**Arabidopsis** transcription factor TCP4 represses chlorophyll biosynthesis to prevent petal greening

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**ABSTRACT**

Green petals pose a challenge for pollinators to distinguish flowers from leaves, but they are valuable as a specialty flower trait. However, little is understood about the molecular mechanisms that underlie the development of green petals. Here, we report that CINCINNATA (CIN)-like TEOSINTE BRANCHED 1/CYCLOIDEA/PCF (TCP) proteins play key roles in the control of petal color. The septuple tcp2/3/4/5/10/13/17 mutant produced flowers with green petals due to chlorophyll accumulation. Expression of TCP4 complemented the petal phenotype of tcp2/3/4/5/10/13/17. We found that chloroplasts were converted into leucoplasts in the distal parts of wild-type petals but not in the proximal parts during flower development, whereas plastid conversion was compromised in the distal parts of tcp2/3/4/5/10/13/17 petals. TCP4 and most CIN-like TCPs were predominantly expressed in distal petal regions, consistent with the green–white pattern in wild-type petals and the petal greening observed in the distal parts of tcp2/3/4/5/10/13/17 petals. RNA-sequencing data revealed that most chlorophyll biosynthesis genes were downregulated in the white distal parts of wild-type petals, but these genes had elevated expression in the distal green parts of tcp2/3/4/5/10/13/17 petals and the green proximal parts of wild-type petals. We revealed that TCP4 repressed chlorophyll biosynthesis by directly binding to the promoters of PROTOCHLOROPHYLLIDE REDUCTASE (PORB), DIVINYL REDUCTASE (DVR), and SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1), which are known to promote petal greening. We found that the conversion of chloroplasts to leucoplasts and the green coloration in the proximal parts of petals appeared to be conserved among plant species. Our findings uncover a major molecular mechanism that underpins the formation of petal color patterns and provide a foundation for the breeding of plants with green flowers.

**Key words:** flower development, petal greening, plastid conversion, chlorophyll biosynthesis, TCP transcription factors

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**INTRODUCTION**

The repression of green petal color is very important for the adaptation of plants with insect-pollinated flowers because green petals make it more difficult for insects to differentiate flowers from green leaves; however, green flowers are considered to be highly desirable by the ornamental horticulture industry (Ohmiya, 2018). Because of their uniqueness and beauty, green-flowered cultivars, including chrysanthemums, carnations, and roses, are generally more expensive for consumers than flowers with more typical colors (Ohmiya et al., 2014; Ohmiya, 2018; Fu et al., 2021). The green color of petals is produced by chlorophyll in chloroplasts. In *Arabidopsis*, the petals in early unopened flower buds are all green owing to the presence of chloroplasts (Pyke and Page, 1998). Then, at a later developmental stage, the distal parts of *Arabidopsis* petals become white through the conversion of chloroplasts into leucoplasts, whereas the proximal regions of the petals stay green, even when the flowers are mature (Pyke and Page, 1998). Elucidating how the color pattern is formed and how the chloroplasts are re-differentiated into leucoplasts will improve our understanding of the evolution of flower development and provide methods for the breeding of ornamental flowers. However, a lack of *Arabidopsis* mutants with green petals has precluded the comprehensive analysis of the molecular mechanisms by...
which plants repress green petal color and promote the conversion of chloroplasts into leucoplasts.

White leucoplasts and green chloroplasts can be interconverted in a tissue-specific manner (Choi et al., 2021). For example, chlorophyll biosynthesis triggered by light signals causes the conversion of leucoplasts into chloroplasts in the parenchyma tissues beneath the periderm in potato tubers (Tanios et al., 2020), whereas the loss of chlorophyll leads to the conversion of chloroplasts into leucoplasts in the above-mentioned petal tissues (Pyke and Page, 1998; Irish, 2008). Chlorophyll biosynthesis starts from the formation of 5-aminolevulinic acid in a branch of the tetrapyrrole biosynthesis pathway (Tanaka and Tanaka, 2007). After a series of reactions, the rate-limiting enzyme protochlorophyllide oxidoreductase (PORB), generates monomethyl chlorophyllide a from protochlorophyllide. Finally, chlorophyll synthase catalyzes the formation of chlorophyll a. Chlorophyll a is converted into chlorophyllide a by chlorophyllide a oxygenase (CAO) in chlorophyll recycling (Tanaka and Tanaka, 2007). During chlorophyll degradation, the first committed enzyme is magnesium dechelatase (STAY GREEN, SGR), which removes the central Mg²⁺ from chlorophyll a. Pheophorbide a oxygenase (PAO) further releases chlorophyll a from the pigment-protein complex (Christ et al., 2014).

Chlorophyll metabolism and chloroplast biogenesis are tightly regulated by transcription factors in a spatial and temporal manner in different tissues. For example, the DNA-binding proteins WHIRLY 1 (WHY1) and WHY3 protect the plastid genome and regulate chlorophyll accumulation and chloroplast biogenesis in leaves (Maréchal et al., 2009). HOMEBOX PROTEIN 25 and REPRODUCTIVE MERISTEM 7 (REM7) transcription factors promote the accumulation of chlorophyll and the conversion of leucoplasts into chloroplasts in roots (Hanano et al., 2020).

