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阿拉伯芥 TCP4 與 HDA6 交互作用並調控開花與葉片發育

TCP4 interacts with HDA6 and regulates flowering and leaf development in *Arabidopsis*

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中文摘要

阿拉伯芥中 TCP 家族的成員參與在種子萌發、葉片老化等植物生長過程，而 Class II 的 TCP 成員則會藉由調控生長中的葉原基(leaf primordia)邊緣細胞分裂程度來影響植物的葉片型態。通過酵母菌雙雜交以及雙分子螢光互補實驗，我們發現 HDA6 會和許多 TCP 轉綠因子有交互作用。另外， *tcp4* 突變株會有晚開花的現象。*tcp4* 與 *hda6* 突變株 *axe1-5* 的雙突變株 *tcp4-2/axe1-5* 比起 *tcp4* 和 *axe1-5* 單突變株會有更晚開花的現象，並且葉片捲曲和鋸齒的性狀也更加明顯。而在 *tcp4*，*hda6* 與 *as1* 或是與 *as2* 的三突變株中，植物的葉片範圍也比單突變或是雙突變株小，葉子的鋸齒亦更明顯。在 *tcp4* 和 *axe1-5* 單突變株以及 *tcp4-2/axe1-5* 雙突變株中，調控葉片發育的基因 *KNATM* 以及開花時間基因 *FLC*，*MAF4* 和 *MAF5* 的表現量均會上升。從這些結果我們知道 TCP4 和 HDA6 可能會藉由共同調控下游基因來影響阿拉伯芥的開花時間以及葉片型態。

相關字：阿拉伯芥；組蛋白去乙醯酶; HDA6; TCP4; 開花時間；葉片發育

Abstract

TCP transcription factors participate in diverse plant developmental processes such as seed germination and leaf senescence. Class II TCPs influence leaf morphogenesis by regulating cell proliferation at the margins of the developing leaf primordia in *Arabidopsis.* We found that HDA6 can interact with many TCPs in yeast two-hybrid assays and bimolecular fluorescence complementation assays. In addition, *tcp4* mutants have a late flowering phenotype under long day conditions. The double mutant of *tcp4* and *hda6* (*axe1-5*), *tcp4-2/axe1-5,* shows enhanced delayed flowering phenotypes and displays severe serration and curling leaf phenotypes compared to the *tcp4* and *axe1-5* single mutants. Furthermore, in the triple mutant of *tcp4*, *axe1-5*, *as1-1* or *as2-1*, the plant sizes are smaller and their leaves are more serrated compared to *tcp4-2/axe1-5* double mutants. The expression of the leaf development regulating gene *KNATM* and flowering time control genes *FLC*, *MAF4* and *MAF5* are increased in *tcp4* and *axe1-5* mutants compared to wild type. These data suggest that *TCP4* and *HDA6* might influence the flowering time and leaf development by acting cooperatively to regulate downstream gene expression in *Arabidopsis*.

Key words: *Arabidopsis*, HDA6, TCP4, flowering time, leaf morphology

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List of Abbreviations

ABA: Abscisic acid

AS1: ASYMMETRIC LEAVES 1

AS2: ASYMMETRIC LEAVES 2

BiFC: Bimolecular Fluorescence Complementation

CO: COSTANT

GI: GIGANTEA

FLC: Flowering locus C

HDA: Histone deacetylase

KNAT: KNOTTED-LIKE FROM *ARABIDOPSIS THALIANA*

KNATM: KNOX *ARABIDOPSIS THALIANA* MEINOX

MAF: MADS affecting flowering

MS: Muraswhing and Skoog

FT: FLOWERING LOCUS T

SOC1: Suppressor of overexpression of CO 1

TCP: TEOSINTE BRANCHED1/CYCLOIDEA/PCF

UBQ: Ubiquitin

Introduction

The *TCP* gene family

The *TCP* gene family is a small group of plant genes encoding proteins with the TCP domain, a 59-amino acid basic helix–loop–helix (bHLH) motif that allows DNA binding and protein–protein interactions (Kosugi and Ohashi, 1997; Cubas et al., 1999). The TCP domain was initially identified in four proteins encoded by four unrelated genes, from which the name 'TCP' was derived: *TEOSINTE BRANCHED1* (*TB1*) in maize (*Zea mays*), *CYCLOIDEA* (*CYC*) in *Antirrhinum (Luo et al., 1996)*, and *PROLIFERATING CELL FACTORS 1* and *2* (*PCF1* and *PCF2*) from rice (*Oryza sativa*).

In *Arabidopsis*, there are 24 TCPs which can be distinguished into two types of TCP proteins based on differences within their TCP domains: class I (also known as PCF class or TCP-P class) and class II (also known as TCP-C class). The TCP domain of class I has four-amino acid deletion relative to class II proteins. The TCP domain mediates the binding of the TCP proteins to GC-rich DNA sequence motifs in vitro (Kosugi and Ohashi, 2002). Class II can be further subdivided into two clades: the CIN clade and the CYC/TB1 clade, based on the differences of TCP domain. The

TCP family transcription factors are among the best-characterized regulators of leaf development and play an essential role in the determination of the leaf size and shape (Nath et al., 2003; Palatnik et al., 2003). At the cellular level, most *TCP* genes modulate cell proliferation in the axillary meristem of plants (Li et al., 2005; Hervé et al., 2009; Martín-Trillo and Cubas, 2010) either by directly controlling the transcription of cyclin or by inducing differentiation of the cells (Nath et al., 2003). In *Arabidopsis*, *TCP2, TCP3, TCP4, TCP10* and *TCP24* have a target site for microRNA (miRNA) *miR319*. Overexpression of *miR319* causes the degradation of these *TCPs* and the generation of crinkled leaves similar to those observed in *tcp* loss-of-function mutants (Palatnik et al., 2003).

