TCP4-dependent induction of CONSTANS transcription requires GIGANTEA in photoperiodic flowering in Arabidopsis

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Abstract

Photoperiod is one of the most reliable environmental cues for plants to regulate flowering timing. In Arabidopsis thaliana, CONSTANS (CO) transcription factor plays a central role in regulating photoperiodic flowering. In contrast to posttranslational regulation of CO protein, still little was known about CO transcriptional regulation. Here we show that the CINCINNATA (CIN) clade of class II TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR (TCP) proteins act as CO activators. Our yeast one-hybrid analysis revealed that class II CIN-TCPs, including TCP4, bind to the CO promoter. TCP4 induces CO expression around dusk by directly associating with the CO promoter in vivo. In addition, TCP4 binds to another flowering regulator, GIGANTEA (GI), in the nucleus, and induces CO expression in a GI-dependent manner. The physical association of TCP4 with the CO promoter was reduced in the gi mutant, suggesting that GI may enhance the DNA-binding ability of TCP4. Our tandem affinity purification coupled with mass spectrometry (TAP-MS) analysis identified all class II CIN-TCPs as the components of the in vivo TCP4 complex, and the gi mutant did not alter the composition of the TCP4 complex. Taken together, our results demonstrate a novel function of CIN-TCPs as photoperiodic flowering regulators, which may contribute to coordinating plant development with flowering regulation.
Author summary

For plant adaptation to seasonal environments, a crucial developmental event is flowering, as proper timing of flowering affects reproductive success. Although plants monitor various environmental parameters to optimize this timing, photoperiod information is important for plants to regulate seasonal flowering time, because changes in photoperiod occur in a predictable manner throughout the year. The model plant Arabidopsis thaliana responds to photoperiodic changes and flowers under long-day conditions. Based on genetic analyses using mutants defective in the photoperiodic flowering response, we learned that the transcription factor referred to as CONSTANS (CO) plays a central role in regulating the timing of flowering by directly controlling the expression of florigen (flowering-inducing substrate) gene. Long-day afternoon expression of CO is critical for this regulation; however, we had limited knowledge of CO transcriptional regulation. Here we identified that a group of plant-specific transcription factors belonging to the TCP gene family function as novel CO transcriptional activators. We demonstrated that TCP transcription factors regulate CO transcription together with known regulators of CO. Our results imply that plants utilize multiple transcription factors to precisely coordinate the expression of the key regulator gene, CO, which will directly affect flowering time.

Introduction

The transition from vegetative to reproductive growth phases at the most appropriate season is crucial for plant reproductive success. One of the most influential environmental cues that induces seasonal response is day length (= photoperiod). In the model plant Arabidopsis thaliana, the growth-phase transition, which is observed as flowering, is promoted in long days (LD). Flowering is induced by the expression of FLOWERING LOCUS T (FT), which is induced specifically at dusk during LD [1–3]. FT encodes a florigen synthesized in leaf phloem companion cells and transported to the shoot apical meristem, where FT protein interacts with the bZIP transcription factor FD and 14-3-3 protein to start orchestrating the expression of the floral identity genes [4–6]. CONSTANS (CO), a transcriptional activator containing two B-box and CO, CO-Like, TOC1 (CCT) domains, is expressed in leaf phloem companion cells and directly activates FT transcription [7–11]. It is widely accepted that day length information is mainly integrated into this pathway by controlling CO functions in Arabidopsis.

Transcriptional regulation of CO is the first crucial step to properly induce photoperiodic flowering [1, 2, 12, 13]. Currently, two groups of transcription factors are known as direct regulators of CO expression; CYCLING DOF FACTOR (CDF) of the DNA binding with One Finger (DOF) family and FLOWERING BHLH (FBH) of the basic Helix-Loop-Helix (bHLH) family [14–16]. In the morning, the expression of CO is tightly repressed by CDFs physically binding to the DOF binding sites in the CO promoter [14, 15]. In early afternoon until dusk, F-box containing blue-light photoreceptor FLAVIN BINDING, KELCH REPEAT, F-BOX 1 (FKF1) forms a complex with GIGANTEA (GI) to degrade CDFs by the 26S proteasome-mediated protein degradation pathway [17]. Once the CDF repressor complex is removed from the CO promoter, FBHs bind to the CO promoter through E-box cis-elements, and strongly activate CO transcription [16]. The induction of CO, together with posttranslational stabilization of CO by multiple photoreceptors, induces FT expression towards dusk in long days [18–23]. However, the results from the mutant analysis of fbh and cdf quadruple mutants implied the existence of additional CO transcriptional regulator(s) especially in the afternoon, when the timing
of CO expression is crucial for day-length discrimination [14, 16]. In order to more comprehensively understand how CO transcription is regulated, we extended our search for other regulators of CO transcription using a large-scale Arabidopsis transcription factor library [24]. We found additional transcription factors that can bind to the CO promoter, including the TEOSINTE BRANCHED 1/ CYCLOIDEA/ PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR (TCP) transcription family.

The TCP genes are evolutionarily conserved plant-specific transcription factor genes, which are named after three of the originally identified members; TEOSINTE BRANCHED 1 (TB1) in ZEA maize, CYCLOIDEA (CYC) in Antirrhinum majus, and PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR 1 (PCF1) and PCF2 in Oryza sativa [25–29]. The TCP protein possesses the non-canonical bHLH motif referred to as the TCP domain, which confers DNA binding and protein-protein interaction capabilities [25]. Based on the sequence variations in the TCP domains, TCP genes are largely classified into two subclasses: class I TCP and class II TCP [30–34]. The class II TCP genes are further categorized into two subgroups: the CIN-CIN-NATA (CIN)-like TCP (CIN-TCP) group ubiquitous in the plant kingdom, and the angiosperm-specific CYC/TB1 group [29]. The CIN-TCP group consists of 8 members (TCP2, 3, 4, 5, 10, 13, 17, and 24), and 5 of them are targeted by microRNA 319/JAW (miR319/JAW) [35]. CIN-TCPs function in a highly redundant manner to control lateral organ development, leaf senescence, and hormone signaling and biosynthesis [36–48]. In leaf development, CIN-TCPs have two functions. One is inhibiting cell proliferation in leaf marginal regions by regulating the cytokinin pathway and cell cycle regulatory genes, and the other is accelerating leaf aging by promoting the biosynthesis of jasmonic acid and upregulation of WRKY53, which positively regulates leaf aging [36, 46, 47, 49–51]. While loss of, or reduced function of CIN-TCPs caused the formation of serrated leaves and retained meristematic activity in the leaf margin, overexpression of the miRNA-resistant form of TCP4 caused reduction in leaf size and earlier onset of leaf senescence [35, 40, 51]. Previously, it was reported that the tcp4 mutation as well as reduced expression of related CIN-TCP genes caused late flowering phenotypes [36, 46]. In addition, overexpression of miRNA-resistant TCP4 shortened the vegetative phase in adult plants and caused early flowering [51]. Although CIN-TCP genes are involved in flowering time regulation, the precise mechanism by which CIN-TCP proteins regulate flowering remained unknown.

Here we identified class II-type TCP4 and its related CIN-TCP transcription factors as new members of CO activators in Arabidopsis. TCP4 directly associated with the CO promoter through TCP binding sites and promoted CO expression around dusk, together with other CO activators, FBHs. In addition, we demonstrated that TCP4 physically interacted with GI, and activated CO in a GI-dependent manner. This genetic dependency of TCP4 on GI to activate CO can be partially explained by the DNA binding ability of TCP4. Moreover, TCP4 interacted with all CIN-TCPs in vivo. Our results demonstrated that the interaction and functional dependency among CO regulators are important mechanisms for precise control of daily expression of CO transcription, which is an important regulation in the photoperiodic flowering pathway in Arabidopsis.

Results

The class II CIN-TCP family of proteins function as CO activators

CO plays a critical role in the Arabidopsis photoperiodic pathway [2, 12, 13]. Although both transcriptional and posttranslational regulation of CO restricts its activity to long-day afternoons, the transcriptional regulation of CO has been less characterized. Currently, the CDF and FBH families of transcription factors are the only known regulators of CO [14–16]. To
identify additional transcription factors that control CO transcription, we searched for ones that could induce the expression of CO promoter (1.5 kb)-driven lacZ reporter in yeast, using a comprehensive Arabidopsis transcription factor library [24]. Our screening identified 22 transcription factors (with more than two-fold induction than controls), including known CO regulators such as FBH3 and CDF2, as potential regulators of CO (S1 Table), validating the effectiveness of this approach. Other than bHLH transcription factors, bZIP transcription factors involved in ABA signaling [52–54] and TCP transcription factors [25] were over-represented in the candidate list (S1 Table). Since some tcp single and multiple mutants, including tcp4, showed later flowering phenotypes in LD [36, 46], and because TCPs are generally transcriptional activators [40, 41], we hypothesized that TCP might regulate flowering time in part by inducing CO transcription. In addition, the promoter sequence comparison of CO homologs in Brassicaceae revealed that the class II TCP binding site (GGACCA [30, 46]) was uniquely enriched among CO homologs and the positions of the elements were highly conserved [55]. This suggested the importance of the TCP contribution to the transcriptional regulation of CO at least in Brassicaceae. The Arabidopsis genome contains 24 TCP genes and these TCP proteins are functionally redundant within the same classes [31–34, 56]. Also, DNA binding sequences for class I and class II TCPs are distinct, but partially overlap [30]. We first confirmed the results of our large-scale yeast one-hybrid screening using some class I and II types of TCP clones in the yeast one-hybrid system (Fig 1A). High induction of LacZ expression regulated by CO promoter was induced in yeast by TCP3 and TCP4, both of which belong to the class II CIN-TCP clade [33], while moderate induction was observed with TCP9, which belongs to class I TCP. (Fig 1A). This induction was still observed when the CO promoter was shortened to the 500-bp fragment, in which one class II TCP binding site exists (Fig 1B and 1C), indicating that TCP3 and TCP4 directly bind to the CO promoter through the TCP binding site in yeast. On the other hand, induction of LacZ activity by TCP9 was not affected when the TCP binding site was mutated (Fig 1C). Because there is no canonical class I TCP binding site within this region of the CO promoter, we speculated that TCP9 may indirectly induce LacZ in yeast.

