Overexpression of TCP8 delays Arabidopsis flowering through a FLOWERING LOCUS C-dependent pathway

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Abstract

Background: Flowering is a key process in the life cycle of plants. The transition from vegetative to reproductive growth is thus under sophisticated regulation by endogenous and environmental signals. The plant-specific Teosinte Branched 1/Cycloidea/Proliferating Cell Factors (TCP) family transcription factors are involved in many biological processes, but their roles in regulating flowering have not been totally elucidated.

Results: We explored the role of Arabidopsis TCP8 in plant development and, especially, in flowering control. Overexpression of TCP8 significantly delayed flowering under both long-day and short-day conditions and dominant repression by TCP8 led to various growth defects. The upregulation of TCP8 led to more accumulated mRNA level of FLOWERING LOCUS C (FLC), a central floral repressor of Arabidopsis. TCP8 functions in an FLC-dependent manner, as TCP8 overexpression in the flc-6 loss-of-function mutant failed to delay flowering. The vernalization treatment could reverse the late flowering phenotype caused by TCP8 overexpression.

Conclusions: Our results provide evidence for a role of TCP8 in flowering control and add to our knowledge of the molecular basis of TCP8 function.

Keywords: TCP8, Flowering, FLC, Vernalization, Arabidopsis

Background

The transition from vegetative to reproductive growth is one of the most important processes in the life cycle of flowering plants. As a result, flowering is under strict and sophisticated regulation by multiple endogenous cues and environmental signals [1]. Many studies using the model plant Arabidopsis thaliana (A. thaliana) have identified major flowering-related genes, these genes were found to be involved in photoperiod, vernalization, gibberellin, aging, temperature, and the autonomous pathways [2–4]. For example, the central floral repressor FLOWERING LOCUS C (FLC) is involved in both the vernalization and the autonomous pathways. During vernalization, FRIGIDA (FRI) activates FLC expression while prolonged cold repressed FLC expression [5, 6]. In the autonomous pathways, FLOWERING CONTROL LOCUS A (FCA), FLOWERING LOCUS PA (FPA), FLOWERING LOCUS KH DOMAIN (FLK), FLOWERING LOCUS Y (FY), LUMINIDEPENDENS (LD), FLOWERING LOCUS VE (FVE) and FLOWERING LOCUS D (FLD) repress FLC expression under both long-day (LD) and short-day (SD) conditions [7, 8]. The MADS-box transcription factor FLC directly binds to the chromatin of floral integrator FLOWERING LOCUS T (FT) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) to repress flowering [9, 10]. FLC acts as a central floral repressor by converging the signal from different pathways in a dose-dependent manner [11]. Its expression is under sophisticated control at both transcriptional and posttranscriptional levels by diverse regulate factors [12–14]. Through massive studies, the FLC locus not only shows the delicate regulation involving flowering control, but also provides an important platform for discovering epigenetic regulation of gene expression [14].

Members of the Teosinte Branched 1/Cycloidea/Proliferating Cell Factors (TCP) family are plant-specific transcription factors involved in many biological processes including...
flowering [15]. The Arabidopsis TCP family consists of 24 genes and can be further divided into two clades, class I and class II, based on their sequence features [16]. Involvement of the TCPs from both classes in regulating flowering has been reported in previous studies. For example, TCP15 promotes flowering by directly regulating SOCI expression [17], and TCP4 functions as a transcriptional activator by directly binding to the CONSTANS (CO) promoter to induce flowering [18, 19]. Several TCPs can regulate flowering through circadian-related pathways. For example, TCP20 and TCP22 are positive regulators of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), while TCP21 represses CCA1 expression [22, 23]. Despite the importance, the molecular mechanism underlying the TCP-FLC interaction remains to be elucidated.

Our previous work demonstrated TCP8, a member of class I TCPs, directly binds to the ISOCHORISMATE SYNTHASE 1 promoter to activate plant immune response [24]. Besides, TCP8 also binds and activates promoter of EF-TU RECEPTOR in planta during pathogenesis [25]. In addition, TCP8 interacts with NON-EXPRESSER of PR GENES 1 and SUPPRESSOR OF rps4-RLD1 via protein-protein interaction after pathogen attack [26, 27]. These results clearly showed a role of TCP8 participating in plant-pathogen interaction. Other studies have also suggested TCP8 may have roles during plant development and yield determination [28–33], but its function in regulating flowering time is not clear. In this study, we investigate the role of TCP8 in flowering control by overexpression. We found that TCP8 overexpression delays plant flowering in an FLC-dependent manner. Furthermore, TCP8 and its homologs are indispensable for plant development.