Previous study have shown that TCP4 can activate the expression of *LIPOXYGENASE2* (*LOX2*), which catalyzes the first steps of JA synthesis in plants, and directly influence JA biosynthesis (Schommer et al., 2008). TCP4 was reported to repress cell proliferation by directly activating the expression of *miR396b* and *ICK1/KRP1*, a core gene involved in the progression of the cell cycle (Inze and De Veylder, 2006; Gutierrez, 2009; Schommer et al., 2014). High levels of *ICK1/KRP1* inhibit cell proliferation and reduce leaf growth (Wang et al., 2000). In addition, *miR396* represses *GRFs,* the positive regulators of leaf growth (Kim et al., 2003;

Horiguchi et al., 2005; Rodriguez et al., 2010).

Histone deacetylases in *Arabidopsis*

Chromatin is composed of both DNA and proteins and is responsible for storing heritable information in cells. Chromatin is highly organized and consists of four core histone proteins, H2A, H2B, H3 and H4, forming into octameric protein complexes containing two molecules of each of the four core histones. Specific histone modifications at certain residues of the amino-terminal tails of H3 and H4 constitute the "histone code" that instructs the chromatin to adopt either "open" or "closed" configurations, thereby regulating the availability of cis-regulatory elements of genes to transcriptional machinery. Histone Deacetylases (HDAs) are histone modification proteins regulating gene expression by histone deacetylation at the epigenetic level. Eukaryotic HDAs can be subdivided into three classes: RPD3/HDA1, SIR2 and HD2. In *Arabidopsis*, RPD3/HDA1 family HDAs can be further divided into three classes: I, II, and IV. Class I has HDA6, HDA7, HDA9, HDA10, HDA17, and HDA19; Class II has HDA5, HDA8, HDA14, HDA15, and HDA18; whereas Class IV has HDA2 only (Hollender and Liu, 2008).

In *Arabidopsis*, two of the most studied HDAs are HDA19 and HDA6. HDA6

participates in the ABA responsive pathway and salt stress responses. In the *hda6* mutant, *axe1-5*, germination rates under ABA and salt stress are lower compared to wild type (Chen et al., 2010). Furthermore, the expression of ABA and abiotic responsive genes including *ABA Insensitive 1* (*ABI1*), *ABI2*, *3-Keto-Acyl-coa Thiolase 1* (*KAT1*), *KAT2*, *Dehydration-Responsive Element-Binding Protein 2A* (*DREB2A*), *Responsive to Desiccation 29A* (*RD29A*), and *RD29B* are decreased in the *axe1-5* mutant (Chen et al., 2010). HDA6 also controls leaf development by repressing the expression of *KNOX* genes (Luo et al., 2012). HDA6 interacts with ASYMMETRIC LEAVES 1 (AS1), a MYB-type transcription repressor that controls leaf development. *as1-1/axe1-5* and *as2-1/axe1-5* double mutants show severe serrated leaf and short petiole phenotypes compared to the single mutants. In the *hda6* mutant, *axe1-5*, *KNOX* genes which function in meristem establishment and maintenance and internode growth were up-regulated and hyperacetylated. Both AS1 and HDA6 target to *KNAT1*, *KNAT2*, and *KNATM* and regulate their expression (Ikezaki et al., 2010; Luo et al., 2012).

In *Arabidopsis*, several pathways can control the transition of the vegetative stage to the reproductive stage including the gibberellin, photoperiod, vernalization and autonomous pathways. The photoperiod pathway responds to seasonal changes in day

length, and the vernalization pathway responds to prolonged exposure to cold. The autonomous and gibberellin-pathways are responsible for responding to endogenous signals. In addition, light quality, ambient temperature, biotic stress and abiotic stresses also contribute to floral transition in plants (Jarillo and Pineiro, 2011; Srikanth and Schmid, 2011; Steinbach and Hennig, 2014). HDA6 also plays an important role in flowering time regulation. It has been reported that HDA6 interacts with FLOWERING LOCUS D (FLD) (Yu et al., 2011) and represses the expression of *FLOWERING LOCUS C* (*FLC), MADS AFFECTING FLOWERING 4 (MAF4),* and *MAF5.* In *hda6* and *fld* mutants, the levels of histone H3 acetylation and H3K4 trimethylation at *FLC*, *MAF4*, and *MAF5* are increased. Furthermore, HDA6 can also interact with FVE and represses the expression of *FLC* expression (Gu et al., 2011; Yu et al., 2011).

In this study, we found that TCP4 could interact with HDA6 by using yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. Both *tcp4* and *hda6* mutants showed delayed flowering and serrated leaf phenotypes. The interaction of TCP4 and HDA6 during plant developing processes in *Arabidopsis* was analyzed.