We next investigated whether TCP3 and TCP4 function as CO activators in vivo. We generated transgenic plants in which either the TCP3 or TCP4 coding sequence was overexpressed (35S:TCP3 and 35S:TCP4, S1A to S1D Fig) to analyze the expression profiles of CO in LD and short days (SD). The CO expression levels in both the 35S:TCP3 and 35S:TCP4 lines were elevated several fold at its peak, without changing the overall diurnal expression patterns in LD and SD (Fig 2A and 2B, S1E and S1G Fig), indicating that both TCP3 and TCP4 can induce CO expression. Even though we did not observe the clear binding of other class II CIN-TCPs to the CO promoter in yeast, overexpression of TCP10, which is a close homolog of TCP3 and TCP4, also elevated the CO transcriptional levels (S1I and S1J Fig). These results imply that TCP3, TCP4, and TCP10 function as transcriptional activators of CO. We also analyzed the expression patterns of FT in these lines, and found that FT levels in these lines were similar to that in WT (Fig 2C and 2D, S1F and S1H Fig). CO protein is negatively regulated posttranslationally [22]. This result may indicate that the induction levels of CO mRNA in the 35S:TCP lines were not sufficient to further increase the levels of CO, which consequently increased the FT levels. Consistent with the FT levels, flowering time of these lines resembled wild type plants (Fig 2E and 2F).

Our yeast one-hybrid assay results, as well as the increased CO levels observed in these 35S:TCP lines, suggest that some of the class II TCPs (TCP3, TCP4, and TCP10, etc.) may function as CO activators in vivo. To assess this possibility, we studied whether these TCP genes are expressed in the same tissues where CO is expressed. We examined the spatial expression...
patterns of these TCP genes using histochemical GUS staining analysis. GUS activity in TCP3:GUS, TCP4:GUS, and TCP10:GUS plants were detected in leaf vascular tissues where CO is expressed (Fig 3A to 3C, [9, 40, 57]), indicating that these TCP proteins could exist where CO transcription occurs. Next, to investigate whether TCP binds to the CO promoter in vivo, we performed chromatin immunoprecipitation (ChIP) assay. We used TCP4 as a representative class II CIN-TCP, as it is the most characterized class II TCP protein [42, 46, 47, 51]. Because most class II CIN-TCP transcripts are regulated by miR319/JAW, we generated plants that express the miR319-resistant form of TCP4 cDNA (mTCP4 [35]) translationally fused to the 3xFLAG-6xHis (3F6H) epitope tag in phloem companion cells under the control of SUCROSE-PROTON SYMPOTER 2 (SUC2) promoter (SUC2:mTCP4-3F6H, S2 Fig). Significant induction of CO was not observed in SUC2:mTCP4-3F6H, potentially due to a relatively low expression level of mTCP4-3F6H in this line (S2A and S2B Fig). ChIP-qPCR analysis showed that mTCP4-3F6H specifically associated with amplicons 1, 3, 5, 6, and 7 of the CO promoter, all of which contain or are adjacent to class II TCP binding sites (Fig 3D and 3E). TCP4 protein was constitutively expressed in the line (S2C Fig), and we did not observe clear changes in the
binding of TCP4 to these regions among the time points analyzed (Fig 3D and 3E), indicating that there is no obvious time-dependent binding of TCP4 to the CO promoter.

To analyze whether the direct binding of TCP4 to the TCP binding site in the CO promoter is important for induction of CO, we utilized a CO promoter-controlled luciferase (Luc) reporter with/without the TCP binding site mutation in a transient expression system in N. benthamiana (Fig 3F). When a similar amount of TCP4 protein was expressed (Fig 3G), it significantly induced the expression of the Luc reporter controlled by either about 1 kb or 500 bp of the CO promoters (pCO 1000 and pCO 500), both of which contain one TCP binding site (Fig 3F and 3H). When the TCP binding site was mutated in the pCO 500 reporter [pCO 500

Fig 2. TCP4 and TCP3 activates the transcription of CO. (A to D) Gene expression patterns of CO (A and B) and FT (C and D) in LD in 35S::TCP4 (A and C), 35S::TCP3 (B and D) in comparison to wild type (WT) are shown. Bars above the traces represent light conditions; open and filled bars represent day and night, respectively. Time indicates hours (h) after light onset within a day. All expression data normalized against IPP2 are shown relative to the average expression values of each gene in WT. Significant differences from WT are indicated by asterisks (p<0.05, Dunnett’s test). Data represent means ± SEM (n = 3).

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TCP4 physically associates with CO promoter and activates its transcription. (A to C) Histochiometric GUS staining images from plants harboring the GUS reporter controlled by the class II TCP promoters. Images from whole seedlings, cotyledons, and the first set of leaves in TCP3:GUS (A), TCP4:GUS (B), and TCP10:GUS (C) plants grown in LD are shown. Scale bar, 0.5 mm. (D) A diagram of the CO locus and the locations of 7 amplicons used in ChIP analysis. The gray box represents 5'-UTR and the white box represents the first exon. Red boxes represent class II TCP binding sites (GGACC). (E) Results of ChIP analysis using SUC2:mTCP4-3F6H plants against the CO promoter harvested at different times of day are shown. 10-day-old plants grown in LD were harvested at Zeitgeber time (ZT) 4, 13, and 22. The UBQ10 locus was used as a control. Significant differences from WT harvested on the same ZT are indicated by asterisks (one-tailed Student's t-test, p<0.005). Data represent means ± SEM (n = 4). (F) A schematic diagram of different lengths of the CO TCP4 activates CONSTANS transcription for photoperiodic flowering.
promoter with the location of the TCP-binding site used in (H) is depicted. Red boxes represent TCP binding sites and a pink box represents a mutated TCP binding site (TCPmut). (G) The representative western blot images of effectors (GFP and mTCP4) and the reference [Renilla Luc (RLuc)] in each combination analyzed in (H) and quantitative results of the effector levels (relative to RLuc levels) obtained from 3 independent biological replicates are shown. (H) The results of the luciferase reporter assay in N. benthamiana are shown. The effects of TCP4 on firefly luciferase (Luc) activities controlled by 1,000-bp of the CO promoter (gray bars), 500-bp (white bars), and 500-bp with a mutation on the TCP-binding site (dark gray bars) are tested. The activities of firefly Luc were normalized by the activities of RLuc. Asterisks denote significant differences in each combination (Bonferroni-corrected student’s t-test, p<0.05). Data represent means ± SEM (n = 3).

TCP4 activates CONSTANS transcription for photoperiodic flowering

TCP4 was no longer able to induce the expression of the Luc reporter (Fig 3H). These results indicate that TCP4 activates the transcription of the CO promoter-controlled Luc reporter mainly through direct binding to the TCP binding site. Taken together, these results suggest that TCP4 associates with the CO promoter through the TCP binding sites to induce CO transcription in vivo.

TCP4-related class II CIN-TCPs have overlapping functions in plant development [40, 56]. To confirm that class II TCPs, including TCP3 and TCP4, are involved in flowering regulation, we analyzed flowering phenotypes in single, double and higher order tcp mutants. In our growth conditions, the tcp4 single mutant showed a quite subtle late-flowering phenotype in LD (Fig 4A). The degree of the late flowering phenotype became obvious in LD (but not in SD) when the mutations of related class II TCP genes were integrated into tcp4 (Fig 4A and 4B and S3A Fig), indicating that the class II TCP genes redundantly regulate flowering time in LD. This is in agreement with the previous report showing that loss-of-function alleles for the tcp4 single mutant or multiple class II CIN-TCP mutants caused late flowering in LD [36, 46].

We next analyzed the expression levels of CO and FT in these mutants. Although CO expression levels were not largely affected in single and double mutants of tcp3 and tcp4, CO expression levels were significantly decreased in higher order tcp mutants [tcp 3 4 10 and tcp 3 4 5 10 13 (tcp-Q)] especially from afternoon to evening [Zeitgeber time 10 (ZT10) to ZT19] in both LD and SD (Figs 4C, 4D and S3B). These results suggest that TCP3, TCP4, and their closer homologs in the class II CIN-TCPs redundantly activate CO transcription from afternoon to evening. Peak FT expression level in the tcp4 single mutant was reduced to almost two thirds of the wild-type level, but not in tcp3 (Fig 4E). The FT levels were further reduced in the higher order tcp mutants in LD and in SD (Fig 4E and 4F and S3C Fig). These results indicate that class II TCP may work as an activator of FT. However, we did not find any class II TCP binding sites on the FT promoter, therefore, we predicted that the effects of class II TCP mutations on FT levels are likely indirect.