Some transcription factors have been recently shown to promote chlorophyll accumulation in petals. Overexpression of CONSTANS-like 16 (COL16) or SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) leads to green petals in petunia, tobacco, or Arabidopsis (Ohmiya et al., 2019; Wang et al., 2019). However, the molecular mechanisms underlying plastid conversion and chlorophyll metabolism in various organs remain largely unknown. In particular, a lack of Arabidopsis mutants with green petals has precluded the comprehensive analysis of the mechanisms that control chlorophyll metabolism in petals.

TEOSINTE BRANCHED 1/CYCOIDEA/PCF (TCP) proteins are highly conserved plant-specific transcription factors. The Arabidopsis genome contains 13 Class I TCPs and 11 Class II TCPs, which are further grouped into 8 CINCINNATA (CIN)-like TCPs and 3 TB1/CYC-like TCPs. Among the 24 Arabidopsis TCPs, TCP2, TCP3, TCP4, TCP5, TCP10, TCP13, TCP17, and TCP24 are CIN-like TCPs (Martin-Trillo and Cubas, 2010; Lan and Qin, 2020). CIN-like TCPs have been found to promote plant adaptation in the control of leaf and flower development (Crawford et al., 2004; Koyama et al., 2007, 2010; Huang and Irish, 2015; Zhao et al., 2020), trichome formation (Vadde et al., 2019; Lan et al., 2021), photomorphogenesis (Dong et al., 2019), thermomorphogenesis (Han et al., 2019; Zhou et al., 2019), and other processes in a dosage-dependent and highly redundant manner (Lan and Qin, 2020). To date, CIN-like TCP transcription factors have not been reported to participate in the regulation of petal greening and plastid conversion. Here, we show that the TCP4 transcription factor controls the green color of petals by repressing the expression of key genes in chlorophyll biosynthesis.

**RESULTS**

CIN-like TCP transcription factors regulate the transition from green to white in petals

To overcome the redundancy of CIN-like TCPs, we generated a septime tcp2/3/4/5/10/13/17 mutant. We observed that the petals of the septime mutant remained green, in contrast to the white petals of the wild-type control (Figures 1A–1D). To our knowledge, no loss-of-function mutants with green petals have been reported in Arabidopsis. Therefore, we analyzed the tcp2/3/4/5/10/13/17 mutant in detail to reveal the underlying mechanisms that control the transition from green to white in Arabidopsis petals. We first transformed the tcp2/3/4/5/10/13/17 mutant with TCP4pro-TCP4-FLAG or TCP10pro-TCP10-FLAG, in which a FLAG tag was fused to TCP4 or TCP10 genomic DNA and driven by a 2821-bp TCP4 promoter or a 986-bp TCP10 promoter (Lan et al., 2021). Although both TCP4pro-TCP4-FLAG and TCP10pro-TCP10-FLAG completely complemented the ectopic trichome phenotype of tcp2/3/4/5/10/13/17 (Lan et al., 2021), TCP4pro-TCP4-FLAG but not TCP10pro-TCP10-FLAG completely complemented the petal greening of tcp2/3/4/5/10/13/17 (Figure 1E and 1F, Supplemental Figure 1). This result indicates that TCP4 plays a key role in the green-to-white color shift during petal development. We then collected tissue samples from the distal and proximal parts of petals for chlorophyll analysis. The distal parts of tcp2/3/4/5/10/13/17 petals, but not wild-type control petals, clearly contained a significant amount of chlorophyll, and chlorophyll was detected in the proximal part of both tcp2/3/4/5/10/13/17 and wild-type flowers (Figure 1G and 1H), suggesting that the green color of tcp2/3/4/5/10/13/17 petals was caused by chlorophyll accumulation. We next observed plastids in different regions of petals from mature flowers using transmission electron microscopy (Figure 1I). In the proximal petal region, both tcp2/3/4/5/10/13/17 and wild-type control petals contained chloroplasts with clear thylakoid membranes but without obvious starch grains (Figure 1J and 1K). Fewer thylakoid membranes were present in the chloroplasts in the middle green-to-white transition region of wild-type petals, and starch grains were found to have accumulated (Figure 1L), whereas thylakoid structures were abundant in tcp2/3/4/5/10/13/17 chloroplasts (Figure 1M). In the distal region of wild-type petals, chloroplasts were differentiated into leucoplasts that contained several starch grains but lacked thylakoids (Figure 1N). By contrast, thylakoid membranes were observed in the chloroplasts in the distal region of tcp2/3/4/5/10/13/17 petals (Figure 1O), indicating that the conversion of chloroplasts into leucoplasts was compromised in the distal regions of these petals. We also observed the transition of chloroplasts into leucoplasts during the early stages of flower development using chlorophyll autofluorescence assays and microscopy (Pyke and Page, 1998; Alvarez-Buylla et al., 2010). At stage 9, whole petals were previously reported to show clear chlorophyll autofluorescence. Indeed, at stage 9 in this study, small petals (one-third of
Figure 1. CIN-like TCP transcription factors prevent greening in distal region of petals.

(A–D) The petals of mature flowers from wild type (A and C) and tcp2/3/4/5/10/13/17 (B and D).

(E and F) The phenotype of green petals in tcp2/3/4/5/10/13/17 (E) was complemented by transforming TCP4pro-TCP4-FLAG into the septuple mutant (F). Scale bars, 1 mm. The phenotypes of inflorescences and petals were observed with a Leica M205 FCA stereoscope.