**Results**

**TCP8 is ubiquitously expressed during plant development**

To characterize the tissue-specific expression pattern of TCP8 at different developmental stages in detail, a 1.5 kb promoter region upstream of the start codon of TCP8 was fused with the GUS gene and the construct was transformed into Columbia-0 (Col-0). Several independent transgenic lines exhibited similar patterns of GUS expression. TCP8 showed a ubiquitous expression pattern in the transgenic plants—GUS signal was mainly detected in the vascular bundles in cotyledons, primary roots, hypocotyls and rosette leaves throughout development (Fig. 1a, d, e). Relatively higher TCP8 expression was detected in leaf primordia and stomatal guard cells (Fig. 1b, c), suggesting a potential role of TCP8 in tissue initiation and stomatal function. We then validated the histochemical GUS staining results by real-time quantitative PCR (RT-qPCR). Consistently, TCP8 expression was detected in all the tissues tested—including rosette leaves, cauline leaves, stem, inflorescence and root (Fig. 1f). Taken together, these results revealed the ubiquitous expression pattern of TCP8 throughout development and a potentially function in flowering.

**TCP8 overexpression delayed flowering under both LD and SD conditions**

To further investigate the role of TCP8, we overexpressed TCP8 using the constitutive 35S cauliflower mosaic virus
promoter (35S) in Col-0 and homozygous T3 progeny were analyzed. The 35S::TCP8 plants showed a clear late-flowering phenotype compared with wild-type (WT) Col-0 under the long-day (LD) condition, and the extent of delay well correlated with the relative transcription levels of TCP8 (Fig. 2a, b). A similar late-flowering phenotype was also observed for the 35S::TCP8 plants under the short-day (SD) condition (Fig. 2d). It is well accepted that late-flowering plants often generate more rosette leaves before flowering. Indeed, the TCP8 overexpression plants generated more rosette leaves than control plants both under LD and SD (Fig. 2c), demonstrating that TCP8 is a bona fide regulator of Arabidopsis flowering. Moreover, we observed retarded growth with the 35S::TCP8 individuals, although the final plant height of 35S::TCP8 were comparable to those of the WT (Additional file 1: Figure S1).

Next, we expressed TCP8 in Col-0 with the native promoter of TCP8 to eliminate the effect of ectopically expressed TCP8 on flowering. The pTCP8::TCP8 plants phenocopied 35S::TCP8 plants by showing a delayed flowering compared with the WT control (Additional file 2: Figure S2), although the late-flowering phenotype was less obvious compared with the 35S::TCP8 individuals, probably due to a lower expression levels of TCP8 under its native promoter. Collectively, these data provide evidence for a role of TCP8 in regulating plant flowering.

**Overexpression of TCP8 up-regulates FLC mRNA level**

The delayed flowering phenotype of 35S::TCP8 plants under both LD and SD conditions suggest a putative role of TCP8 in the autonomous pathways. Thus, we
examined the transcription levels of FLC—a master repressor of flowering in the autonomous pathways—in the 35S::TCP8 lines. Consistent with our hypothesis, FLC transcription level in the 35S::TCP8 lines increased by over two folds compared with WT control during early developmental stages (Fig. 3a). This result suggests that TCP8 may control flowering by upregulating FLC mRNA level.

Vernalization has been shown as an effective way to reduce native FLC expression, so we checked whether vernalization could rescue the late-flowering phenotype of TCP8 overexpression plants. As expected, vernalization greatly rescued the delayed flowering of TCP8 overexpression plants (Fig. 3b). We observed no obvious difference in the number of rosette leaves between the 35S::TCP8 and control plants in the vernalization group (Fig. 3c), confirming a potential role of FLC in TCP8-mediated flowering control. It is of interest to note that the number of days after germination for flowering of TCP8 overexpression plants were still more than WT, presumably owing to the retarded growth caused by TCP8 overexpression (Fig. 3b). That is, vernalization specifically rescued the late-flowering phenotype caused by TCP8 overexpression, but had no effect on its growth hindering effect.