TCP and FBH1 act additively to induce CO

Our results imply that some class II CIN-TCPs (such as TCP3, TCP4, and TCP10) are transcriptional activators of CO. As we previously identified the other group of CO transcriptional activators, FBHs [16], we next aimed to analyze the functional relationship between two groups of CO transcriptional activators, TCPs and FBHs. We first generated the tcp fbh septic mutant in which three similar TCPs (tcp 3, 4, and 10) and four FBHs (fbh1, 2, 3, and 4) were mutated, and analyzed CO expression patterns in the mutant in LD (Fig 5A and S4 Fig). The CO expression levels during the afternoon in the tcp fbh septic mutant were slightly lower than those in the fbh quadruple mutant (Fig 5A). This suggests that FBH and TCP regulate CO in an additive manner during LD afternoon, whereas FBHs, but not TCPs, play major roles in CO transcription at night. We also analyzed FT expression in these lines. While the FT levels were not much affected in the fbh quadruple mutant, introducing three tcp mutations
largely repressed FT in the tcp fbh mutant (Fig 5B), which is similar to the phenotype of the tcp 3 4 10 triple mutant (Fig 4F). To further investigate the genetic relationship between FBH1 and CIN-TCPs, we generated lines that overexpressed similar levels of FBH1 in WT and tcp-Q background (Fig 5C and S5A Fig). Overexpression of FBH1 in tcp-Q completely failed to accelerate flowering time, and the 35S:FBH1/tcp-Q plants showed a similar flowering phenotype to the tcp-Q plants (Fig 5D). To investigate the molecular mechanism underlying this phenotype, the expressions of CO and FT were analyzed. FBH1 was still able to activate CO in the tcp-Q background, although the induction was weakened in the afternoon (Fig 5E and S5B Fig). Together with the additive effect of fbh and tcp mutations on the CO expression profile during daytime in LD (Fig 5A), these results suggest that CIN-TCPs and FBHs additively induce CO transcription mainly during the afternoon in LD. In contrast, changes in FBH1 expression in the tcp-Q background had little effect on FT expression (Fig 5F and S5C Fig). Similar to the

Fig 4. Class II CIN-TCP proteins redundantly function as transcriptional activators of CO. (A and B) Flowering phenotypes of single, double (A), and higher order tcp mutants (B) in LD were analyzed. Total number of rosette leaves and cauline leaves generated from the main stem were counted when plants bolted. Significant differences are indicated by asterisks (HSD test; *p<0.05, **p<0.01, ***p<0.001). Data represent means ± SEM (n=16). (C to F) Gene expression patterns of CO (C and D) and FT (E and F) in LD in single, double (C and E), and higher order tcp mutants (D and F) are shown. Significant differences from WT values are indicated by asterisks (p<0.05, Dunnett’s test). Data represent means ± SEM (n=3).
A minimal change in FT expression observed in 35S:TCP lines (Fig 2C and 2D), the induction of CO levels in the 35S:FBH1 lines might not be high enough to further induce FT. In addition, the epistatic effect of higher order tcp mutation on FT expression in fbh tcp septuple and in the 35S:FBH1/tcp-Q lines suggest that CIN-TCPs may have unknown indirect roles in the induction of FT, which may be independent of CO transcriptional regulation.

TCP4 activates CO in GI-dependent manner

To investigate whether other known factors that regulate CO transcription also work together with TCP4 and/or FBH1, we performed yeast two-hybrid analysis and found that GI interacts with both TCP4 and FBH1 mainly through its N-terminal region (Fig 6A and S6A Fig). To validate these interactions in plant cells, we performed a bimolecular fluorescence
TCP4 activates CONSTANS transcription for photoperiodic flowering

complementation (BiFC) assay in *N. benthamiana* leaves. When TCP4-YFP was expressed *in planta*, fluorescent signals of TCP4-YFP localized in nuclear speckles and nucleoli (Fig 6B). Reconstituted split YFP signals derived from the combination of YFP\textsuperscript{n}-mTCP4 and YFP\textsuperscript{c}-GI constructs were also observed in nuclear speckles (Fig 6C). A broad nuclear signal (without nucleoli) was observed when GI-YFP\textsuperscript{n} and FBH1-YFP\textsuperscript{c} were co-infiltrated (S6B Fig). None of these constructs reconstituted YFP signals when co-infiltrated with the nuclear-localized form of GST (GST\textsubscript{NLS}) fused to YFP\textsuperscript{n} or YFP\textsuperscript{c} (Fig 6C and S6B Fig). As a control, YFP\textsuperscript{n}/YFP\textsuperscript{c} fused GST\textsubscript{NLS} proteins dimerized in the nucleus (Fig 6C), as GST forms a dimer [58]. These results suggest that GI physically interacts with TCP4 and FBH1 in the nucleus. Physical interaction between TCP4 and GI was also confirmed by co-immunoprecipitation assay *in planta* (Fig 6D).

To assess the contribution of GI to FBH1 and TCP4 functions, we generated transgenic lines in which similar amounts of FBH1 or TCP4 are expressed with or without the gi mutation (Fig 7A, S6C and S7A Figs). As previously reported, 35S:FBH1 flowered slightly early in LD.
The 35S::FBH1/gi-2 plants flowered earlier than the gi-2 mutant but later than the 35S::FBH1 plants (S6D Fig). In 35S::FBH1/gi-2, CO induction was largely compromised as compared to that in 35S::FBH1 (S6E and S6F Fig). However, especially at night (from ZT16 to ZT1), FBH1 was still able to partially activate CO in the gi-2 background (S6E and S6F Fig). FT expression in 35S::FBH1/gi-2 was almost identical to that in gi-2 (S6G Fig). As 35S::FBH1/ gi-2 flowered earlier than gi-2, we wondered whether other floral integrator genes may be expressed higher in 35S::FBH1/gi-2 than in gi-2. We therefore analyzed the expression levels of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), which is one of the major floral integrators downstream in the photoperiodic pathway [8, 59–61]. In 35S::FBH1/gi-2, the expression of SOC1 was significantly upregulated compared to that in gi-2 (S6H Fig). This may contribute to accelerated flowering time in the 35S::FBH1/gi-2 plants compared to the gi-2 mutant. These results suggest that GI is not essential for FBH1 to activate CO, although GI affects FBH1 activity.

Next, we analyzed the effect of TCP4 overexpression in the gi-2 background. In contrast to the phenotype observed in 35S::FBH1/gi-2, the flowering phenotype of the lines expressing 35S::TCP4-3F6H in the gi-2 background were almost identical to gi-2 (Fig 7B). In addition, CO induction observed in 35S::TCP4-3F6H was completely abolished in 35S::TCP4-3F6H/ gi-2 (Fig 7C and S7B Fig). FT expression profiles in the 35S::TCP4-3F6H/ gi-2 lines were identical to those in the gi-2 mutant (Fig 7D and S7C Fig). These results suggest that TCP4 requires GI to
activate CO. Taken together, these results indicate that TCP4 physically interacts with GI to activate CO in a GI-dependent manner, whereas FBH1 is partially independent of GI to activate CO.

**GI enhances TCP4 binding onto the CO promoter**

As our genetic analysis indicates that TCP4-dependent induction of CO may require functional GI, we sought to elucidate the mechanism by which GI directly regulates the function of TCP4 for CO transcriptional regulation. GI functions as a molecular hub to connect various environmental signals into the flowering pathway [62–65]. GI often affects the stabilities of its interacting proteins [14, 17, 66, 67]. In addition, a recent report showed that GI has a general chaperone activity and facilitates proper protein folding in its interacting partners [68]. Moreover, GI interacts with several transcription factors, which function in the flowering pathway, in the nucleus and also physically associates with the CO and FT promoter regions [17, 69]. Therefore, we speculated the following three possibilities to explain how GI regulates TCP4 activity. 1) GI may stabilize TCP4 protein. 2) GI may recruit other components that are important for the transcriptional activity of TCP4. 3) GI may enhance the DNA-binding of TCP4 to the CO promoter. To test the first possibility, we analyzed the daily expression profiles of TCP4 protein in the 35S:TCP4-3F6H lines with/without the gi mutation. Although TCP4-3F6H protein tended to accumulate slightly less in the gi-2 mutant background than in WT background, the overall protein amount was not significantly different between the two genetic backgrounds (Fig 8A and 8B, S7D and S7E Fig).