(G and H) The absorption spectra of extractions from the distal and proximal petal regions from wild-type (G) and tcp2/3/4/5/10/13/17 (H) flowers. The tissues were collected by dissecting petals into distal and proximal parts. The peaks at 430–450 nm (red light) and 640–660 nm (blue-violet light) indicate the absorption spectrum of chlorophylls.

(I–O) Transmission electron microscopy analysis of plastids from petals. The proximal, middle, and distal regions of the petal of a wild-type flower are depicted (I). The structures of plastids from the proximal, middle, and distal regions of petals from wild-type (J, L, and N) and tcp2/3/4/5/10/13/17 flowers are shown (K, M, and O). The proximal petal regions of wild-type and tcp2/3/4/5/10/13/17 flowers all contained chloroplasts with clear thylakoid membranes (J and K). In the distal petal regions of wild-type flowers, chloroplasts were converted into leucoplasts (N), whereas thylakoid structures in chloroplasts were maintained in tcp2/3/4/5/10/13/17 petals (O). Scale bars, 0.1 mm in (I), 0.2 μm in (J–O).

(P–W) Confocal microscopy analysis of chlorophyll autofluorescence in wild-type (P, R, T, and V) and tcp2/3/4/5/10/13/17 (Q, S, U, and W) petals during flower development. Chlorophyll autofluorescence in chloroplasts was observed with a Zeiss Axio Imager M2 microscope using the RFP channel. Early flowers at developmental stage 9 (P and Q), stage 10 (R and S), stage 11 (T and U), and stage 12 (V and W) are shown. A clear disappearance of chlorophyll autofluorescence was observed in the distal petals at flower stages 11 and 12 in wild-type (T and V) petals but not in tcp2/3/4/5/10/13/17 petals (U and W). Scale bars, 50 μm.
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stamen height) were green in color and showed autofluorescence in all regions in both wild-type and tcp2/3/4/5/10/13/17 flowers (Supplemental Figure 2A, 2C, and 2E and Figure 1P). The petals of wild-type flowers at stage 10 were green in color, but they showed less autofluorescence in the distal region than in the proximal region (Supplemental Figure 2A, 2C, and 2F and Figure 1R). By contrast, the intensity of chlorophyll autofluorescence was similar in the distal and proximal regions of tcp2/3/4/5/10/13/17 petals at stages 9 and 10 (Supplemental Figure 2B, 2D, 2J, and 2K and Figure 1Q and 1S). At stages 11 and 12, the distal regions of wild-type petals were clearly white in color, and chlorophyll autofluorescence disappeared in a corresponding manner (Supplemental Figure 2A, 2C, and 2G–2I and Figure 1T and 1V). However, the green color and chlorophyll autofluorescence were maintained in most areas of the distal part of tcp2/3/4/5/10/13/17 petals at stages 11 and 12 (Supplemental Figure 2B, 2D, and 2L–2N and Figure 1U and 1W). These observations demonstrate that TCPs control the conversion of chloroplasts into leucoplasts as early as flower stage 10.

TCPs 2/3/4/5/10/13/17 are redundant in the control of petal greening

To determine the redundant function of CIN-like TCPs in the control of petal chloroplast conversion, we analyzed the ratios of green region lengths of the petals of tcp mutants that contained different combinations of tcp mutations. The green-to-white ratios of the petals of tcp4 and tcp10 single mutants were each approximately 60%, which was comparable to that of the wild-type control petals (Supplemental Figure 3A–3C and 3J). However, the green-to-white ratio of the tcp4/10 double mutant was 71.5% (Supplemental Figure 3D and 3J). The tcp3/4/10 triple mutant, tcp3/4/5/10 quadruple mutant, and tcp3/4/5/10/13 quintuple mutant had petals that were approximately 89% green in color (Supplemental Figure 3E–3G and 3J), and almost all areas of the petals of the tcp2/3/4/5/10/13 sextuple mutant and the tcp2/3/4/5/10/13/17 septuple mutant were green, with green-to-white ratios of 92.9% and 96.2%, respectively (Supplemental Figure 3H–3J). These results demonstrate that the ratio of green-to-white color in Arabidopsis petals increased as the number of tcp mutants increased, indicating that TCPs play additive and redundant roles in controlling the conversion of chloroplasts into leucoplasts during petal development.

The expression of TCP4 is developmentally regulated during petal growth

To better understand the role of TCP4 in the regulation of green petal color, we analyzed the expression pattern of TCP4 protein in petals during flower development. The TCP4pro-TCP4-GUS construct, in which a 2818-bp-long TCP4 promoter was used to drive a β-glucuronidase (GUS) reporter gene fusion with TCP4 genomic DNA, was transformed into Arabidopsis (Lan et al., 2021). Subsequently, stable TCP4pro-TCP4-GUS transgenic plants were used for GUS staining analysis. The inflorescences showed clear GUS activity, with stronger GUS staining from flower stage 10 to stage 12 (Figure 2A). The flowers were dissected to enable observation of GUS activity in the petals. At stage 7, no TCP4 expression was observed in the petals of small flower buds (Figure 2B). However, weak GUS staining began to appear at the petal tip at stage 8 (Figure 2C). At stage 9, strong GUS staining was observed in approximately one-third of the petal length in the distal region (Figure 2D and 2E). From stage 10 to stage 12, GUS activity extended from the tip of the petal to the base, and it covered more than half of the petal length in the distal region (Figure 2F–2K). At stage 13, GUS staining began to fade. During the early part of stage 13, TCP4 protein was mainly found in the vasculature of petals (Figure 2L and 2M), and no expression of TCP4 was observed in the petals during the late part of stage 13 (Figure 2N). TCP4 protein expression was not observed in the proximal petal region, where the green color was maintained at all flower developmental stages (Figure 2B–2N). The expression pattern of TCP4 protein is consistent with that of TCP4 gene transcription reported previously (Li et al., 2016). These data indicate that the expression pattern of TCP4 protein is developmentally regulated and corresponds well to the green-to-white color shift and the final green-white color pattern of petals, providing strong evidence that TCP4 acts as a positive regulator in the conversion of chloroplasts into leucoplasts in petals.