Materials and Methods

Plant materials

Arabidopsis thaliana plants were germinated and grown in 22℃ under long day (LD) (16 h light /8 h dark cycle) or short day (SD) (8 h light /16 h dark cycle) conditions. The loss-of-function mutants, *tcp4-1* and *tcp4-2*, were T-DNA insertion mutants (Sarvepalli and Nath, 2011). The gain-of-function mutant, *35S:mTCP4-CFP*, was obtained by transforming the *35S:mTCP4-102* contructs into Col-0 wild type plants. The *hda6* mutant line, *axe1-5*, is a splicing mutant that has a base change at the third intron splice site (+1636) resulting in two *HDA6* transcripts with altered lengths (Murfett et al., 2001). *as1-1* and *as2-1* mutants are kindly provided by Dr. Jun-Yi Yang (National Chung Hsing University). All of the mutant lines used in this study are listed in Table 1.

Quick DNA extraction

1. Cut one rosette leaf from *Arabidopsis* plants and put into a 1.5 ml tube, and add 400 μl DNA extraction buffer.
2. Use micro-pestle to grind the leaf evenly.

3. Centrifuge at 12,000 g for 5 min. Then, transfer 300 μl supernatant into a new tube.
4. Add 300 μl volume of isopropanol and mix well.
5. Centrifuge at 12,000 g for 5 min.
6. Remove the supernatant and add 200 μl of 75% ethanol to resuspend the DNA pellet.
7. Centrifuge at 12,000 g for 5 min again.
8. Discard 75% ethanol and air dry the DNA pellet. Dissolve the pellet in 40 μl ddH_2O.

RNA isolation

1. Collect about 0.2 g *Arabidopsis* plants and put into a mortar with liquid nitrogen, ground the sample with a pestle.
2. Add 1 ml of TRIzol Reagent (Invitrogen; Catalogue no.15596-018) to dissolve the leaf powder and transfer the mixture into a 1.5 ml tube.
3. Add 200 μl of chloroform per 1 ml of TRIzol Reagent into the extraction mixture, shake the tube vigorously by vortex and incubate for 5 min at room temperature.
4. Centrifuge the tube at 12,000 g (SIGMA 2-16) at 4℃ for 15 min.

5. After centrifugation, the extraction mixture is separated into a lower red phenol-chloroform phase, an inter-phase, and the colorless upper aqueous phase. RNA will remain exclusively in the aqueous phase, and the volume of the aqueous phase should be about 70% of the volume of TRIzol Reagent used for homogenization.
6. Transfer 500 μl of aqueous phase liquid to a new tube, and precipitate RNA from the aqueous phase by added the same volume of the isopropanol at room temperature for 10 min.
7. Centrifuge 12,000 g at 4℃ for 10 min and discard the supernatant, keep the pellet.
8. Use 1 ml of 70% ethanol (DEPC-treated water prepared) to wash the pellet clearly, and centrifuge 7,500 g at 4℃ for 5 min.
9. Remove the supernatant and air-dry the remaining pellet briefly for 10-15 min
10. Finally, dissolve RNA with 35 μl DEPC-treated water and incubate the tube at 55 ℃ for 10 min.

DNase treatment

1. Take 8 μl of RNA sample into the new tube, add 1 μl of 10X DNase buffer and

1 μl of DNase (Promaga; Catalogue no. M6101), then incubate the mix at 37℃ for 30 min to remove the DNA.

2. Add 1 μl stop solution into RNA sample to stop the reaction of DNase treatment, then continue incubate at 65℃ for 10 min.
3. The RNA sample can be stored in a -80℃ freezer, or used directly for the RT-PCR analysis.

Quantitative RT-PCR analysis

1. 2 microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65ºC for 10 min.
2. cDNA was synthesized in a volume of 20 μl that contained 4 μl of M-MLV (Moloney Murine Leukemia Virus) 5X reverse transcriptase buffer (Promega; Catalogue no. M531A), 4 μl of 2.5 mM dNTP (dATP, dCTP, dGTP, dTTP), 3 μl of 10 mM oligo (dT) primer, 25 U RNasin ribonuclease inhibitor (Promega; Catalogue no. N251B), 200 U M-MLV reverse transcriptase (Promega; Catalogue no. M170B) at 37ºC for 1 hr 20 min.
3. cDNAs obtained from reverse-transcription were used as templates to run real-time PCR. The following components were added to a reaction tube: 9 μL of

iQ™ SYBR Green Supermix solution (Catalogue no. 170-8882, Bio-Rad), 1 μL of 5 μM specific primer pairs and 8 μL of the diluted template.

4. Thermo cycling conditions were 95℃ for 3 minutes followed by 40 cycles of 95 ℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 20 seconds, with a melting curve detected at 95℃ for 1 minute, 55℃ for 1 minute and detected the denature time from 55℃ to 95℃.
5. Each sample was quantified at least triplicate and normalized using *Ubiquitin 10* (*UBQ10*) as an internal control. The gene-specific primer pairs for quantitative RT-PCR are listed in Table 3.

Bimolecular Fluorescence Complementation (BiFC) assays

A. Plasmids construction

To generate the constructions for bimolecular fluorescence complementation assays, full length coding sequences of TCP3, TCP4, TCP9, TCP14, TCP20, HDA5, HDA6, HDA9, HDA15 and HDA19 were PCR-amplified. PCR products were subcloned into the pENTR/SD/D- TOPO or pCR8/GW/TOPO vectors and then recombinated to the pEarleyGate201-YN and pEarleyGate202-YC vectors. The constructions were sequenced and confirmed by the Vector NTI Suite

program (InforMax Inc., Bethesda, MD, USA).