To test the second possibility, we next investigated whether GI affects the composition of the TCP4 complex. We performed tandem affinity purification coupled with mass spectrometry (TAP-MS) analysis to identify components of TCP4 complex in vivo, and explored the effect of the loss of gi on the composition. We purified a functional TCP4-3F6H protein complex from 2-week-old sample (35S:TCP4-3F6H and 35S:TCP4-3F6H/gi-2) lines harvested at ZT13 when the daytime CO peaks. We identified peptides derived from approximately 30 different proteins as TCP4 interacting proteins (S2 Table). Among those, all the transcription factors identified belonged to the class II CIN-TCP family (Table 1). We confirmed these interactions by BiFC assay, showing that TCP4 interacted with all class II CIN-TCP proteins in the nucleus (Fig 8C and S8 Fig). Most combinations between mTCP4 and class II CIN-TCPs (except for mTCP4-TCP3 and occasionally mTCP4-TCP17 interactions) formed nuclear speckles (Fig 8C). Previously, mainly based on co-expression and yeast two-hybrid analyses, it was suggested that TCP may work as heterodimers formed among proteins belonging to the same class [56]. Our TAP-MS and BiFC results nicely validated the presence of multiple heterodimers in vivo. Our TCP4 interactor list did not contain GI, indicating that GI may not be a major interactor of TCP4. Also, we did not find a significant difference in the composition of the TCP4 complex between the WT and gi-2 backgrounds (Table 1 and S2 Table), suggesting that interactions between TCP4 and other CIN-TCPs were not affected by the presence of GI. The MS analysis also provided information regarding the presence of protein modifications such as phosphorylation and ubiquitination. We identified nine phosphorylated residues and two ubiquitinated residues in the TCP4 peptides (S9 Fig and S3 Table). Interestingly, one of the clusters of phosphorylation sites matches the consensus of the casein kinase II phosphorylation sites (S/T-X-X-D/E), indicating that TCP4 may be phosphorylated by casein kinase II in vivo. We further analyzed whether GI modulates these post-translational modifications of TCP4. None of these modifications showed a clear difference between WT and gi-2 background (S3 Table). These results indicate that, potentially, changing protein complex composition or protein modification of TCP4 is not how GI regulates TCP4 activity.
To assess the third possibility, we tested whether GI affects the DNA binding of TCP4 by performing ChIP assay at ZT16, when the amount of TCP4-3F6H protein in WT and gi-2 background was similar (Fig 8A and 8B). We found that the binding of the TCP4-3F6H protein to the CO promoter was reduced in gi-2 on amplicons 1, 2, 5, and 7 (Fig 8D). Less binding of TCP4-3F6H to the CO promoter in gi-2 was also observed on amplicon 1 at ZT13 (S10 Fig).

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Fig 8. GI enhances TCP4-binding to the CO promoter. (A) The representative protein profiles of TCP4-3F6H overexpression in WT and gi-2 background grown in LD are shown. Histone H3 protein was used as the loading control. The numbers above the images indicate time (h) after light onset within a day. (B) The quantified results of the TCP4-3F6H protein profiles shown in (A) are obtained from 3 independent biological replicates. Significant differences are indicated by asterisks (Student’s t-test, p<0.05). Data represent means ± SEM. (C) BiFC assay results of interactions among CIN-TCP proteins are shown. The full length of TCP2, 3, 5, 10, 13, 17, and 24 fused to YFP were co-expressed with YFP-mTCP4 in N. benthamiana leaf epidermal cells. Scale bar, 20 μm. (D) Results of ChIP analysis using 35S:TCP4-3F6H in either WT and gi-2 backgrounds harvested at ZT16 are shown. Amplicons located in the CO promoter are described in Fig 3D. Significant differences are indicated by asterisks (Student’s t-test, p<0.05). Data represent means ± SEM (n = 4).
Notably, both at the ZT13 and ZT16 time points, significant differences in the DNA binding were observed in the distal part of the TCP binding site (amplicon 1), where the highest amount of GI bound within the CO promoter [17]. These results suggest that GI may bind to TCP4 on the CO promoter to enhance DNA-binding of TCP4, which may play an important role in the activation of CO transcription. Although GI may enhance TCP4 binding to specific regions in the CO promoter, DNA binding of TCP4-3F6H was not completely lost in gi-2. This also suggests that there may be other unknown mechanisms of GI that contribute to the epistasis effect of the gi-2 mutation on TCP4-dependent CO transcriptional regulation.

**Discussion**

**TCP4 and its close homologs function as transcriptional activators of CO**

Transcriptional regulation of CO is one of the key steps in the photoperiodic flowering pathway [3, 11]. Comparative analysis of the promoter regions of the CO/COL genes in Brassicaceae revealed that the CO promoter contains three blocks of highly conserved regions, and these regions are involved in generating daily expression patterns of CO and flowering time regulation [55]. Within these regions, several cis-elements were particularly conserved. These were Dof binding sites, class II TCP binding sites, and E-box elements [55]. It was previously shown that CDFs and FBHs bind to Dof binding sites and E-box elements, respectively [14–16]; however, it remained elusive whether and how class II TCPs regulate CO transcription. In addition, misregulation of TCP4 affected flowering time, but the underlying mechanism was unknown [36, 46, 51]. In this study, we demonstrated that TCP4 and other class II CIN-TCPs are novel transcriptional activators of CO in Arabidopsis. TCP4, and likely other class II CIN-TCPs, physically associate with the CO promoter through multiple TCP binding sites, and promote CO expression mainly around dusk (Figs 1 to 4 and S1 Fig). Previously, based on protein-protein interaction in heterologous systems, structural analysis, and co-expression analysis, it was proposed that TCP4 forms a stable homodimer as well as heterodimers with other CIN-TCPs [30, 56, 70]. We successfully demonstrated that functional TCP4 forms heterodimers with all members of class II CIN-TCPs in vivo using a TAP-MS approach and a BiFC assay (Fig 8 and Table 1). Some potential TCP4-interacting proteins are chloroplast-localized proteins (S2 Table). A previous report showed that TCP13 is localized in chloroplasts and regulates expression of genes encoded in the chloroplast genome [71]. Although we did not observe either a TCP4-YFP signal or TCP4-TCP13 direct interaction in chloroplasts (Figs
6 and 8), potentially, a small portion of TCP4 may also be localized and function in chloroplasts, if these interactions are reproducible. Structural analysis of the TCP domain of TCP4 showed that mutations in the domain, which are located in a predicted dimer interface, abolish the DNA binding ability of TCP4 and fail to rescue tcp4 mutant phenotype, indicating that dimerization plays an important role in its function [70]. We also found that miR319-targeted homologs of CIN-TCPs are present more abundantly in the TCP4 complex (Table 1). TCP10, which was the most abundant class II CIN-TCP found in the TCP4 complex, is predicted to function in a highly redundant manner with TCP4 (Table 1) [56]. BiFC analysis indicated that TCP4 interacts with class II CIN-TCPs and GI in nuclear speckles (Figs 6 and 8). These results indicate that TCP4 preferentially interacts with functionally redundant homologs of class II CIN-TCPs in the specific foci within the nucleus to regulate CO transcription as well as leaf development [35, 39–41, 44, 56].

**Functional network among CO regulators**

In this study, we sought to investigate the regulatory network of CO regulators, especially among the positive regulators, TCP4, FBH1, and GI. Our results derived from genetic analysis using higher orders of tcp fbh mutants demonstrated that TCPs and FBHs additively activate CO transcription during the daytime, especially from ZT4 to ZT16 time points (Fig 5 and S5 Fig). BiFC analysis suggested that both FBH1 and TCP4 interact with GI in nucleus, but interaction patterns were not completely identical (Fig 6 and S6 Fig). In addition, FBH1 and TCP4 showed a difference in functional dependency on GI. While the presence of functional GI is not essential for FBH1-dependent induction of CO, TCP4 seemed to require GI function to activate CO transcription (Fig 7, S6 and S7 Figs). Therefore, FBH1 and TCP4 may regulate CO through different mechanisms although both of them interact and work together with GI.

Interestingly, overexpression of neither FBHs nor TCPs drastically changed the temporal expression patterns of CO, although the amplitude of CO expression was increased (Figs 2, 5 and 7 and S5 to S7 Figs) [16]. In contrast, overexpression of CO repressors, CDFs, and loss-of-function of cdf multiple mutants can change the daily expression patterns of CO [14, 15]. This suggests that repressors may play a more important role in creating the daily expression patterns of CO, while activators ensure that a certain amount of CO is transcribed near dusk. A similar mechanism is proposed in the plant circadian clock, where activators are not essential for generating circadian oscillation by themselves, but they can confer the robustness of the oscillation under a wide range of environmental conditions [72, 73].

**TCP4 requires GI to activate CO transcription**

Our results demonstrated that TCP4 physically interacts with GI and activates CO in a GI-dependent manner (Figs 6 and 7 and S7 Fig). In addition, TCP4 and GI partially share their target sites on the CO promoter (amplicons 1, 5, and 6) [17] (Figs 3 and 8 and S10 Fig). Furthermore, the amount of TCP4 associated with the specific regions of the CO promoter was reduced in gi-2, especially where GI was most abundant on the CO promoter (Fig 8 and S10 Fig) [17]. We propose that TCP4-GI complex formation may be recruited to the class II TCP binding sites on the CO promoter and/or stabilize the association of TCP4 with the CO promoter. Recently, it was shown that GI acts as a co-chaperone with HSP90 to facilitate the maturation of its interacting partner, ZEITLUPE (ZTL) [68]. It might be possible that the gi-2 mutation partially affects TCP4 protein maturation to maintain its association with the CO promoter or to fully function as a CO transcriptional activator. ZTL protein abundance is significantly reduced in the gi mutants likely due to the destabilization of misfolded ZTL protein [68]. However, the protein levels of TCP4 were not largely affected by the gi-2 mutation (Fig 8.
and S7 Fig), indicating that, even though GI may potentially affect TCP4 protein maturation, the effect of GI on TCP4 protein is different from that on ZTL. In LD, TCP4 induced CO expression mainly from mid-day to dusk (Figs 2 and 4). Although the expression of TCP3 and TCP4 peaks around ZT10 to 13 in LD (S1 and S2 Figs), both protein stability and DNA binding ability of TCP4 are almost consistent at ZT4, ZT13, and ZT22 (Fig 3 and S2 Fig). On the other hand, transcriptional and post-translational regulation of GI results in GI protein accumulation towards dusk [17, 74, 75]. In addition, GI changes subnuclear localization throughout the day [76]. These potential time-dependent functions of GI may enhance the activity of TCP4 at specific times of day.