TCPs 2/3/4/5/10/13/17 mainly repress gene expression during petal development

To determine the molecular mechanisms by which TCP4 controls green-white color pattern formation and plastid conversion in petals, we collected proximal and distal petal tissues from wild-type and tcp2/3/4/5/10/13/17 septuple mutant flowers for RNA sequencing (RNA-seq) with three replicates. The transcriptome analysis comparing the green proximal tissue and white distal tissue of wild-type flowers (WT proximal versus WT distal, green versus white) revealed 4383 differentially expressed genes (DEGs, false discovery rate <0.01; fold change >2.0 or <−2.0), including 2790 upregulated genes and 1593 downregulated genes (Supplemental Figure 4A and Figure 3A; Supplemental Table 1), suggesting that gene repression was predominant during the conversion of chloroplasts into leucoplasts in distal white tissues. However, only 1206 upregulated genes and 1166 downregulated genes were found in the comparison of tcp2/3/4/5/10/13 proximal tissue and tcp2/3/4/5/10/13/17 distal tissue (tcp proximal versus tcp distal, green versus green) (Supplemental Figure 4B and Figure 3A; Supplemental Table 2). More than 56% (1584/2790) of the genes that were downregulated in the distal tissue of wild-type petals did not show lower expression levels in the green distal petals of tcp2/3/4/5/10/13/17 petals, indicating that TCPs play important roles in repressing gene expression during the conversion of chloroplasts into leucoplasts in petals. Consistent with these results, 4175 upregulated genes and 1110 downregulated genes were found in the comparison of tcp2/3/4/5/10/13 distal tissue and wild-type distal tissue (tcp distal versus WT distal, green versus white) (Supplemental Figure 4D and Figure 3A; Supplemental Table 3). In addition, 2968 upregulated genes and 702 downregulated genes were found in the comparison of tcp2/3/4/5/10/13/17 proximal tissue and wild-type proximal tissue (tcp proximal versus WT proximal, green versus green) (Supplemental Figure 4C and Figure 3A; Supplemental Table 4), further confirming that TCPs 2/3/4/5/10/13 play pivotal roles in regulating gene expression, and particularly in repressing expression, during petal development. Indeed, the RNA-seq analysis showed that wild-type petals expressed 22 TCP family
Interestingly, most CIN-like TCP genes, including TCP4, TCP10, TCP3, TCP5, TCP13, and TCP2, showed higher expression levels in the distal parts of petals, whereas most Class I TCP genes, including TCP23, TCP14, and TCP15, had higher expression levels in the proximal parts of petals (Figure 3B). The observed predominant expression of TCP4 and other CIN-like TCP genes in the distal petal region is consistent with the expression pattern of TCP4 protein revealed by the TCP4pro-TCP4-GUS reporter, as well as the distribution of green and white coloration in petals (Figures 1A, 1I, and 2). To identify the downstream genes responsible for the green color of Arabidopsis petals, we analyzed overlapping DEGs. The DEG analysis revealed 1133 upregulated genes and 315 downregulated genes that were differentially expressed in the comparisons of WT proximal versus WT distal expression and tcp distal versus WT distal expression (Figure 3C; Supplemental Tables 5 and 6), and subsequent analysis was focused on these two sets of green versus white DEGs. Gene ontology (GO) analysis showed that genes related to chlorophyll biosynthesis, as well as chloroplast and photosynthesis pathways (Waters and Langdale, 2009; Zhou et al., 2011; Ohmiya et al., 2017; Sun et al., 2019; Fu et al., 2021), were highly enriched in the set of 1133 overlapping upregulated genes (Figure 3D; Supplemental Figure 5), whereas no genes related to chlorophyll or plastids were enriched in the set of 315 overlapping downregulated genes (Supplemental Table 6), suggesting that TCPs 2/3/4/5/10/13/17 repress genes related to chloroplasts in distal petal tissue.

TCPs 2/3/4/5/10/13/17 promote petal color conversion by repressing chlorophyll biosynthesis-related genes.