To further investigate the molecular mechanism of TCP8 in controlling flowering, we tested the binding of TCP8 to FLC promoter in TCP8-GFP transgenic plants [24] using the chromatin immunoprecipitation (ChIP) assay and the in vitro electrophoretic mobility shift assay (EMSA) (Additional file 3: Figure S3). Neither the ChIP assay nor the EMSA detected an interaction between TCP8 and the FLC promoter, suggesting that TCP8 indirectly regulated FLC expression. Considering the Col-0 ecotype contains a fri-null allele, TCP8 may regulate genes upstream of FLC in the autonomous pathway, which were thought to cooperatively repress FLC expression. Therefore, we measured the relative expression levels of FCA, FPA, FLK, FY, LD, FVE, and FLD in the TCP8 overexpression seedlings and observed moderate decreases in FCA, FLK, LD and FLD expression (Additional file 4: Figure S4a). In addition, a group of antisense long noncoding transcripts termed COOLAIR are reported to regulate FLC expression level [34]. But we did not detect a significant change of COOLAIR levels in TCP8 overexpression seedlings (Additional file 4: Figure S4b). Taken together, these analyses revealed that TCP8 overexpression indirectly upregulated FLC mRNA level, probably through the downregulation of a set of autonomous genes.

**FLC is required for TCP8-mediated flowering control**

To validate the role of FLC in TCP8-regulated flowering, we overexpressed TCP8 in an FLC loss-of-function mutant, flc-6. TCP8 overexpression had no obvious effect on the flowering of flc-6—no significant difference in rosette leaves number was observed between the flc-6 and 35S::TCP8/flc-6 individuals—despite high expression of TCP8 was detected in 35S::TCP8/flc-6 plants (Fig. 4a-c). We then crossed the
35S::TCP8 transformants in Col-0 with flc-6. In the F2 segregation population, TCP8 overexpression in flc-6 background showed comparative flowering time with flc-6, while TCP8 overexpression in FLC WT background showed significant delayed flowering (Fig. 4d-f), suggesting that a functional FLC gene is required for TCP8 function. We obtained similar results in pTCP8:TCP8 and flc-6 crossed F2 progeny (Additional file 5: Figure S5). Therefore, FLC is essential for the TCP8-mediated flowering control.

**Dominant repression by TCP8 leads to various growth defects**

A TCP8 mutant generated by T-DNA insertion (tcp8–1, CS875709) was obtained from ABRC to investigate the
role of TCP8 in flowering. However, we observed no visible defect in flowering and development (Additional file 6: Figure S6), which may be owing to the functional redundancy among TCP family members, as proposed in previous studies [35]. To overcome this redundancy, we fused the EAR motif to the C-terminus of TCP8 and overexpressed the TCP8-EAR fusion protein in Col-0 to mediate dominant repression by TCP8 (Fig. 5a). The 35S::TCP8-EAR plants exhibited severe developmental defects—the growth of most plants was arrested at the seedling stage (Fig. 5c). The survived 35S:TCP8-EAR seedlings failed to develop normal leaves (Fig. 5d). Similarly, we observed a moderate level of growth defect with the pTCP8::TCP8-EAR seedlings, which had dark green leaves, hypostatic cotyledons, and shorter primary roots (Fig. 5e, f); some pTCP8::TCP8-EAR seedlings could develop curved true leaves but few made to the reproductive stage (Fig. 5h). The adult plants of pTCP8::TCP8-EAR were considerably smaller than Col-0 and could not develop a normal inflorescence—the three outer whorls of the pTCP8::TCP8-EAR flower were fused and the irregular gynoecia was exposed (Fig. 5i, j). The pTCP8::TCP8-EAR plants also failed to develop viable seeds. Taken together, these results point to an indispensable role of TCP8 in maintaining normal plant growth and flower development.