Other than homo- and hetero-dimerization of TCPs, TCPs interact with other proteins as well. In the cytokinin signaling pathway, TCP4 requires chromatin remodeling factor BRAHMA to activate the expression of ARR6 [49]. In addition, CIN-TCPs interact with the transcriptional repressor TCP INTERACTOR CONTAINING EAR MOTIF PROTEIN 1 (TIE1), which also interacts with co-repressor TOPLESS, and this interaction modifies the transcriptional activities of CIN-TCPs [77]. Another member of CIN-TCP (TCP24) interacts with ARMADILLO BTB ARABIDOPSIS PROTEIN1, which may function as a member of ubiquitin E3 ligase [78, 79]. Our TAP-MS analysis of the in vivo TCP4 complex identified members of class II CIN-TCPs as TCP4 interactors, but neither chromatin remodeling factors nor other components involved in transcriptional machineries were identified (S2 Table). Similarly, GI was not in the list of the interactors (S2 Table). The TAP procedure is composed of dual affinity purification processes to reduce false positives; therefore, it is designed to detect strong and major protein-protein interactions [80, 81]. This implies that interactions with those potential known co-regulators occur under very limited conditions or transiently, or that the expression levels of the co-regulators are so low that they may not exist in all TCP4 complexes.

To affect TCP4 function, GI may also indirectly affect the DNA binding ability of TCP4 by regulating the stability of CDF1 and its homologues and changing the accessibility of the CO promoter. In LD, GI interacts with the F-box protein, FKF1 in a blue-light dependent manner and leads CDF1 for ubiquitination and proteasome-mediated degradation around dusk [15, 17]. CDF1 and its homologues function as strong CO repressors and their binding sites are closely localized to TCP binding sites [14, 15, 55]. Since the gi mutation stabilizes CDF1 and CDF2 throughout the day [14, 17], this may change the chromatin structure and inhibit the accessibility of the CO promoter. It is noteworthy that introducing the gi mutation to cdf1 2 3 5 still reduced CO expression especially around dusk in LD, suggesting that GI works with additional CO activators, other than CDFs, to regulate CO expression in the dusk of LD [14]. Although the contributions of uncharacterized CDF homologs need to be examined, we propose that TCP4 and likely other class II CIN-TCPs are GI-associated proteins that induce CO expression in the late afternoon of LD.

**TCP4 integrates plant developmental signals into photoperiodic flowering**

It is well known that TCP4 and other class II CIN-TCPs play critical roles in various plant developmental processes [44, 46, 47, 49, 51]. In this study, we demonstrated a novel function of class II CIN-TCP as a photoperiodic flowering regulator. In addition to direct activation of CO expression, TCP4 may indirectly activate FT expression through unknown mechanisms. Loss of tcp4 significantly reduced FT expression on ZT16 without having much of an effect on CO expression at dusk (Fig 4), suggesting that FT regulation by TCP4 might be independent from the CO-FT pathway. A previous study suggested the involvement of the SQUAMOSA
PROMOTER BINDING-LIKE (SPL) family in TCP4-mediated FT regulation. SPL3 and SPL9 are direct and indirect activators of FT, and both of them are targeted by miR156, whose expression declines in an age-dependent manner [82–84]. SPL3 is downregulated in the tcp4 mutant and jaw-D, plants that overexpress miR319a [35, 46, 51]. Furthermore, TCP4 is capable of binding to the TCP binding site (TGGTCC) located in the upstream region of SPL3 promoter in vitro [51], implying the function of TCP4 as a direct activator of SPL3. In leaf development, SPL9 physically interacts with TCP4 and interferes with complex formation between TCP4 and CUP SHAPED COTYLEDON (CUC2) [45]. It is possible that TCP4 may enhance SPL9 stability through physical interaction, which causes FT accumulation. Given that TCP4 promotes onset of cell differentiation and leaf senescence [46, 51], TCP4 may promote flowering response by directly activating CO expression and potentially indirectly promoting FT expression as leaves mature during the course of plant development.

Our results indicated that TCP4 preferentially interacts with miR319-targeted CIN-TCPs and that they work together (Figs 4 and 8 and Table 1). Previous studies indicated that miR319 expression is induced by multiple stresses such as drought, salt or cold temperatures [85, 86]. In addition, overexpression of rice miR319 confers salt tolerance in creeping bentgrass, Agrostis stolonifera [87]. Although the role of miR319 in stress response remains largely unknown, it is possible that the miR319-TCP pathway affects flowering in response to environmental stress. Therefore, our findings imply that TCP4 may connect plant development or possibly stress responses to the photoperiodic flowering pathway.

Materials and methods

Plant materials and growth conditions

The Colombia-0 (Col-0) accession was used as a wild type for all experiments. The tcp3-1, tcp4-1, tcp5-1 tcp10-1, tcp13-2, ffb2-1 and fbb3-1 mutant lines were described previously [16, 40]. The higher order tcp mutants (tcp-3-1 tcp4-1 tcp5-1 tcp10-1 tcp13-2 tcp-Q) were described previously [41]. To generate TCP3, TCP4 and TCP10 overexpressing transgenic plants, the coding regions of each TCP gene were amplified from cDNA derived from LD-grown wild-type plants using the following primers (sequences underlined are necessary for pENTR/D-TOPO cloning): 5'-CACCATGGAACCAGATACGACCATTTC-3' and 5'-TTAATGGCGAGAATCGGATGAAGC-3' for TCP3, 5'-CACCATGTCTGACGATCTCCA-3' and 5'-ATGGCGGAAATAGAGGAAGC-3' for TCP4, 5'-CACCATGGGACTTAAAGGATATAGCGTC-3' and 5'-TTAGAGGTGTGAGTTTGGAGGAG-3' for TCP10. The amplified TCP cDNAs were cloned into the pENTR/D-TOPO vector (Life technologies). After the sequences of the TCP genes were confirmed, each TCP gene was transferred, using Gateway LR clonase II (Life technologies), to pB7WG2 binary vector [88], which contains the CaMV 35S promoter-driven expression cassette. These constructs were introduced to Agrobacterium strain ABL and then transformed to wild-type plants (Col-0) possessing the pCO:GUS reporter gene [9] using conventional floral-infiltration methods. The target site of microRNA319/JAW (miR319/JAW) in the TCP4 coding sequences was replaced with a non-target sequence by site-directed mutagenesis with the following primers (5'-CTCAGGAGGTGCCCTTGCAAAGGATACGATCGACGATGATTACGCTGCGATGTCTGACGACCAATTCCA-3' and 5'-CTAGAGGTGTGAGTTTGGAGGAG-3') (designated as mTCP4). The coding region of mTCP4 was amplified using the following primers (5'-CACCATGGGTTACCCATGATCGACGACCAATTCCA-3' and 5'-CTAATTGGGAAATAGAGGAAG-3', the underlined sequences encode HA epitope tag) and cloned into the pENTR/D-TOPO vector (named pENTR-HA-mTCP4). To generate the TCP4-3F6H and mTCP4-3F6H construct, the coding regions of wild-type TCP4 and mTCP4 were amplified using the following...
primers (5’-CAGCCATGGGCTGACGACC AATTCCATC-3’ and 5’-ATGGGATCCGAGAAATAGAGGAAGC-3’), and inserted into the NcoI-BamHI site of the pRTL2-3F6H vector designed for the in-frame fusion of the 3xFLAG-6xHis sequence to the 3’ region of a gene (named pENTR- TCP4-3F6H and pENTR-mTCP4-3F6H, respectively) [89]. To generate the SUCROSE-PROTON SYMPORTER 2 (SUC2) promoter driven mTCP4-3F6H construct, 2.3 kb of the SUC2 5’ upstream promoter region was amplified using the following primers (5’-GGTG CATAATGATGGAACAAAGC-3’ and 5’-ATTTGACAAACAAGAAAGTAAGAAAA-3’), and cloned into pRNTR500 vector (Life Technologies). Both SUC2 promoter and mTCP4-3F6H coding sequences were transferred to R4pGWB501 binary vector [90] using Gateway LR clonase II (Life technologies). The resultant plasmid SUC2: mTCP4-3F6H was introduced into Col-0. To make the tcp fbh septuple (tcp3 tcp4 tcp10 fbh1 fbh 2 fbh 3 fbh4) mutant line, the fbh2-1 fbh3-1 double mutant and tcp3-1 tcp4-1 tcp10-1 triple mutant lines were first crossed to make the fbh2-1 fbh3-1 tcp3-1 tcp4-1 tcp10-1 quintuple mutant. Artificial microRNA (amiR) constructs of FBH1 and FBH4 [16] were tandemly fused and cloned into the pENTR/D-TOPO. The resultant cassette amiR-FBH1-amiR-FBH4 was transferred into the pH7WG2 binary vector [88] to generate 35S:tandem amiR-FBH1-amiR-FBH4 by using Gateway LR clonase II. Finally, the construct was transformed into ffbh2-1 ffbh3-1 tcp3-1 tcp4-1 tcp10-1 quintuple mutant lines. All homozygote lines were selected by several antibiotic-resistant markers and confirmed by genomic PCR. To generate FBH1 overexpressing plants, a pENTR/D-TOPO vector harboring the coding region of FBH1 [16] was transferred to the pK7WG2 or pH7WG2 vectors [88] to generate 35S:FBH1, which was then transformed into both Col-0 harboring SUC2: NTF and ACT2:BirA [91], and the tcp-Q mutant [41]. To generate FBH1 overexpressing lines in the gi-2 mutant, 35S:FBH1 #24 [16] was crossed with the gi-2 mutant. To generate the 35S: TCP4-3F6H construct, pENTR-TCP4-3F6H was introduced into the pB7WG2 binary vector [88] to generate 35S:TCP4-3F6H, which was then transformed into Col-0 harboring CO:GUS and the gi-2 mutant. Transgenic plants were selected on culture media containing appropriate antibiotics, and all experiments were carried out using T3-T4 homozygous plants that have a single insertion of T-DNA.