CIN-like TCP genes may control petal development by regulating petal color, morphology, polarity, and other processes (Lan and Qin, 2020). Because the green color of petals is produced by chlorophyll, we assessed the expression levels of all genes encoding enzymes involved in chlorophyll biosynthesis, recycling, and degradation based on the RNA-seq data.
Heatmap analysis showed that almost all genes encoding enzymes related to the chlorophyll biosynthesis pathway were repressed in the distal tissue of wild-type petals and expressed at higher levels in the green proximal tissue of wild-type petals, as well as the green distal tissue of tcp2/3/4/5/10/13/17 petals (Figure 4A; Supplemental Table 5). Surprisingly, some genes related to chlorophyll recycling or degradation were also repressed in the white petal tissue of wild-type flowers (Figure 4A; Supplemental Table 5). This finding indicates that the color shift in the petals is a result of decreased chlorophyll biosynthesis rather than increased chlorophyll degradation. Because TCP5 was previously reported to regulate ethylene biosynthesis and ethylene is an important phytohormone that functions in chlorophyll catabolism and degradation (Qiu et al., 2015; van Es et al., 2018), we investigated whether ethylene could participate in the prevention of petal greening by searching our RNA-seq data for genes related to ethylene biosynthesis and signaling (Binder, 2020). The result showed that the ethylene biosynthesis gene ACC-oxidase 2 (ACO2) was induced in the green petal tissues compared with the white petal tissues of the wild type, whereas the expression levels of the other ethylene biosynthesis and signaling genes were not consistently altered in the green tissues (Supplemental Figure 7A), suggesting that ethylene may not play a key role in the prevention of petal greening. Analysis of the expression levels of transcription factor (TF) genes showed that genes encoding SOC1, COL16, WHY1, WHY3, and REM7, as well as other TFs known to positively regulate chlorophyll biosynthesis (Marechal et al., 2009; Kobayashi et al., 2012, 2017; Bastakis et al., 2018; Ohmiya et al., 2019; Wang et al., 2019; Hanano et al., 2020; Luo et al., 2021), were predominantly expressed in the green petal tissue of wild-type and tcp2/3/4/5/10/13/17 flowers (Figure 4B; Supplemental Table 5). These results suggest that TCPs 2/3/4/5/10/13/17 control petal color conversion by inhibiting the expression of multiple genes that positively regulate chlorophyll biosynthesis.
To provide an explanation for the phenomenon in which inhibition of chlorophyll biosynthesis in Arabidopsis flowers results in white petals rather than yellow petals, we analyzed the expression of carotenoid biosynthesis genes in petals using our RNA-seq data (Rosas-Saavedra and Stange, 2016). Heatmap analysis showed that almost all the carotenoid biosynthesis genes were repressed in the white distal tissue of wild-type petals (Supplemental Figure 7B), consistent with the whitening of Arabidopsis petals. These data indicate that TCPs 2/3/4/5/10/13/17 inhibit both chlorophyll and carotenoid biosynthesis during the prevention of petal greening.

TCP4 regulates key genes in chlorophyll biosynthesis by binding directly to their promoters

To further confirm that TCPs 2/3/4/5/10/13/17 regulate green petal color by repressing chlorophyll biosynthesis and not by promoting chlorophyll degradation, we used quantitative real-time PCR to measure the expression levels of selected genes involved in chlorophyll biosynthesis or degradation in the proximal and distal tissues of wild-type and tcp2/3/4/5/10/13/17 petals. All six selected chlorophyll biosynthesis genes were expressed at lower levels in white petal tissue (Figure 5A–5G): CHLH and CHL2 (encoding magnesium [Mg] chelatase subunits H and I, which are critical for chlorophyll biosynthesis) (Brzezowski et al., 2016), CRD1 (encoding Mg-protoporphyrinogen IX monomethylster cyclase, which is involved in isocyclic ring formation during protochlorophyllide biosynthesis) (Tottey et al., 2003), PORB and PORC (encoding protochlorophyllide reductases) (Frick et al., 2003), DVR (encoding DVR) (Wang et al., 2013), and CAO (encoding CAO, which is involved in biosynthesis of chlorophyllide b) (Wojtowicz et al., 2021). By contrast, the expression levels of chlorophyll degradation genes, including SGR (Shimoda et al., 2016) and PAO (Aubry et al., 2008), were comparable in white and green petal tissues, except that PAO was also upregulated in distal tcp2/3/4/5/10/13/17 petal tissue (Ohmiya et al., 2017; Wang and Grimm, 2021) (Figure 5H and 5I). The expression level of SOC1 was also decreased in white petal tissue, but it was increased in the green petals of tcp2/3/4/5/10/13/17 flowers (Figure 5J), consistent with a previous report that SOC1 overexpression leads to green petals (Wang et al., 2019). To determine whether CIN-like TCPs directly regulate these chlorophyll biosynthesis-related genes, we analyzed our previously collected TCP4 chromatin immunoprecipitation sequencing (ChIP-seq) data using dark-grown seedlings (Dong et al., 2019). The promoter regions of PORB, DVR, and SOC1 were bound by TCP4, and potential TCP4-binding sites were found in the promoters of these genes (Figure 5K–5M). We then confirmed the binding of TCP4 to the promoters by ChIP-PCR assays using flowers. The results showed that TCP4 was significantly enriched near cis elements bound by TCP4 (Figure 5N–5P). We further confirmed that TCP4 bound directly to cis elements by an electrophoretic mobility shift assay (EMSA) (Figure 5Q). These results clearly demonstrate that TCP4 promotes the conversion of chloroplasts into leucoplasts by repressing chlorophyll biosynthesis genes directly or indirectly through its influence on other TFs, such as SOC1.

The prevention of petal greening may occur ubiquitously in different plant species

To determine whether the conversion of chloroplasts into leucoplasts during petal development is ubiquitous, we observed some easily accessible flowers of peppers from a vegetable garden, lilies from a flower vase, potted Kalanchoe blossfeldiana, and Orychophragmus violaceus from the campus of Peking University. The results of these observations showed that petals in the early flower buds of each flower were all green (Supplemental Figure 8). The green petal color gradually faded as the flowers grew (Supplemental Figure 8). Interestingly, red or...
Figure 5. TCP4 directly regulates chlorophyll biosynthesis genes and the transcription factor SOC1 to control petal greening.