B. Protoplast isolation

1. 5 mL fresh enzyme solution was prepared with heat treatment at 55℃ to promote the dissolution.

Enzyme solution

Reagent	**Amount**	**Final concentration**
Cellulase R10[a]	0.15 g	1%
Macerozyme R10[b]	0.03 g	0.25%
0.8 M Mannitol	5 ml	0.4 M
0.2 M KCl	1 ml	20 mM
0.1 M MES[c]	2 ml	20 mM
ddH_2O	to 10 ml	

[a]Cellulase R10 (ONOZUKA; Cat# 28302), [b] Macerozyme R10 (SERVA; Cat# 12710), and [c] MES: Monohydrate (J.T. Baker; Cat# 4014-00).

2. After cooling the enzyme solution to room temperature, 50 μl of 1 M $CaCl_2$ and 50 μl of 10% BSA were added into the 5 mL of enzyme solution.

3. The Arabidopsis rosette leaves were cut into a small pieces soaking in the enzyme solution.

4. The digestion was carried for 5 hours in light with gently shacking at 50 rpm.

5. The enzyme solution was transferred into a 5 mL tube and centrifuged at 100 g at

room temperature for 3 min.

6. The supernatant was removed, and protoplasts were washed by 4 mL of W5 buffer twice with centrifuge at 100 g at room temperature for 1 min.

W5 buffer

Reagent	Amount	Final concentration
3M NaCl	10.3 ml	154 mM
1M $CaCl_2$	25 ml	125 mM
0.2 M KCl	5 ml	5 mM
0.1 M MES	4 ml	2 mM
0.1 M glucose	10 ml	5 mM
ddH_2O	to 200 ml	

7. The protoplasts were resuspended in another 2~3 mL of W5 buffer and kept on ice for 30 min.

C. Protoplast PEG transfection

1. The W5 buffer was removed by centrifugation at 100 g at room temperature for 1 min, and the protoplasts were resuspended in an equal volume of MMg solution.

MMg solution

Reagent	Amount	Final concentration
0.8 M Mannitol	5 ml	0.4 M
1M $MgCl_2$	0.15 ml	15 mM
0.1 M MES	0.4 ml	4 mM
ddH_2O	to 10 ml	

2. For 200 μl of protoplasts (~$4x10^5$), 20 μl of plasmid DNA (10~20 μg) was added in a 5 mL tube and mixed well.

3. 220 μl of PEG/Ca (final PEG concentration=20%) solution was added in the mixture.

PEG/Ca solution

Reagent	**Amount**	**Final concentration**
PEG4000[a]	2 g	40%
0.8 M Mannitol	1.25 ml	0.2 M
1M $CaCl_2$	0.5 ml	0.1 M
ddH_2O	to 5 ml	

[a] PEG: polyethylene glycol 4000 (Fluka; Cat# 81240)

4. The tube was incubated at room temperature for 5 min.

5. The protoplasts were diluted with 440 μl of W5 buffer and centrifuged at 100 g at room temperature for 2 min to remove the PEG solution.

6. The protoplasts were resuspended in another 100 μl of W5 buffer and incubated under light for 16-24 hours.

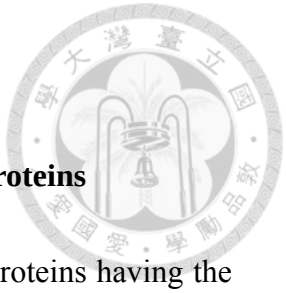
7. Transfected cells were imaged using TCS SP5 (Leica) Confocal Spectral Microscope Imaging System.

Yeast two-hybrid assays

Yeast two hybrid assays were performed according to the instruction for the Matchmaker GAL4-based two hybrid system 3 (Clontech).

1. The gene coding sequences of *TCP4*, *HDA5* and *HDA6* were subcloned into pGBKT7 and pGADT7 vectors.
2. AD and BD constructs were transformed into yeast strain AH109 by the lithium acetate method and yeast cells were grown on a minimal medium/-Leu-Trp-His according to the manufacturer's instructions (Clonthech).
3. Transformed colonies were plated onto a minimal medium/-Leu-Trp-His for three generation to test for possible interactions between TCP4 and HDA5 and HDA6.
4. Positive clones were PCR-amplified with pAD-3' and pAD5' primer pairs and sequenced by using the AD- 3' sequencing primer.

Results



Phylogenic analysis and domain structure comparison of TCP proteins

The TCP gene family is a small group of plant genes encoding proteins having the TCP domain, a 59-amino acid basic helix–loop–helix (bHLH) motif that allows DNA binding and protein–protein interactions (Kosugi, S. and Ohashi, Y. 1997; Cubas, P. et al 1999). Based on the differences of their TCP domains, TCP proteins can be distinguished into two types: Class I and Class II. Class II can be further subdivided into two clades: the CIN clade and the CYC/TB1 clade (Navaud. et al., 2007; Cubas, 2002) (Figure 1).

TCP4 belongs to the Class II CIN clade and shares highest sequence similarity with TCP3. Besides the TCP domain, both of *TCP3* and *TCP4* contain a miR319 target site, which can be recognized and down-regulated by miR319 in *Arabidopsis* (Figure 2). In addition, *TCP2*, *TCP10* and *TCP24* can also be regulated by miR319 (Palatnik et al., 2003).