Flowering time experiment

For flowering time analysis, seeds were sown on the soil (Sunshine Mix #4; Sun Gro Horticulture) directly and stratified for 2–3 days at 4˚C in darkness to synchronize the timing of germination. Plants were grown at 22˚C in LD (16 h light/8 h dark) or SD (8 h light/16 h dark) conditions. Light was provided by full-spectrum white fluorescent light bulbs (F017/950/24” Octron; Osram Sylvania) with a fluence rate of 60–90 μmol m⁻² s⁻¹ in LD and 75–115 μmol m⁻² s⁻¹ in SD. Flowering time was measured by counting the number of rosette and cauline leaves on the main stem when they bolted. The experiments were repeated at least twice with at least 8 individual plants and similar results were obtained. The results are means ± standard errors of means (SEM).

Yeast one-hybrid analysis

All reporter strains were generated in the yeast strain YM4271 according to manufacturer protocol (Clontech). 1.5 kb (1547 bp) and 500 bp of CO promoter fragments were amplified using the following primers (5’-CAGGTACCTGGGAAGAGAAGTGCGGTGTAAGC-3’ and 5’-CCGAGATACCTGAACAGTTATC-3’ for 1.5 kb of the CO promoter, 5’-CAC CGAGATACCTGGGAAGAGAAGTGCGGTGTAAGTTGTTGTGTAAGTTTGG-3’ for 500 bp of the CO promoter) and cloned into pENTR500 vector (Life technologies), then transferred to pMW3 (pLacZi vector containing the gateway cassette [92]) following the manufacturer protocol (Life technologies). To generate mutated TCP binding cis-
element reporter constructs, the TCP binding site GGACCAC (-263 to -257) located on the CO promoter was mutated to GGAACTC by PCR based site-directed mutagenesis. To generate translational fusions to the GAL4 activation domain (AD), the coding sequences of TCPs were amplified with the primers shown in Supplemental S4 Table, cloned in pENTR/D-TOPO, and subsequently transferred into pDEST22 (Life technologies). The pDEST22-MCS plasmid containing the pBluescriptII multi-cloning sites and pDSET22-FBH3 [16] were used as the negative and positive control, respectively. Amplified sequences in all reporter and effector constructs were confirmed by sequencing. The transformation of Arabidopsis transcription factor library was performed in a 96-well format as previously described [93]. Transformation of AD constructs into the reporter strains and measurement of the β-galactosidase (β-gal) activity were performed in a 96-well format as previously described [94].

Yeast two-hybrid analysis

To test protein-protein interactions in yeast, the cDNAs encoding full length of TCP4 and FBH1 were cloned into pENTR/D-TOPO (Life technologies) and then transferred to pASGW-attR bait vector through LR reaction [95]. The constructs for full-length GI, N-terminal half of GI (GI-N; amino acid residues 1–391), and C-terminal of GI (GI-C; amino acid residues 382–1173) and protocols for yeast two-hybrid assays were described previously [17].

RNA preparation and gene expression analyses

Seedlings were grown on plates containing 1x Linsmaier and Skoog media (Caisson) and 3% sucrose in LD, SD for 10 days and harvested at 3 h intervals from 1 h after the onset of light (ZT1) to ZT22. To quantify the mRNA of genes involved in flowering regulation, total RNA was isolated from seedlings using illustra RNAspin Mini kit (GE Healthcare). To synthesize cDNA, 2 μg of total RNA was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). cDNA was diluted to 5 times its volume with water, and 2 μl each of diluted cDNA was used for quantitative PCR (qPCR) analysis. The qPCR was performed in the buffer consisting of 1× ExTaq buffer (Takara Bio USA), 0.1 mM Hepes-Na pH 7.5, 1× SYBR-Green (Molecular Probes), 10 nM fluorescein (Bio-Rad), 0.1% (w/v) tween-20, 5% (v/v) DMSO, 100 μg/mL BSA, 0.2 mM dNTPs, 250 nM primers and 1 U Taq DNA polymerase (New England Biolabs) using a MyiQ real-time detection system (Bio-Rad). The primers sequences used for amplification are shown in S4 Table. ISOPENTENYL PYROPHOSPHATE / DIMETHYLALLYL PYROPHOSPHATE ISOMERASE (IPP2) was used as an internal control for normalization. To amplify CO, TCP3, TCP4, and FBH4, we used the following 3-step PCR program: 1 min at 95°C, followed by 40–50 cycles of 10 sec at 95°C, 20 sec at 52–58°C, 20 sec at 72°C. To amplify the remaining genes, we used the following 2 step PCR program: 1 min at 95°C, followed by 40–50 cycles of 10 sec at 95°C, 20 sec at 60°C. Each data value shown in the figures is the mean values derived from three biological replicates and the value of each sample in each replicate is the average of values obtained from two technical replicated PCR reactions. Error bars indicate the SEM from three independent biological replicates.

Analysis of tissue expressions (GUS staining)

For the construction of the TCP4:GUS reporter gene, the 5′ upstream region of the TCP4 coding sequence (-3063 to -1, the translation initiation site was counted as +1) was amplified from Col-0 genomic DNA using the following primers (5′-CACCTGACTAAATGTGTTAACCAAC CAATG-3′ and 5′-GGTACACAATTTGCAGAGCAGC-3′) and cloned into the pENTR/D-TOPO vector, then transferred to the pGBW3 binary vector [96]. Transgenic lines carrying TCP3:GUS, TCP5:GUS and TCP10:GUS reporters were described previously [40]. Transgenic
Plants were grown in LD conditions for 12 days and fixed with cold 90% acetone on ice for 10–15 min. Subsequently, plants were incubated at 37°C in the staining buffer [0.5 mM X-Gluc, 50 mM sodium phosphate pH 7.2, 0.5 mM of K₄Fe(CN)₆ and 0.5 mM K₃Fe(CN)₆]. After staining, the samples were bleached and dehydrated with a sequence of buffers: 30% ethanol, fixing solution (50% ethanol, 5% acetic acid and 3.7% formaldehyde), 80%, and 100% ethanol for 30 min each. The staining patterns of GUS activity of more than 20 individual T₁ transgenic plants were analyzed, and the data from the T₂, T₃, and T₄ population plants which showed the representative staining patterns of the T₁ populations are shown.

**Chromatin immunoprecipitation assays**

Approximately 1.5 to 2 g (fresh weight) of 10-day-old seedlings harvested at the indicated time point were ground into fine powder in liquid N₂, and then homogenized in 10 ml of the nuclei extraction buffer [0.4 M sucrose, 10 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 μM MG-132, 1mM Na₃VO₄, 1mM NaF, and Complete protease inhibitor cocktail tablets (Roche)]. Cross-linking reaction was performed by treating with 1% formaldehyde for 10 min at 4°C. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.15 M and incubating at 4°C for 5 min. After filtration with miracloth, and centrifugation at 10,000 g at 4°C for 10 min, nuclei-containing pellets were washed twice in the nuclei wash buffer [0.25 M sucrose, 10 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1% (w/v) Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 50 μM MG-132, 1mM Na₃VO₄, 1mM NaF, and Complete protease inhibitor cocktail tablets]. Isolated nuclei were lysed in the nuclei lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, 1 mM PMSF, and Complete protease inhibitor cocktail tablets) and sonicated to shear DNA to an average size of 500 to 1,000 bp. The chromatin solution was diluted to 1 ml with ChIP dilution buffer (16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, 0.1 mM PMSF, 50 μM MG-132, 1mM Na₃VO₄, 1mM NaF, and Complete protease inhibitor cocktail tablets). Immunoprecipitation was performed using Dynabeads Protein G (Life technologies). The beads were pretreated with anti-FLAG antibody (A8592, Sigma) and incubated with chromatin solution in an ultrasonic water-bath for 20 min, followed by 1.5-h incubation at 4°C. After washing with low salt buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA), high salt buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA) and TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), immunocomplexes were eluted from beads, reverse cross-linked at 65°C overnight, and treated with proteinase K that digests all proteins. DNA was extracted and eluted in 140 μl of volume using QIAquick PCR purification kit (Qiagen), according to manufacturer protocol. 3 μl aliquots were used for qPCR reaction. The forward and reverse primer pairs that were used to amplify the genome sequence designated as CO amplicon 1 to amplicon 7 are shown in S4 Table. The three-step PCR cycling program was used as follows: 1 min at 95°C, followed by 60 cycles of 10 sec at 95°C, melting temperatures (57°C for amplicons 1–4 and 60°C for amplicons 5–7 and UBQ) for 20 sec, and 72°C extension for 15 sec. The immunoprecipitation efficiency (%) against the total input was calculated for each amplicon using the following formula: 0.02×2^(Ct input-Ct ChIP) ×100.