(A–J) The relative expression levels of selected genes related to chlorophyll biosynthesis (A–G), selected genes related to chlorophyll degradation (H and I), and SOC1 (J) in the proximal and distal tissues of wild-type and tcp2/3/4/5/10/13/17 petals were determined by qRT-PCR. The expression level of each gene in the distal region of wild-type petals was set to 1.0. UBQ5 was used as the control gene for expression level normalization. The data are the mean ± SD of three biological replicates. The asterisks indicate the significance of the comparison of gene expression level between green tissues (the proximal tissues of the wild type, the proximal tissues of tcp2/3/4/5/10/13/17, and the distal tissues of tcp2/3/4/5/10/13/17) and white tissues (the distal tissues of the wild type) based on Student’s t-test (**p < 0.001).

(K–M) TCP4 binds directly to the promoter regions of PORB, DVR, and SOC1. Chromatin immunoprecipitation sequencing (ChIP-seq) using 35S-Myc-mTCP4 transgenic plants indicates that TCP4 binds to the promoter regions of PORB (K), DVR (L), and SOC1 (M). Peak graphs show the ChIP-seq raw reads at potential TCP4-binding sites. The black bars indicate the genomic coding regions of the genes. The white arrows indicate the transcriptional direction. Scale bars, 100 bp. The schematic diagrams show the upstream regions of PORB, DVR, and SOC1. The long black lines indicate the promoter regions of genes. The vertical red lines indicate the potential TCP4-binding motif GGACCA. The blue arrows indicate the transcriptional start sites. PORB-I, –127 to –122 bp; DVR-I, –1411 to –1406 bp; SOC1-I, –4261 to –4256 bp; SOC1-II, –2871 to –2866 bp; SOC1-III, –1700 to –1695 bp; SOC1-IV, –1543 to –1538 bp.

(legend continued on next page)
Green petals controlled by TCP4

purple coloration began to emerge from the petal tip after the petals had whitened (Supplemental Figure 8K, 8O, and 8U), suggesting that decreased chlorophyll abundance may be a prerequisite for the accumulation of other pigments in petals. In addition, the observed flowers also maintained their green coloration in the proximal petal portion (Supplemental Figure 8K, 8O, and 8U). We also observed the plastids of O. violaceus during petal growth. During the early flower stage, the petals contained chloroplasts with clear thylakoids. As the flowers grew, the chloroplasts transformed into leucoplasts and then chromoplasts without thylakoids (Supplemental Figure 8V–8X), similar to our findings in Arabidopsis petals (Figure 1J, 1L, and 1N).

DISCUSSION

In this paper, we demonstrate that CIN-like TCP TFs repress petal greenness by directly and indirectly inhibiting chlorophyll biosynthesis. We propose a model for the function of TCP4 in the control of green petals (Figure 6A). On one hand, TCP4 binds directly to the promoters of chlorophyll biosynthesis genes such as PORB and DVR to downregulate their expression. On the other hand, TCP4 directly inhibits SOC1 and possibly other TFs to indirectly inhibit the expression of chlorophyll biosynthesis genes. Downregulation of chlorophyll biosynthesis possibly leads to the conversion of chloroplasts into leucoplasts, producing a shift in petal color from green to white (Figure 6A). Disruption of TCP functions in tcp multiple mutants may release the repression of some unknown TFs that positively regulate chlorophyll biosynthesis (Figure 6B), causing the formation of green petals.

The CIN-like TCPs play a critical role during petal growth and development. It has been reported that CIN-like TCPs have redundant functions in the control of petal curvature in Arabidopsis (Koyama et al., 2011). Disruption of the functions of TCP5, TCP13, and TCP17 by artificial microRNA increases petal width and thus alters petal shape, whereas overexpression of TCP5 leads to narrow petals (Huang and Irish, 2015; Li et al., 2016; van Es et al., 2016). The roles of CIN-like TCPs in the regulation of petal growth and morphology may be conserved. For example, in Antirrhinum, disruption of CIN genes causes defective growth of lobes in the distal petal regions (Crawford et al., 2004). The silencing of a CIN-like TCP gene, PhLA, in Petunia hybrida leads to increased petal curvature (Chen et al., 2020b). In addition, the expression of TCP3-SRDX, in which the Arabidopsis TCP3 gene is fused to a sequence encoding the Ethylene-responsive element-binding factor-associated amphiphilic repression motif, causes wavy margins in the dorsal petals of Torenia fournieri and smaller petals of ray flowers in Chrysanthemum morifolium (Narumi et al., 2011). CIN-like TCPs control petal growth and morphology by affecting the cell proliferation and differentiation of petal epidermal cells. Disruption of the functions of CIN-like TCPs results in the replacement of conical petal cells with flat ones (Crawford et al., 2004; Koyama et al., 2011). Interestingly, the expression of TCP3-SRDX also reduces the accumulation of purple anthocyanin pigmentation in the distal regions of Torenia petals, whereas it converts white into green in the petals of Chrysanthemum (Narumi et al., 2011). In this paper, our genetic data strongly indicate that CIN-like TCPs, including TCP5, TCP6, DVR, and SOC1, have higher expression levels in proximal petal regions. Detailed analysis of TCP4 promoter reveals that TCP4 is not expressed in petals at early or mature flower developmental stages, whereas most Class I TCP genes have higher expression in the distal petal regions from flower stage 9 to stage 12 (Li et al., 2016). CIN genes in Antirrhinum begin to be expressed in flower buds at developmental stage 7 and are also specifically expressed in distal petal regions during flower development (Crawford et al., 2004). In this paper, we found that most CIN-like TCP genes are predominantly expressed in the distal regions of petals, whereas most Class I TCP genes have higher expression in proximal petal regions. The spatial-temporal expression pattern of TCP4 protein is consistent with that of the TCP4 gene previously identified using the TCP4 promoter (Li et al., 2016). Interestingly, the expression pattern of TCP4 protein nicely coincides with the spatial and temporal pattern of green coloration in petals during flower development. This is also observed in the expression of TCP4 in the distal petal region, corresponding to the white distal petal region, and the lack of TCP4 expression in the proximal petal regions coincides with the green petal areas in Arabidopsis flowers. These data support the notion that TCP TFs are important for suppressing green color and forming the distal white and proximal green pattern in petals.