TCPs can interact with HDAs

We further analyzed whether TCPs can interact with RPD3-type HDAs. BiFC assays showed that the Class I TCPs, TCP9, TCP14 and TCP20 interacted with HDA5

in *Arabidopsis* protoplasts. In addition, TCP14 also interacted with HDA6, HDA15 and HDA19, but TCP20 interacted with HDA15 (Figure 3A, 3B and 3C). The class II TCPs, TCP3 and TCP4 interacted with HDA5 and HDA6 (Figure 4A and 4B). In addition, TCP3 and TCP4 also interacted with HDA19 and HDA9, respectively (Figure 4A and 4B). The interaction of TCPs with HDAs was summarized in the Table 4.

Yeast-two-hybrid assays were also used to further confirm the interaction of TCP4 with HDA5 and HDA6. As shown in Figure 5, TCP4 interacted with HDA6 in yeast cells. To identify the interaction domains of TCP4 responsible for the interaction with HDA6, we divided TCP4 into two parts: the N terminal part which contains the TCP domain and the C terminal part. Results show that HDA6 can interact with the C terminal part of TCP4, suggesting that their interaction is independent with the DNA binding activity of TCP4 (Figure 5).

The expression of *TCP4* in different tissues of *Arabidopsis*

To understand the function of TCP4, we used eFP Browser to analyze the expression pattern of *TCP4*. *TCP4* is highly expressed in leaves and flowers, but lowly expressed in seeds and shoot apices (Figure 6A). We also used qRT-PCR to test

the expression of *TCP4*. The high level of *TCP4* transcript was detected in rosette leaves, cauline leaves and flowers (Figure 6B).

Identification of T-DNA insertion mutants of *TCP4*

Two T-DNA insertion mutant lines, *tcp4-1* and *tcp4-2* (Sarvepalli and Nath, 2011), were used to study the function of TCP4. The T-DNA insertions of *tcp4-1* and *tcp4-2* are both located at the exon of *TCP4* (Figure 7A). Homozygous *tcp4-1* and *tcp4-2* were screened by PCR analysis (Figure 7B). In both *tcp4-1* and *tcp4-2* mutants, the full length *TCP4* transcript could not be detected, suggesting that they are knockout mutants (Figure 7C).

***tcp4* mutants show delayed flowering under long day conditions**

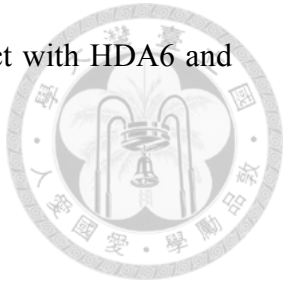
Under LD conditions, the bolting time was delayed and the rosette leaf number was increased of in *tcp4-1* and *tcp4-2* mutants compare with Col wild type (Figure 8A and 8B). Under SD conditions, although the bolting time of *tcp4-1* and *tcp4-2* were slightly delayed, there was no significant difference in the rosette leaf number among the mutants and Col (Figure 8C and 8D).

Delayed flowering time of *tcp4-1*and *tcp4-2* under LD conditions suggests the participation of TCP4 in the photoperiod pathway. qRT-PCR analyses were carried out to detect the expression of *COSTANT (CO)* and *GIGANTEA (GI).* The expression of both *CO* and *GI* has no significant difference between wild type and *tcp4* mutants (Figure 9).

The *tcp4 hda6* double mutant shows a severe late-flowering phenotype

HDA6 promotes flowering in *Arabidopsis* by repressing *FLC, MAF4* and *MAF5* (Yu et al., 2008). We generated *tcp4-2/axe1-5* double mutants by crossing *tcp4-2* with *axe1-5*. The *tcp4-2/axe1-5* double mutant displayed an enhanced delayed-flowering phenotype compared with the single mutants (Figure 10A-B). The expression of *FLC*, *MAF4* and *MAF5*, in wild type, *tcp4-1*, *tcp4-2*, *axe1-5* and *tcp4-2/axe1-5* was detected. Compared to the wild type, the expression of *FLC*, *MAF4* and *MAF5* were slightly increased in *tcp4* and *axe1-5* mutants. In the *tcp4-2/axe1-5* double mutant, the expression of *FLC*, *MAF4* and *MAF5* were also increased compared to the single mutants. The expression of the downstream gene *FT* was decreased *tcp4*, *axe1-5* and their double mutants, which is consisting of the delayed flowering phenotype of those

mutants (Figure 11). These results suggested that TCP4 can interact with HDA6 and co-regulate the flowering time by repress *FLC*, *MAF4*, *MAF5*.



The leaf phenotype of the *tcp4* and *hda6* double mutant

In addition to the delayed flowering phenotype, *tcp4* mutants also show a curling leaf phenotype. The *tcp4-1* and *tcp4-2* seedlings displayed downward curling cotyledons at the seedling stage, and the *tcp4-1/axe1-5* and *tcp4-2/axe1-5* double mutants showed severe downward curling cotyledons (Figure 12A and12B). In the vegetative stage, *tcp4-1* and *tcp4-2* displayed a serration and curling leaf phenotype, and *tcp4-1/axe1-5*, *tcp4-2/axe1-5* had an enhanced severe serrated leaf phenotype (Figure 12C).