**Transient luciferase reporter assays and co-immunoprecipitation assays in Nicotiana benthamiana**

To amplify the CO promoter sequence, we used different combinations of forward primers [for -1 kbp_F (5’-CACCCTACAAGTGTCGTTTGTATTAG-3’), -500bps_F (5’-CACCTCTACCTTTGTATAGGTAGT-3’)] with reverse primer (5’-AAAGCTTATATCTGGTGAGAGA-3’). Amplified PCR products were cloned into pENTR/D-TOPO vector. CO promoter
fragments were transferred to firefly luciferase (Luc) vector pFLASH (Gateway compatible version of pPZPomegaLUC™ [97]) to generate reporter plasmids by using LR clonase II. To generate effector plasmids, pENTR-HA-mTCP4 was transferred to pB7WG2 vector [88] to generate 35S:HA-mTCP4 by using LR clonase II plus. 35S:GFP (used as negative control of effector plasmids) and 35S:Renilla Luc (RLuc, used as internal control to measure transient expression efficiency) were described previously [98]. N. benthamiana samples were harvested three days after the transfection. The expression levels of HA-mTCP4, GFP, and RLuc were analyzed by western blot using a HRP-conjugated anti-HA antibody (3F10, Roche), a HRP-conjugated anti-GFP antibody (ab6673, Abcam), and an anti-Renilla Luc antibody (PA532210, Life technologies), respectively. Total protein was extracted using 2x Laemmli sample buffer, and the same amount of total protein was loaded to each lane. Luc reporter activity was analyzed using the Dual-Luciferase Assay System (Promega) based on manufacturer instructions. Soluble proteins were extracted with Passive Lysis Buffer (Promega) supplemented by Complete Protease Inhibitor Mixture tablets (Roche). The luminescence of firefly LUC and Renilla LUC were analyzed using a Victor3 V multiwall plate reader (Perkin-Elmer).

To generate GI overexpression construct for transient co-IP assays, the full length of GI cDNA without the stop codon was amplified and inserted into the pENTR/D-TOPO vector. After sequences were verified, the GI cDNA was transferred into the pB7HFc vector harboring 6xHis-3xFLAG (6H3F) sequences designed for in-frame fusion to the 3' region of a gene [99] by LR reaction. 35S:HA-mTCP4 and 35S:GI-6H3F constructs were infiltrated into approximately 3-week-old N. benthamiana plants grown in LD conditions and subjected to co-IP experiments as previously described [67].

**BiFC assay in N. benthamiana leaf epidermal cells**

To generate binary vectors for the BiFC assay, the coding sequences of CIN-TCPs, FBH1 [16], and GI [17] were amplified with the primers shown in S4 Table and cloned in pENTR/D-TOPO, and subsequently transferred into pSITE-3C1/N1 binary vectors to generate in-frame fusion of either the N-terminal or C-terminal half of EYFP [100]. The nuclear-localized form of GST-tag (GST<sub>NLS</sub>), which was generated from the plasmid pGEX-4T1, was used as the negative control. H2B-RFP was used for the nuclear marker [98]. Approximately 20-day-old N. benthamiana plants grown in LD at 22˚C were used for agroinfiltration. The Agrobacterium strain GV3130 containing plasmids of interest was grown to the stationary phase. Bacterial cells were harvested by centrifugation and resuspended to OD<sub>600</sub> of 0.4 in MES buffer [10 mM MgCl<sub>2</sub>, 10 mM MES pH 5.6, 150 μM acetosyringone (Sigma)]. After 4 h of incubation at room temperature in MES buffer, Agrobacterium solution was infiltrated into the abaxial air spaces of leaf tissue using 1-mL syringes. Two to three days after infiltration, YFP and RFP images of the tissue were analyzed with a confocal laser scanning microscope (TCS SP5; Leica Microsystems). Approximately 50 to 100 cells that express H2B-RFP were observed in each combination. We called positive interactions when more than 70% of the H2B-RFP positive cells showed reconstituted specific YFP signals, whereas no signals were observed from the combinations with GST<sub>NLS</sub>.

**Protein extraction and immunoblot analysis**

Total protein was extracted from seedlings grown under LD and harvested at 4h intervals from ZT0 to ZT20 on day 10. Whole protein extract was extracted using a buffer containing 50 mM Na-phosphate pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 5 mM EDTA, 1 mM DTT, 0.1% Triton X-100, 50 μM MG-132, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM NaF, and Pierce Protease Inhibitor Tablets, EDTA-free. Approximately 30 μg protein in each sample was run in 12% SDS-PAGE gels, and
transferred to Nitrocellulose membranes (Bio-Rad). TCP4-3F6H proteins were detected by using a HRP-conjugated anti-FLAG antibody (A8592, Sigma), whereas histone H3 protein was detected by anti-histone H3 antibody (ab1791, Abcam), followed by a HRP-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific). Immunoreactive proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and Amersham ECL Select Western Blotting Detection Reagent (GE healthcare). For protein quantification, signals from immunoblotted membranes incubated in chemiluminescent detection reagents were imaged and quantified by a high sensitivity cooled CCD camera system (NightOWL, Berthold) and the IndiGo program (Berthold). Histone H3 was used for normalization of a protein in whole extract.

Tandem affinity purification coupled mass spectrometry (TAP-MS) analysis

TAP-MS analysis using functional TCP4-3F6H protein was performed based on the previously described methods with slight modification [67]. Approximately 10 g of $^{35}S$:TCP4-3F6H lines in both WT and gi-2 background were harvested on ZT13 of day 14. To exclude nonspecific binding, Col-0 seedlings without any transgenes were used as a control. Samples were ground into fine powder in liquid nitrogen and resuspended in SII buffer [100 mM Na-P buffer pH 7.4, 150 mM KCl, 5 mM EDTA, 5mM EGTA, 1% Triton X-100, 0.1% Sodium deoxycholate, Complete Protease Inhibitor Mixture Tablet, EDTA-free (Roche), PhosStop Phosphatase Inhibitor Mixture (Roche), 100 μM PMSF, and 50 μM MG-132]. Samples were sonicated and filtered twice through 0.45-μm pore size PVDF membrane (Millipore), followed by centrifugation to remove cell debris. The clear supernatant was incubated with anti-FLAG antibody (A8592, Sigma)-bound Dynabeads (Invitrogen). Once the TCP4-3F6H proteins were captured by the Dynabeads, the beads were washed three times with SII buffer with 0.1% Triton, and without the deoxycholate, protease and phosphate inhibitor mixtures. The rest of the purification steps were same as previously described [67].

On-bead digestion for TCP4-interacting proteins was performed using the following methods. Disulfide bonds of proteins bound to Dynabeads were reduced using 50 μl of 0.2% PPS surfactant (Expedeon) in 100 mM ammonium bicarbonate with 1 μl 500 mM Tris (2-carboxyethyl) phosphine hydrochloride (Thermo Fisher Scientific) for one hour at 60°C. The sample was cooled to room temperature and the cysteine thiols alkylated by the addition of 1.1 μl of 500 mM iodoacetamide for 20 min. The beads were then digested with the addition of 1 μg trypsin overnight at 37°C with constant agitation. The supernatant containing the tryptic peptides were removed, and combined with a methanol wash of the beads. The combined supernatants were dried using vacuum centrifugation, and resuspended in 15 μl of 0.1% trifluoroacetic acid prior to LCMS analysis.

All mass spectrometry was performed on a LTQ-FT (Thermo Fisher Scientific). Three microliters of sample digest were loaded from the autosampler onto a 150-μm Kasil fritted trap packed with 3 u Dr. Maisch ReproSil-Pur C18-AQ beads to a bed length of 2 cm at a flow rate of 2 μl/min. After loading, the trap was brought on-line with a pulled fused-silica capillary tip (75-μm i.d.) also packed with the same Dr. Maisch beads mounted in an in-house constructed microspray source and placed in line with a Waters Nanoacquity binary UPLC pump plus autosampler. Peptides were eluted off the column using a gradient of 2–35% acetonitrile in 0.1% formic acid over 60 min, followed by 35–60% acetonitrile over 5 min at a flow rate of 250 μl/min. The mass spectrometer was operated using data dependent acquisition (DDA) where a maximum of seven MS/MS spectra were acquired per MS spectrum. The resolution for MS was 100,000 at m/z 400, and for MS/MS the linear ion trap provided unit resolution. The automatic gain control targets for MS in the FT was $1 \times 10^6$, whereas for MS/MS it was
8000, and the maximum fill times were 20 and 80 ms, respectively. The MS/MS spectra were acquired using an isolation width of 2 \( m/z \) and a normalized collision energy (NCE) of 35. MS/MS acquisitions were prevented for precursor charge states of 1, or if the charge state could not be discerned from the MS spectrum. Dynamic exclusion (including all isotope peaks) was set for 30 sec.