Discussion

Functional redundancy often hinders the analysis of the TCP family members [15]. For example, TCP8, TCP14, and TCP15 are functionally redundant in regulating development and immune response [25–28, 30], however, TCP8 and TCP15 may play different roles in controlling flowering. Overexpression of TCP15 promoted early flowering and the tcp15 loss-of-function mutant showed delayed flowering [17]. In the present study, the tcp8 single mutant had no obvious phenotype but TCP8 overexpression led to delayed flowering (Fig. 2b–d). According
to previous studies, TCP15 directly activates SOCI expression [17], and TCP8 functions upstream of FLC (Fig. 4), suggesting that TCP8 functions upstream of SOCI. In contrast to that observed with the immune response, TCP8 and TCP15 may have no redundancy in flowering regulation. TCP23 overexpression could also delay flowering under LD condition [36], suggesting that TCP23 and TCP8 may have overlapping functions in flowering control. To overcome the functional redundancy among TCP members, the chimeric TCP8-EAR protein was expressed in WT Col-0 to introduce dominant TCP8 repression, which resulted in various growth defects in the transgenic plants (Fig. 5). Compared with TCP14 and TCP15 dominant repression [37–39], the TCP8-EAR plants exhibited more severe developmental defects. This observation is consistent with the ubiquitous expression pattern of TCP8 during seedling development (Fig. 1).

TCP8 overexpression delayed flowering under both LD and SD conditions and TCP8 functions upstream of FLC, suggesting that TCP8 may play a role in the autonomous pathways. Consistent with this hypothesis, we found that the expression levels of several known autonomous pathway regulators, including FCA, FLK, LD, and FLD, were downregulated in the TCP8 overexpression lines (Additional file 4: Figure S4a), suggesting that TCP8 may indirectly affect FLC expression through regulating these genes. Consistent with this assumption, we failed to detect a direct interaction between TCP8 and the promoter of FLC in the EMSA and ChIP assay. Previous studies have suggested that the autonomous pathway factors mainly regulate FLC expression via post-transcriptional RNA-processing and epigenetic mechanisms [40–42]. Future work is required to characterize the interaction between TCP8 and FLC in detail. TCP14 and TCP15 have been shown to interact with MODIFIER OF snc1–1 (MOS1), which directly interacts with Suppressor of FRIGIDA 4—a transcriptional activator of FLC [28, 43]. In this scenario, TCP8 may also regulate FLC mRNA level through interaction with MOS1. Alternatively, TCP8 may compete the interaction between TCP14, TCP15 with MOS1, as protein-protein interactions between TCPs are prevalent [24, 35]. Previous studies have shown that the AP2-domain transcription factors TEMPRANILLO1 (TEM1) and TEM2 inhibit flowering under LD condition through direct transcriptional repression of FT [44]. Moreover, TEMs also regulate flowering involving the gibberellin levels and miR172, both required to orchestrate floral transition under SD conditions [45, 46]. Interestingly, we detected up-regulated levels of TEM1 and TEM2 in TCP8 overexpression lines under LD condition (Additional file 7: Figure S7). Examining the putative relationship between TCP8 and TEMs may provide new clues for their functions.

In this study, we found that TCP8 overexpression inhibited plant growth in a dose-dependent manner (Additional file 1: Figure S1a). The 35S::TCP8 plants showed retarded growth earlier in development but the adult plants established a normal plant height comparable to the wild-type control (Additional file 1: Figure S1b). TCP8 and other TCP genes are known to participate in cell-cycle control, presumably through regulating CYCA1;1 and CYCA2;3 [28, 33, 47]. Thus, the growth inhibition in 35S::TCP8 plants may be related to the misregulation of cell-cycle. Vernalization could reverse the late-flowering phenotype but not the growth inhibition of 35S::TCP8 plants, suggesting independent regulation of flowering time and cell-cycle by TCP8, and TCP8-related cell-cycle regulation has negligible role in flowering control in our study.

Conclusions
Our study demonstrates that TCP8 regulates plant flowering in an FLC-dependent manner. The findings of this study expand our knowledge on the molecular basis of how TCPs, especially TCP8, control flowering time and provide evidence for the interaction between TCP8 and FLC.

Methods
Plant materials and growth conditions
All mutants and transgenic lines used in this study were in the Arabidopsis thaliana ecotype Col-0 background. The T-DNA insertion mutant tcp8–1 (CS875709) was obtained from the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu/) and genotyped as previously reported [24]. For transgenic plants, the corresponding plasmid was transformed into WT Col-0 or flc-6 (SALK_041126) by floral dipping and the transgenic plants were selected by half-strength Murashige and Skoog (MS) medium supplemented with proper antibiotic. For TCP8 overexpression lines, homozygous T3 transgenic plants were used for further analysis. For the co-segregation analysis, 35S::TCP8 or pTCP8::TCP8 plants crossed with flc-6, and the resulting F2 progenies in each population were genotyped. All primers used for genotyping are listed in Additional file 8: Table S1.

