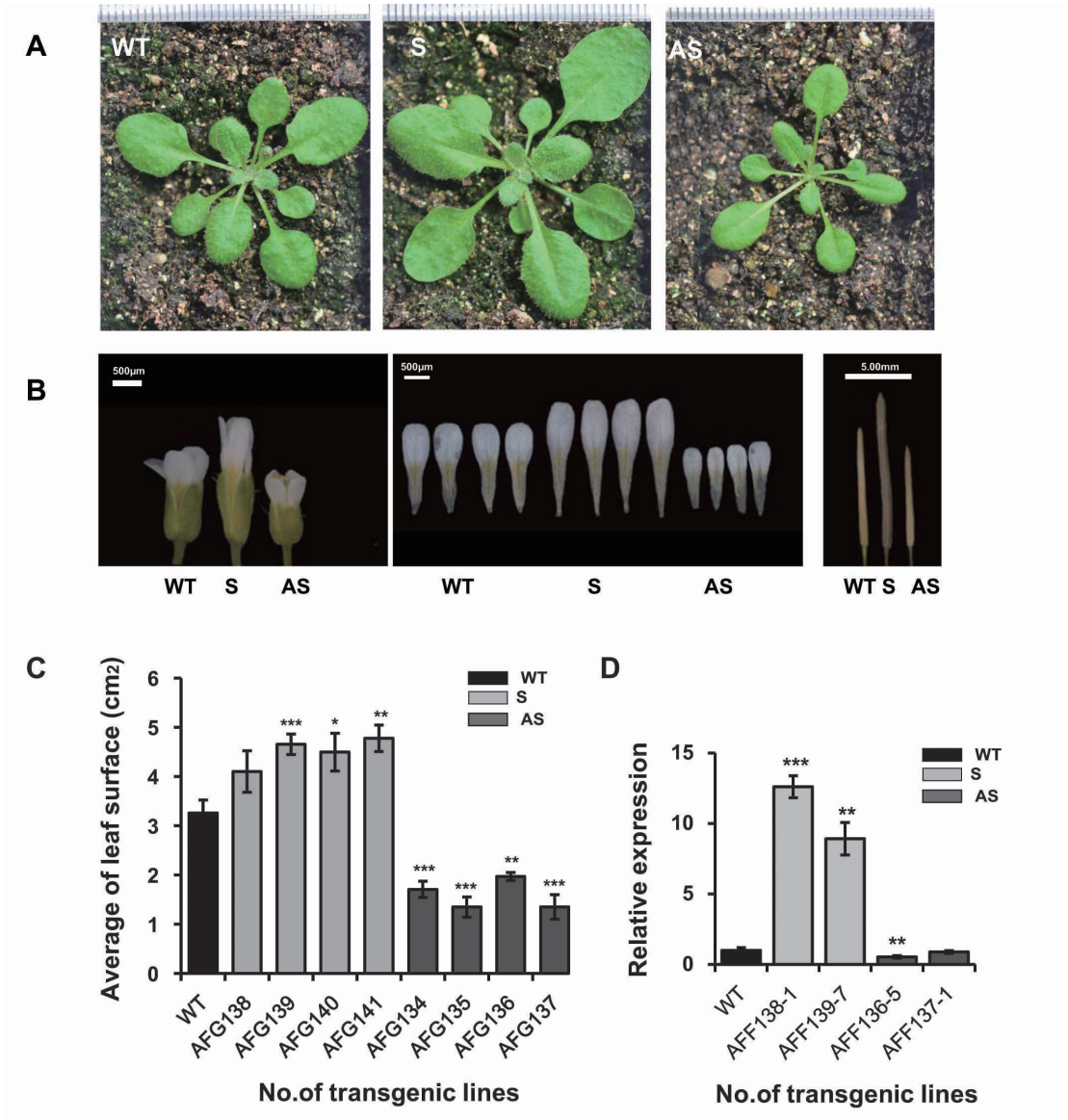

Fig. 10. Phenotype and expression analysis of *GmTCP2* transgenic Arabidopsis. (A) From left to right is the seedling phenotype of the wild type control, sense lines and antisense lines 23 d after sowing (Scale bars, 1.00mm); (B) From left to right is the flower and pod phenotype of the wild type control, sense lines and antisense lines (Scale bars, 500μm; 500μm; 5.00mm); (C) From left to right is average area of the seventh true leaf of the wild type control, sense lines and antisense lines; (D) From left to right is the relative expression of *AtTCP1* in the wild type control, sense lines and antisense lines.TThe qRT-PCR product of was used as a normalizer.(WT, wild type; S, sense; AS, antisense) Asterisk (*) denotes a significant difference between the wild type and transgenic plant respectively (t-tests: * P <0.05;** P <0.01; *** P <0.001).

the formation of the dorsoventral axis. Moreover, in soybean, the fact that the TCP domain transcription factors negatively regulated *CUC*-like genes, suggests that the TCP protein might directly or indirectly interact with the NAC domain protein that regulates organ fusion. However, this hypothesis needs to be verified. Further in-depth analyses of the transcriptional networks of different *CYC*, *RAD* and *DIV*-like genes should be done to provide more information about the development of zygomorphy in soybean.

Arabidopsis transgenic analysis

To analyze whether the *CYC*-like genes affect cell division in *Arabidopsis*, sense and antisense expression lines of *GmTCP1* and *GmTCP2* were generated. The *35S::GmTCP2* plant manifested various morphological abnormalities, especially in the size of various organs. The rosette leaves, floral organs and siliques of *35S::GmTCP2* plants were significantly larger than those of the wild type. In addition, we observed an organ variation phenotype in antisense transgenic lines, which showed the opposite phenotype, with smaller leaves and floral organs (**Fig. 10**).

The surface area of the seventh true leaf of the transgenic lines in *Arabidopsis* was measured and the data showed that the leaf surface area of *35S::GmTCP2* plants was approximately 26 % to 47 % larger than that of the control plants. In contrast,