Previously, it was found that HDA6 can interact with ASYMMETRIC LEAVES 1 (AS1) *in vivo* and *in vitro*, and HDA6 is a part of the AS1-AS2 repressor complex to regulate the *KNOX* expression by binding directly to the chromatin of *KNAT1*, *KNAT2*, and *KNATM* in leaf development (Luo et al., 2012). The expression of *KNAT1*, *KNAT2*, and *KNATM* were analyzed in *tcp4, axe1-5* and t*cp4-2/axe1-5* double mutants (Figure 13). In both *axe1-5* and *tcp4-2/axe1-5* double mutants, the expression of *KNAT1* and *KNATM* were increased. Besides, the expression of *KNATM* in *tcp4*

mutants was also increased. It remains to be determined whether TCP4 is a part of the AS1-HDA6 repressor complex involved in leaf development.

BiFC assays revealed that TCP4 interacted with AS1 and AS2, which can form a complex with HDA6 in *Arabidopsis* (Figure 14). To investigate the genetic interaction of TCP4 with AS1, AS2 and HDA6, we generated the triple mutants, *tcp4-1/as1-1/axe1-5*, *tcp4-2/as1-1/axe1-5*, *tcp4-1/as2-1/axe1-5* and *tcp4-2/as2-1/axe1-5*. Compared with the single and double mutants, the *tcp4/as1-1/axe1-5* and *tcp4/as2-1/axe1-5* triple mutants displayed smaller plant sizes, shorter petioles and enhanced severe phenotypes in leaf serration and curing (Figure 15A-D).

The expression of *KNAT1* was increased in *as1-1* and *axe1-5* single mutants, *tcp4/axe1-5, as1-1/axe1-5* and *as2-1/axe1-5* double mutants, and their triple mutants compared with Col wild type. In addition, the *KNAT1* transcript level of *as1-1* plants was higher than that of *as2-1* and *axe1-5* plants, whereas *as1-1/axe1-5* and *tcp4/as1-1/axe1-5* plants displayed similar level of *KNAT1* expression compare with the *as1-1* single mutant. The *KNAT1* expression level in *tcp4/axe1-5* and *as2-1/axe1-5* was similar but highest in *tcp4/as2-1/axe1-5* (Figure 17A).

Compared with Col wild type, the expression of *KNATM* was increased in all the single, double and triple mutants (Figure 17). Besides, the expression level of *KNATM*

in *tcp4/axe1-5*, *as1-1/axe1-5* and *as2-1/axe1-5* double mutants were higher compared to *tcp4*, *as1-1*, *as2-1* and *axe1-5* single mutants. Furthermore, the *KNATM* expression in *tcp4/as1-1/axe1-5* and *tcp4/as2-1/axe1-5* triple mutants was higher compared to the double mutants, which are consistent with their leaf phenotypes.

A previous study indicated that TCP3 could down-regulate the expression of *AS1* and *AS2* (Koyama et al., 2010). We detected the expression of *AS1* and *AS2* in *tcp4*, *axe1-5* and *tcp4/axe1-5* double mutants. The *AS1* and *AS2* transcripts were increased in the *tcp4* and *axe1-5* single and double mutants compare with Col wild type (Figure 18).

35S::mTCP4-CFP plants display short and aberrant siliques

We also generated transgenic *Arabidopsis* (*35S::mTCP4-CFP*) overexpressing an *miR319*-resistant version of *TCP4* (*mTCP4*) fussed with *CFP* driven by the cauliflower mosaic virus 35S promoter. *35S::mTCP4-CFP* plants displayed short silique (Figure 19A-B), aborted seed (Figure 19C) and aberrant silique (Figure 19D) phenotypes.

Discussion

The TCP protein family can be divided into two classes: class I and class II. Based on the difference in the TCP domain, the class II TCPs can be subdivided into two clades: CIN clade and CYC/TB1 clade (Kosugi and Ohashi, 1997). Through the TCP domain, a 59-amino acid basic helix–loop–helix (bHLH) motif, TCP proteins can interact with other proteins and bind to DNA (Kosugi and Ohashi, 1997; Cubas et al., 1999). The five CIN clade members, *TCP2, TCP3, TCP4, TCP10* and *TCP24,* all have the target site that can be recognized and down-regulated by the *miR319* in *Arabidopsis* (Palatnik et al., 2003). Furthermore, CIN-TCPs can forms tertiary complexes with TPL/TPRs which are known transcriptional corepressors with HDA19 to regulate leaf development (Tao et al., 2013). In our study, we found that TCP4 interacted with several RPD3-type HDAs in *Arabidopsis* protoplasts, including HDA5, HDA6 and HDA9, suggesting that they may form a protein complex to regulate the gene expression.

The *jawD* mutant is a *miR319* overexpression line in which *TCP2, TCP3, TCP4, TCP10* and *TCP24* are all downregulated (Palatnik et al., 2003). This mutant shows a crinkled and serrated leaf phenotype with an altered senescence behavior which can be rescued by treating with jasmonate. In the *jawD* mutant, the expression of *LOX2* is

decreased and the level of JA is reduced (Schommer et al., 2008). In this study, we found that *tcp4* mutants and *jawD* show delayed flowering under LD conditions (Figure 8A-D, Sup 1). Delayed flowering time of *tcp4-1*, *tcp4-2* under LD but not SD conditions suggests the participation of TCP4 in the photoperiod pathway. It was reported that TCP4 represses cell proliferation by directly activating the expression of *mir396b* and *ICK1/KRP1*, a core gene involved in the progression of the cell cycle (Inze and De Veylder, 2006; Gutierrez, 2009 Schommer et al., 2014). The delayed bolting time in LD might due to the influence of TCP4 in cell proliferation.