Imbibed seeds were sowed and stratified in a cold room at 4°C for 3 days to break dormancy and then transferred to 22 ± 1°C at a light intensity of approximately 120 μmol m⁻² s⁻¹, with a 16 h light/8 h dark photoperiod for LD and 8 h light/16 h dark photoperiod for SD. The number of rosette leaves was counted after the main stem has bolted to 2 cm. For the vernalization treatment, the imbibed seeds were grown on half-strength MS medium at 4°C for 4 weeks under dim light and then transferred to 22 ± 1°C and grown under LD condition.
Vector construction
A 1582 bp promoter fragment upstream of the TCP8 translation start site was amplified by PCR using primers pTCP8–1582-S/A, and then cloned into the pCAMBIA1301 (GeneBank accession number AF234297) vector through KpnI/BglII double digestion to generate the pTCP8::GUS construct (the TCP8 promoter-driven GUS expression construct). For TCP8 overexpression, the coding sequence (CDS) of TCP8 was amplified using primers OE-TCP8-S/A and cloned into the pCHF3 vector by SacI/BamHI double digestion to generate the 35S::TCP8 vector. For pTCP8::TCP8 vector construction, a DNA fragment containing the TCP8 coding region as well as the 1582 bp promoter region was amplified from Col-0 genomic DNA using primers pTCP8-TCP8-S/A and cloned into the pCAMBIA1301 through PstI/BstBI double digestion. To construct the 35S::TCP8-EAR vector, DNA fragment containing the EAR motif was synthesized using primers EAR-S/A and cloned into the 35S::TCP8 vector via BamHI/PstI double digestion. To construct the pTCP8::TCP8-EAR vector, a 1351 bp promoter fragment upstream of the TCP8 translation start site was amplified using primers pTCP8–1351-S/A and cloned into the 35S::TCP8-EAR vector through EcoRI/Sacl double digestion. Primers used for vector construction are summarized in Additional file 8: Table S1.

Histochemical GUS assays
For the histochemical detection of GUS activity, plant tissues were immersed in the 5-Bromo-4-Chloro-3-indolyl β-D-glucuronic acid (X-Gluc) solution (containing 750 mg ml⁻¹ X-Gluc, 0.2 mM K₃Fe(CN)₆, 0.2 mM K₄Fe(CN)₈, and 0.2% Triton X-100, pH = 7.2) in the vacuum for 15 min at room temperature and incubated at 37 °C overnight. The samples were then washed with 70% ethanol several times until transparent before examined under the microscope.

RNA extraction and quantitative real-time PCR
To investigate the transcript profile of TCP8, different tissues of 40-day-old WT Col-0 plants grown under LD condition were harvested. For detecting the transcript levels of TCP8 in Col-0 and the 35S::TCP8, rosette leaves were collected. To detect the transcript levels of FLC and genes involved in the autonomous pathways, ten-day-old seedlings grown under LD condition were collected. Total RNA was extracted from the tissues of Col-0 and the transgenic lines using the TRIzol reagent (Invitrogen), DNase I (TaKaRa) was used to wipe out genomic DNA. Two micrograms of total RNA of each sample was used for first-strand cDNA synthesis with M-MLV reverse transcriptase (TaKaRa).

RT-qPCR was performed as previously described [48]. Briefly, reverse-transcribed cDNA was used as the template for RT-qPCR. The RT-qPCR reactions were performed using SYBR Green (TaKaRa) on an iCycler (Bio-Rad) following the manufacturer’s instructions. ACTIN2 was used as the endogenous control for normalizing the transcript levels of the tested genes. All experiments were performed in three independent biological replicates and three technical replicates. Data are represented as mean ± standard deviation (S.D.). Asterisks indicate significant differences relative to control (*P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test). Primers used for RT-qPCR are listed in Additional file 8: Table S1.

Supplementary information
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transcription levels of genes detected. The transcription level of each gene in Col-0 was arbitrarily set to 1. (Student’s t test: * P < 0.05).

Additional file 8: Table S1. Primers used in this paper.

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Authors’ contributions
XYW, BXM and BKK designed the study. XYW, XTX, XWM, LYZ and JCZ performed the experiments. XYW, XTX and BKK analyzed the data. BXM, XYW and BKK contributed to writing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analyzed during the current study are available from the corresponding author (X. Wang) on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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