that of the antisense lines was 40 % to 59 % smaller (**Fig. 10C**). These results indicated that *GmTCP2* was able to induce abnormal development in various organs, suggesting that TCP transcription factors were critical for the morphogenesis of lateral shoot organs. Compared with the transgenic plants that expressed *GmTCP2*, we did not find any differences in the phenotype of *GmTCP1* transgenic plants.

The *Arabidopsis* genome encodes 24 predicted TCP proteins, of which, based on the structure of the DNA binding domain, 11 are grouped as a CYC/TB1 subgroup and 13 as a PCF subgroup (7, 17). The TCP domain of the CYC/TB1 subgroup affects floral organ asymmetry, axillary meristem development, branching, and leaf shape via the regulation of cell proliferation and/or growth (22). The AtTCP1 protein, which affects brassinosteroid biosynthesis by regulating the transcription of DWARF4 protein, is the sole CYC homologue in the CYC/TB1 subgroup. It has an asymmetric expression pattern transiently in the dorsal domain of floral meristems, as well as in the adaxial region of axillary shoot meristems (6, 15). The transgenic lines in *Arabidopsis* supported the hypothesis that the TCP family performs a specific function in proliferating tissues where TCP transcription factors may be recruited to combine with other proteins to influence cell division and growth, thus generating the original morphological traits (**Fig. 10**). This suggestion is in agreement with previous reports that *TCP* genes are involved in the control of the development of leaves (24, 26).

Conclusions

In this study, we demonstrate that functional copies of the GmTCP1 and GmTCP2 proteins maintain the dorsal identity in flower meristems in *Glycine max*. We believe that the *CYC*-like genes perform a crucial function in the control of the typical zygomorphic flowers in soybean, and the two copies of the *CYC*-like genes may define functional divergence during petal development. *CYC*-like genes are implicated in the development of typical bilaterally symmetrical flowers in soybean. The transgenic lines in *Arabidopsis* indicate that the TCP transcription factors play a role in proliferating tissues to influence cell division and growth.

Acknowledgements

We thank Professor Edward Mignot (Shandong University) for critical comments on the manuscript. This work was supported by the National Nature Science Foundation of China (Grant No. 31171571) and a grant from the Natural Science Foundation of Shandong Province of China for Distinguished Young Scholars (Grant No. JQ200909).