The *hda6* mutant *axe1-5* also shows delayed flowering phenotype. *tcp4-2/axe1-5* double mutants displayed a more severe delayed-flowering phenotype compared with the *tcp4-2* and *axe1-5* single mutants (Figure 10A-B). *FLC* is a key regulator in flowering time control and acts in the autonomous pathway by repressing *FT* and *SOC1* (Kobayashi et al., 1999; Lee et al., 2000). *MAF4* and *MAF5* are *FLC* homologs and are known to be involved in flowering (Ratcliffe et al., 2001; Ratcliffe et al., 2003). The expression of *FLC*, *MAF4* and *MAF5* were increased in *tcp4, axe1-5* and *tcp4/axe1-5* double mutants, while the expression of *FT* was decreased in these mutants (Figure 11). These results suggested that TCP4 can interact with HDA6 and they may co-regulate the flowering time by repressing *FLC*, *MAF4*, and *MAF5*.

TCP proteins have been characterized as regulators of leaf development by tuning cell division, differentiation, and expansion (Nath et al., 2003; Kuchen et al., 2012). AS1, a MYB-like protein, induces cell differentiation and represses the expression of *KNOX* genes by forming a complex with AS2 (Byrne et al., 2000; Semiarti et al., 2001; Guo et al., 2008). TCP3, a CIN-like TCP in *Arabidopsis*, regulates the expression of *KNOX* genes by interacting with AS2 (Li et al., 2012). It was reported that HDA6 interacts with AS1 and represses the expression of *KNAT1, KNAT2* and *KNATM* by directly binding to their chromatin (Luo et al., 2012). Here we found that *tcp4* and *axe1-5* mutants also showed the serrated and curling leaf phenotype (Figure 11-12). The expression of *KNAT1* and *KNATM* was increased in *tcp4* mutants (Figure 13), indicating that TCP4 could regulate these genes. TCP4 also could interact with AS1 and AS2 in *Arabidopsis* protoplasts (Figure 14). The *tcp4-1/as1-1/axe1-5, tcp4-2/as1-1/axe1-5, tcp4-1/as2-1/axe1-5* and *tcp4-2/as2-1/axe1-5* triple mutants displayed smaller plant sizes, shorter petioles and enhanced leaf serration compared to the single and double mutants (Figure 15). The expression of *KNATM* was also increased in the triple mutants. Furthermore, TCP4 interacts with AS1, AS2 and HDA6, indicating the participation of TCP4 in the AS1-HDA6 repressor complex during the regulation of *KNATM* (Figure 17B). TCP3 interacts with AS2 to repress the

expression of *KNOX* genes (Li et al., 2012). In addition, TCP3 also induces the expression of *AS1* and *AS2* (Koyama et al., 2010). We also analyzed the expression of *AS1* and *AS2* in *tcp4* mutants. Surprisingly, the expression of *AS1* and *AS2* are increased in *tcp4*, *axe1-5* and *tcp4/axe1-5* double mutants (Figure 18). It remains to be determined whether TCP4 regulates the expression of *AS1* and *AS2* directly or indirectly.

In summary, our study showed that TCP4 can interact with HDA6 and is involved in flowering time control by regulating the expression of *FLC, MAF4* and *MAF5*. Furthermore, TCP4 also interacts with AS1 and AS2 involved in leaf development by controlling the transcript level of *KNAT1* and *KNATM*.

Tables

Table 1. Mutant lines used in this study

Gene	AGI[#]	Mutation line	Mutation	Mutation site	Reference
HDA6	*AT5G63110*	*axe1-5*	Point mutation	+1636G→A	Murfett et al., 2001
TCP4	*AT3G15030*	*tcp4-1*	T-DNA insertion	Exon	Sarvepalli and Nath, 2011
TCP4	*AT3G15030*	*tcp4-2*	T-DNA insertion	Exon	Sarvepalli and Nath, 2011
AS1	*AT2G37630*	*as1-1*	X-ray (CS3374)	unknown	Yang et al., 2008
AS2	*AT1G65620*	*as2-1*	X-ray (CS3117)	unknown	Yang et al., 2008

Table 2. Primers used for T-DNA lines screening

Primer Name	Primer Sequence (5'~3')
LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
TCP4-T-F	ATGTCTGACGACCAATTCCATC
TCP4-T-R	ATGGCGAGAAATAGAGGAAGCAG