Our findings illuminate the molecular mechanisms by which plants form proximal green and distal white colors, as well as

(N–P) ChiP–PCR assays using tissue from 3SS-Myc-mTCP4 flowers confirm that TCP4 regulates PORB (N), DVR (Q), and SOC1 (P) by binding directly to cis elements in their promoters. Promoter regions containing the TCP4-binding motif were amplified with specific primer pairs as shown in the schematic diagrams (K–M). The relative enrichment of the wild-type petals was set to 1.0. The T3 transposon locus was used as a negative control. The data are the mean ± SD of three biological replicates. The asterisks indicate the significance based on Student’s t-test (**p < 0.001).

(Q) EMSA experiments confirmed that TCP4 binds directly to the promoters of PORB, DVR, and SOC1.
other petal colors, and provide an empirical knowledge base for the breeding of green flowers.

METHODS

Plant materials and growth conditions

The ecotype of Arabidopsis thaliana used in this study was Columbia-0 (Col-0). T-DNA insertion mutants, including tcp2 (SALK_562_D05), tcp3 (wiscDSlox441C3), tcp4 (GABI_363H06), tcp5 (SM_3_29636), tcp10 (SALK_137205), tcp13 (GABI_182B12), and tcp17 (SALK_148580), were crossed to generate tcp multiple mutants. Seedlings were grown on half-strength Murashige and Skoog medium containing sucrose (10 g/L) and agar (8 g/L). After stratification at 4°C for 2 days, plates with seeds were incubated in a chamber under long-day conditions (16 h light/8 h dark) at 22°C for 7 days. The seedlings were then planted in soil and grown in a greenhouse under the growth conditions described above.

The quantitative real-time PCR primers for SOC1 (AT2G45660), DVR (AT5G18660), PORB (AT4G27440), PORC (AT4G27440), CHLH (AT5G13630), CHLI2 (AT5G56940), CAO (AT1G44446), SGR (AT4G22920), and PAO (AT3G44880) are listed in Supplemental Table 7.

Genotyping and gene expression analysis

The tcp T-DNA insertion mutants were genotyped using TCPx-F/TCPx-R primers based on the sequences flanking the T-DNA insertion sites, as well as TCPx-TDNA insertion based on T-DNA vector sequences. The genotyping conditions were described previously (Lan et al., 2021). All primers used in this work are listed in Supplemental Table 7.

For analysis of gene expression, petals were dissected, and proximal and distal tissue samples were isolated from the flowers of wild-type or tcp2/3/4/5/10/13/17 septuple mutants. The petal tissue samples were immediately placed in liquid nitrogen after isolation, and total RNA was extracted using a Plant Total RNA Purification Kit (GeneMark, TR02-150). Total RNA was reverse transcribed using an M-MLV kit (Promega, M170A) in a 20-μl reaction volume. The products were diluted and used as templates for quantitative real-time PCR. A Thermo Fisher QuantStudio 5 real-time system and UltraSYBR Mixture (CWBIO, CW2601M) were used to perform quantitative real-time PCR with three biological and technical replicates. The cycling conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The relative expression levels of genes were calculated based on the 2^{-ΔΔCt} (cycle threshold) method (Livak and Schmittgen, 2001). The gene expression levels were normalized based on the expression level of the housekeeping gene AtUBQ5.

Generation of binary constructs and transformation

The constructs were generated as described previously (Lan et al., 2021). In brief, the miR319-resistant form of TCP4 (mTCP4) was obtained by PCR-based mutagenesis with the primer pair TCP4-mut-F/TCP4-mut-R. To generate TCP4pro-TCP4-FLAG and TCP10pro-TCP10-FLAG, a 2821-bp promoter region or a 986-bp promoter region with the full-length genomic coding region of TCP4 or TCP10 (lacking the stop codon) was amplified from Arabidopsis genomic DNA and cloned into the pENTR/D-TOPO vector to obtain pENTR-TCP4pg and pENTR-TCP10pg. TCP4pro-TCP4-FLAG or TCP10pro-TCP10-FLAG was finally generated by Gateway Cloning via the LR reaction between pENTR-TCP4pg or pENTR-TCP10pg and pK7FLAGWG0 (Invitrogen). TCP4pro-TCP4-GUS was generated via the LR reaction between pENTR-TCP4pg and pB7GUSWG0 (Lan et al., 2021; Li et al., 2021).