REFERENCES

1. **Citerne H.L., Luo D., Pennington R.T., Coen E., et al.** (2003) Plant Physiol., **131**, 1042-1053.
2. **Clough S.J., Bent A.F.** (1998) Plant J., **16**, 735-743.
3. **Coen E.S., Romero J.M., Doyle S., Elliott R., et al.** (1990) Cell, **63**, 1311-1322.
4. **Corley S.B., Carpenter R., Copsey L., Coen E.** (2005) P. Natl. Acad. Sci. USA, **102**, 5068-5073.
5. **Cubas P.** (2004) Bioessays, **26**, 1175-1184.
6. **Cubas P., Coen E., Zapater J.M.** (2001) Curr. Biol., **11**, 1050-1052.
7. **Cubas P., Lauter N., Doebley J., Coen E.** (1999) Plant J., **18**, 215-222.
8. **Cui M.L., Copsey L., Green A.A., Bangham J.A., et al.** (2010) PLoS Biol., **8**, e1000538.
9. **Endress P.K.** (1998) Sym. Soc. Exp. Biol., **51**, 133-140.
10. **Endress P.K.** (1999) Int. J. Plant Sci., **160**, S3-S23.
11. **Feng X., Zhao Z., Tian Z., Xu S., et al.** (2006) P. Natl. Acad. Sci. USA, **103**, 4970-4975.
12. **Fukuda T., Yokoyama J., Maki M.** (2003) J. Mol. Evol., **57**, 588-597.
13. **Galego L., Almeida J.** (2002) Genes Dev., **16**, 880-891.
14. **Green P.B., Linstead P.** (1990) Protoplasma, **158**, 33-38.
15. **Guo Z., Fujioka S., Blancaflor E.B., Miao S., et al.** (2010) Plant Cell, **22**, 1161-1173.
16. **Howarth D.G., Donoghue M.J.** (2006) P. Natl. Acad. Sci. USA, **103**, 9101-9106.
17. **Kosugi S., Ohashi Y.** (2002) Plant J., **30**, 337-348.
18. **Koyama T., Furutani M., Tasaka M., Ohme-Takagi M.** (2007) Plant Cell, **19**, 473-484.
19. **Koyama T., Mitsuda N., Seki M., Shinozaki K., et al.** (2010) Plant Cell, **22**, 3574-3588.
20. **Luo D., Carpenter R., Copsey L., Vincent C., et al.** (1999) Cell, **99**, 367-376.
21. **Luo D., Carpenter R., Vincent C., Copsey L., et al.** (1996) Nature, **383**, 794-799.
22. **Martín-Trillo M., Cubas P.** (2010) Trends Plant Sci., **15**, 31-39.
23. **Murashige T., Skoog F.** (1962) Physiol. Plant., **15**, 473-497.
24. **Nath U., Crawford B.C., Carpenter R., Coen E.** (2003) Science, **299**, 1404-1407.
25. **Olhoft P.M., Flagel L.E., Donovan C.M., Somers D.A.** (2003) Planta, **216**, 723-735.
26. **Palatnik J.F., Allen E., Wu X., Schommer C., et al.** (2003) Nature, **425**, 257-263.
27. **Preston J.C., Hileman L.C.** (2009) Trends Plant Sci., **14**, 147-154.
28. **Preston J.C., Martinez C.C., Hileman L.C.** (2011) P. Natl. Acad. Sci. USA, **108**, 2343-2348.
29. **Wang Z., Luo Y., Li X., Wang L., et al.** (2008) P. Natl. Acad. Sci. USA, **105**, 10414-10419.
30. **Wei H., Hao R.** (2012) Molecular Plant Breeding, **10**, 155-162.
31. **Weir I., Lu J., Cook H., Causier B., et al.** (2004) Development, **131**, 915-922.
32. **Zhang W., Kramer E.M., Davis C.C.** (2010) P. Natl. Acad. Sci. USA, **107**, 6388-6393.