Peptide identification was performed using Comet, searching against a Uniprot Arabidopsis protein sequence database, and using Percolator with a \( q \)-value cutoff of 0.01. Cysteine residue masses were considered statically modified by iodoacetamide, and methionine dynamically modified by a single oxidation. Precursor mass tolerance was 10 ppm, and product ion tolerance 0.5 Da. The principle of parsimony was used for protein inference. Spectral counts per protein were then analyzed using the Fisher’s Exact test, where alpha was set at 0.01 and multiple hypothesis correction was carried out using the Bonferroni correction. Frequently identified contaminant proteins were subtracted based on previous reports [101].

**Statistical analysis**

Statistical analyses were done using R Statistical Computing software (v3.2.3; R Core Team, 2015). The statistical significance in yeast one hybrid analyses, transient assays in \( N. \) benthamiana, and ChIP analyses was determined using Welch’s \( t \)-test or Student’s \( t \)-test. The statistical significance in gene expression analyses was determined by one-way ANOVA with strains as main effects. Pairwise comparisons were determined using Dunnet’s test on each time point. Student’s \( t \)-test was used to determine the statistical difference in expression of each gene in WT. \( FT \) expression values in SD are shown relative to the WT average expression value in LD shown in Fig 2. Significant differences from WT values are indicated by asterisks (\( p<0.05, \) Dunnett’s test). Data represent means ± SEM (\( n = 3 \)). (I and J) Results of qPCR analysis that show the expression pattern of \( TCP10 \) (I) and \( CO \) (J) in \( 35S:TCP10 \) lines and WT harvested at ZT16 in LD. Significant differences are indicated by asterisks (HSD test; *\( p<0.05 \), **\( p<0.01 \)). Data represent means ± SEM (\( n = 3 \)).

**Supporting information**

**S1 Fig. Gene expression patterns in the 35S:TCP4 and 35S:TCP3 lines under different photoperiod conditions.** (A to D) Results of qPCR analysis that show gene expression patterns of \( TCP4 \) (A and B), \( TCP3 \) (C and D), \( CO \) (E and G) and \( FT \) (F and H) in LD (A and C) and SD (B, D, and E to H) in 35S:TCP4 (A, B, E and F), 35S:TCP3 (C, D, G and H), and wild type (WT) plants. Time indicates hours (h) after light onset within a day. Bars above the traces represent light conditions; open and filled bars represent day and night, respectively. All expression data were normalized against \( IPP2 \) and shown relative to the average expression of each gene in WT. FT expression values in SD are shown relative to the WT average expression value in LD shown in Fig 2. Significant differences from WT values are indicated by asterisks (\( p<0.05, \) Dunnett’s test). Data represent means ± SEM (\( n = 3 \)). (I and J) Results of qPCR analysis that show the expression level of \( TCP10 \) (I) and \( CO \) (J) in 35S:TCP10 lines and WT harvested at ZT16 in LD. Significant differences are indicated by asterisks (HSD test; *\( p<0.05 \), **\( p<0.01 \)). Data represent means ± SEM (\( n = 3 \)).

**S2 Fig. Gene expression patterns and protein profiles in the SUC2:mTCP4-3F6H plants.** (A and B) Results of qPCR analysis that show the gene expression pattern of \( TCP4 \) (A) and \( CO \) (B) in LD. Significant differences from WT are indicated by asterisks (\( p<0.05, \) Student’s \( t \)-test). Data represent means ± SEM (\( n = 3 \)). (C) The representative protein profiles of mTCP4-3F6H in LD. Ponceau S-staining of RbcL is shown as the loading control. Similar results were obtained from 3 independent experiments.
S3 Fig. Flowering phenotypes and gene expression patterns in higher order tcp mutants in SD. (A) Flowering phenotypes of higher order tcp mutants in SD. Total number of rosette leaves and cauline leaves generated from the main stem were counted when plants bolted. ns: no significance (HSD test). Data represent means ± SEM (n=16). (B and C) Results of qPCR analysis that shows gene expression patterns of CO (B) and FT (C) in SD in higher order tcp mutants. FT expression values in SD are shown relative to the WT average expression value in LD shown in Fig 4. Significant differences from WT values are indicated by asterisks (p<0.05, Dunnett’s test). Data represent means ± SEM (n = 3).

S4 Fig. Gene expression patterns in tcp fbh multiple mutants. (A and B) Results of qPCR analysis that shows gene expression patterns of FBH1 (A) and FBH4 (B) in LD. Significant differences from WT are indicated by asterisks (Dunnett’s test, p<0.05). Data represent means ± SEM (n = 3).

S5 Fig. Gene expression patterns and flowering phenotype in the 35S:FBH1 lines in LD. (A to C) Results of qPCR analysis that show gene expression patterns of FBH1 (A), CO (B) and FT (C) in the 35S:FBH1, WT, and tcp-Q plants grown in LD. The data set shown in Fig 5 was used for WT, 35S:FBH1 #9, and tcp-Q. Significant differences between the 35S:FBH1 lines and their background strains are indicated by asterisks (p<0.05, Student’s t-test). Data represent means ± SEM (n = 3).

S6 Fig. Analysis of protein-protein interaction between FBH1 and GI and their genetic interaction. (A) Results of the yeast two-hybrid assay between FBH1 and GI. The full-length of FBH1 and full-length or truncated GI fused to either the DNA-binding domain (DBD) or the activation domain (AD) of Gal4 were tested under selective (–LWH, top) and non-selective (–LW, bottom) conditions. For GI, N and C indicate the amino acid residues1-391 and 382–1173, respectively. (B) BiFC assays of interaction between FBH1 and GI. The full-length of GI fused to the N-terminal half of enhanced YFP (GI-YFPn) and the full-length of FBH1 fused to the C-terminal half of enhanced YFP (FBH1-YFPc) were expressed in N. benthamiana leaf epidermal cells. H2B-RFP was used for the nuclear marker. Images from YFP and RFP channels were merged with bright-field (BF) images. Nuclear-localized form of GST fragments (GST-NLS) fused to YFPn or YFPc were used as negative controls. Scale bar, 20 µm. (C and E to H) Results of qPCR analysis that show gene expression patterns of FBH1 (C), CO (E and F), FT (G), and SOC1 (H) in 35S:FBH1, WT, and gi-2 grown in LD. CO expression profiles shown in (E) were enlarged in (F) to show the difference in lower values within the lines. Significant differences between the 35S:FBH1 lines and their background strains are indicated by asterisks (Student’s t-test, p<0.05). Data represent means ± SEM (n = 3). (D) Flowering phenotype of the 35S:FBH1 plants in LD. Total number of rosette leaves and cauline leaves generated from the main stem were counted when plants bolted. Significant differences are indicated by asterisks (HSD test, *** p<0.001). Data represent means ± SEM (n = 16).

S7 Fig. Gene expression patterns and protein profiles in the 35S:TCP4-3F6H lines in LD. (A to C) Results of qPCR analysis that show gene expression patterns of TCP4 (A), CO (B), and FT (C) in the 35S:TCP4-3F6H, WT, and gi-2 plants grown in LD. The same data set for WT and gi-2 shown in Fig 7 was used. Significant differences between the 35S:TCP4-3F6H lines and their background strains are indicated by asterisks (Student’s t-test, p<0.05). Data represent means ± SEM (n = 3). (D) The representative protein profiles of TCP4-3F6H in WT and
$gi$-2 grown in LD are shown. Histone H3 protein was used as the loading control. The numbers above the images indicate time (h) after light onset within a day. (E) The quantified results of the TCP4-3F6H protein profiles shown in (D) were obtained from 3 independent biological replicates. Significant differences are indicated by asterisks (Student’s $t$-test, $p<0.05$). Data represent means ± SEM ($n=3$).

**(EPS)**

**S8 Fig. Results of BiFC assays of interactions among class II TCP proteins.** Full length of TCP2, 3, 5, 10, 13, 17, and 24 fused to YFP$^c$ were co-overexpressed with GST protein fused to YFP$^n$ in *N. benthamiana* leaf epidermal cells. H2B-RFP was used for a nuclear marker. Images from YFP and RFP channels were merged with bright-field (BF) images. Scale bar, 20 μm.

**(EPS)**

**S9 Fig. Posttranslational modifications of TCP4 identified by TAP-MS analysis.** The peptide coverages for TCP4 protein identified by MS analysis are indicated. The TCP4 peptides identified by MS analysis are highlighted in red. The percent coverage was 73%. The number of peptides recovered is shown in Table 1 and S2 Table. Phosphorylated and ubiquitinated amino acid residues are indicated by light green and light blue boxes, respectively. The putative CKII phosphorylation site is underlined.

**(EPS)**

**S10 Fig. ChIP assays in the 35S:TCP4-3F6H lines at ZT13.** Results of ChIP analysis using 35S:TCP4-3F6H in either WT and $gi$-2 backgrounds harvested on ZT13 are shown. Amplicons located in the CO promoter are described in Fig 3D. Significant differences are indicated by asterisks (Student’s $t$-test, $p<0.05$). Data represent means ± SEM ($n=5$).

**(EPS)**

**S1 Table. Results of the large-scale yeast one-hybrid analysis using the CO promoter.**

**(XLSX)**

**S2 Table. List of TCP4 interacting proteins in WT and $gi$-2 backgrounds identified by the TAP-MS analysis.**

**(XLSX)**

**S3 Table. List of phosphorylated and ubiquitinated residues identified within TCP4 peptide sequences.**

**(XLSX)**

**S4 Table. Primers used in this study.**

**(XLSX)**

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