The constructs were transformed into Arabidopsis by Agrobacterium tumefaciens GV3101 using the floral dip method as described previously (Qin et al., 2005).

Microscopy

To observe chlorophyll autofluorescence in petals, intact petals were dissected from buds at different stages during flower development. Chlorophyll autofluorescence in chloroplasts was observed with a Zeiss Axio Imager M2 microscope using the RFP channel.
Prior to observation of plastids in petals, a fixation solution was prepared with 2.5% (v/v) glutaraldehyde and 2% paraformaldehyde (w/v) in 0.1 M phosphate buffer (pH 7.4). The petals were cut into pieces (smaller than 1 mm³) and fixed in the fixation solution at room temperature for 4 h. The samples were then kept at 4°C overnight. After three washes with 0.1 M phosphate buffer (pH 7.4), the samples were post-fixed with 2% osmium tetroxide (w/v) and 1.5% potassium ferricyanide (w/v) in the same buffer at 4°C for 2 h and washed three times. Next, an epon staining with 2% uranyl acetate (w/v) was performed overnight at 4°C. The samples were then dehydrated by a graded ethanol series, embedded in fresh resin, and polymerized at 65°C for 24 h. Ultrathin (70 nm) sections were collected using a Leica UC7 ultramicrotome equipped with a Diatome diamond knife, after which the sections were post-stained with uranyl acetate and lead citrate. Grids were imaged at 80 kV in a JEOL JEM-1400 transmission electron microscope using a CMOS camera (XAROSA, EMSIS).

The phenotypes of inflorescences and petals were observed with a Leica M205 FCA stereoscope. Petals were dissected from flower buds, and the lengths of the green and white petal regions were analyzed.

**Measurement of chlorophyll**

Proximal and distal tissue samples (0.1 g) were collected from the flower petals and soaked in 1 ml extraction solution (90% acetone:75% ethanol 1:1) for 9 h. The supernatants were analyzed using a DU 800 UV/Visible spectrophotometer with three biological and technical replicates.

**GUS staining**

The GUS staining was performed as described previously (Zhang et al., 2017). In brief, the inflorescences of TCP4pro-TCP4-GUS plants were soaked in GUS staining solution at 37°C overnight and transferred into 75% ethanol. Petal staining was observed using a Leica M205 FCA stereoscope or a Zeiss Axio Imager M2C microscope.

**RNA-seq and data analysis**

RNA-seq was performed with three replicates. RNA extraction, RNA-seq library preparation, and RNA-seq were carried out by GENEWIZ ( Suzhou, China). The libraries were sequenced using an Illumina HiSeq 4000 platform to generate 150-bp paired-end reads. Quality control of the raw reads was performed with fastp (0.12.2) (Chen et al., 2018). The clean reads were mapped to the Arabidopsis reference genome (TAIR10) using STAR (2.8.0a) (Dobin et al., 2013). Expression counts were generated using featureCounts with default parameters (Liao et al., 2014). The R package DESeq2 (v.1.20.0) was used for differential gene expression analysis (Love et al., 2014). Genes with a Bonferroni-adjusted p value ≤ 0.01 and log₂ fold change ≤ −1 or ≥ 1 were considered to be significantly differentially expressed genes. Venn diagrams and heatmaps were constructed using TBtools (Chen et al., 2020a). GO analysis was performed using the GO annotation function of The Arabidopsis Information Resource (Thomas et al., 2003). RNA-seq data from wild-type and tcp2/ tcp4 were obtained from the NCBI BioProject under series no. PRJNA792926.

**Chromatin immunoprecipitation assay**

Raw ChIP-seq data were obtained from the Gene Expression Omnibus (accession no. GSE115589, Dong et al., 2019). Screenshots were obtained using the Integrative Genomics Viewer (v.2.6.2). The data were analyzed as described previously (Lan et al., 2021).

To carry out EMSA, the purified TCP4 DNA binding domain was cloned into PET-21b with a C-terminal His-tag. Next, the vector was transformed into Escherichia coli strain BL21 (DE3). The bacteria were cultured overnight at 18°C, and proteins were induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM. The cells were collected and resuspended in buffer A (25 mM Tris and 1 M NaCl [pH 8.0]). The target protein was eluted with 200–500 mM imidazole using a Ni-NTA chelating column (GE Healthcare, USA). The crude protein was then subjected to size-exclusion chromatography (Superdex 75, GE Healthcare) for the final purification step.

The DNA sequence encoding the TCP4 DNA binding domain was cloned into pET-21b with a C-terminal His-tag. Next, the vector was transformed into Escherichia coli strain BL21 (DE3). The bacteria were cultured overnight at 18°C, and proteins were induced by adding isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM. The cells were collected and resuspended in buffer A (25 mM Tris and 1 M NaCl [pH 8.0]). The target protein was eluted with 200–500 mM imidazole using a Ni-NTA chelating column (GE Healthcare, USA). The crude protein was then subjected to size-exclusion chromatography (Superdex 75, GE Healthcare) for the final purification step.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at Plant Communications Online.

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**AUTHOR CONTRIBUTIONS**

G.Q. supervised and designed the study. X.Z. performed most of the experiments. J.L., H.Y., J.Z., and X.Z. generated the genetic material. Y.Z. and X.S. participated in the EMSA experiments. G.Q., X.Z., J.L., and Y.Q. analyzed the results. G.Q. and X.Z. wrote the manuscript.
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