Table 3. Primers used for RT-PCR

Primer Name	Primer Sequence (5'~3')
TCP4-RT-F	CATCTCCACCGACGATCTCAAC
TCP4-RT-R	GAAGCAGAGGACGGCTTGTGAG
KNAT1-RT1	GGGAAGAGTGACAATATGGG
KNAT1-RT2	TATGGACCGAGACGATAAGG
KNAT2-RT1	TCATCTGACGAGGAACTGAG
KNAT2-RT2	CGTCCATCATATCAAACGGC
KNOT-pr1	AAGAGAATCTCAAGCCACCC
KNOT-pr2	GTTCTGAAGAGGTAGCTTCG
CO-pr1	ACCAGTGCCATAACCAACAG
CO-pr2	GGAACAATCCCATATCCTGTG
GI-pr1	CTGAGTCACTGGTGATTCTC
GI-pr2	GTGTCGAGAAACTGAGTAGG
FT-RT-1	CCCTCTTATAGTAAGCAGAG
FT-RT-2	CTAAAGTCTTCTTCCTCCGC
SOC1-RT-1	AGGATCGAGTCAGCACCAAA
SOC1-RT-2	GGTAACCCAATGAACAATTGC
FLC RT1	TTAGTATCTCCGGCGACTTGAACCCA
FLC RT2	AGATTCTCAACAAGCTTCAACATGAG
MAF4 RT1	ATTAGGTCAGAAGAATTAGTCGGAGAAAAC
MAF4 RT2	CTTGGATGACTTTTCCGTAGCAGGGGGAAG
MAF5 RT1	GGGGATTAGATGTGTCGGAAGAGTGAAG
MAF5 RT2	GATCCTGTCTTCCAAGGTAACACAAAGG

Table 4. Interaction of TCPs with HDAs in BiFC assays

		Class I			**Class II**	
		TCP9	TCP14	TCP20	TCP3	TCP4
Class I	HDA6		√		√	√
	HDA9					√
	HDA19		√		√	
Class II	HDA5	√	√	√	√	√
	HDA15		√	√		

Figures

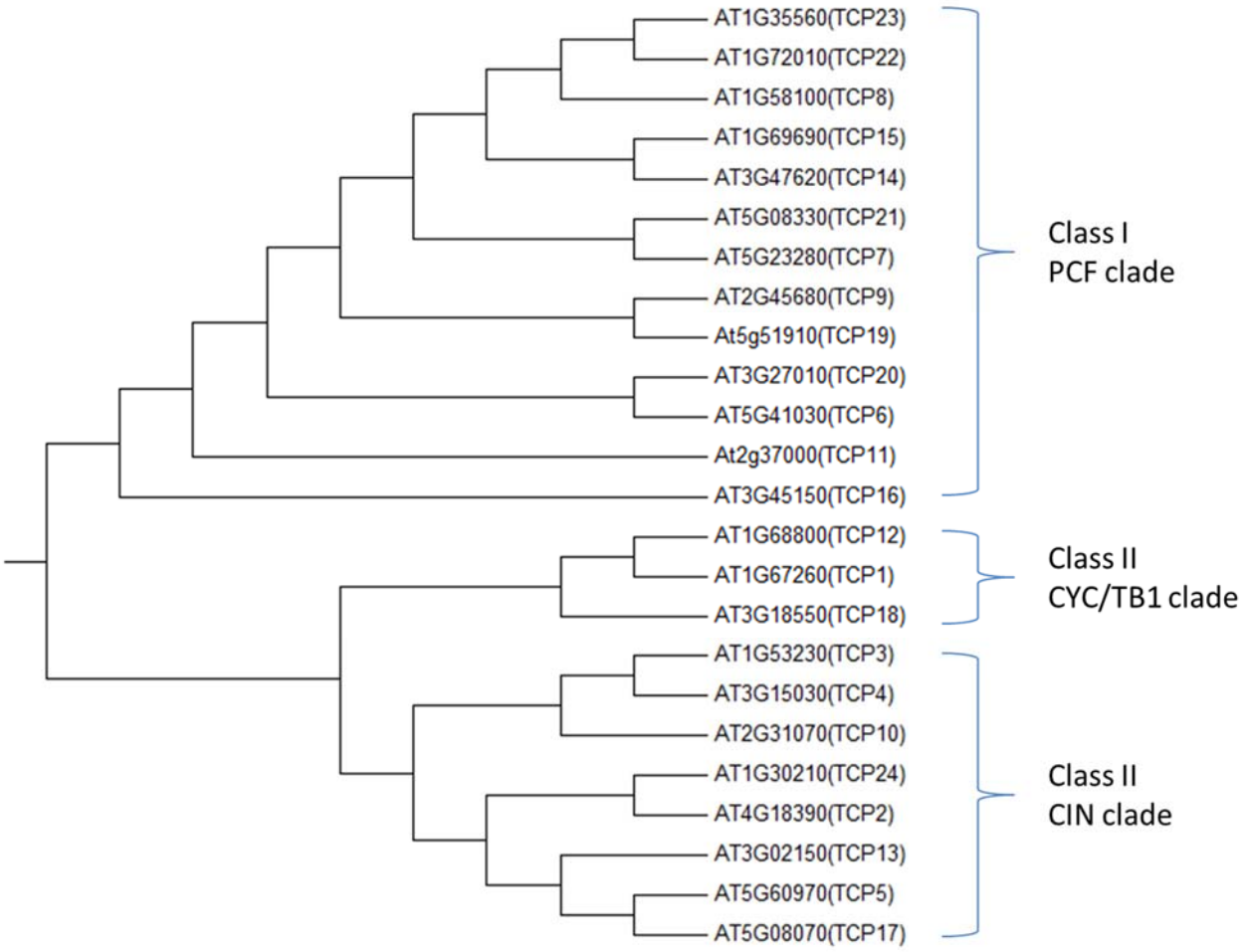
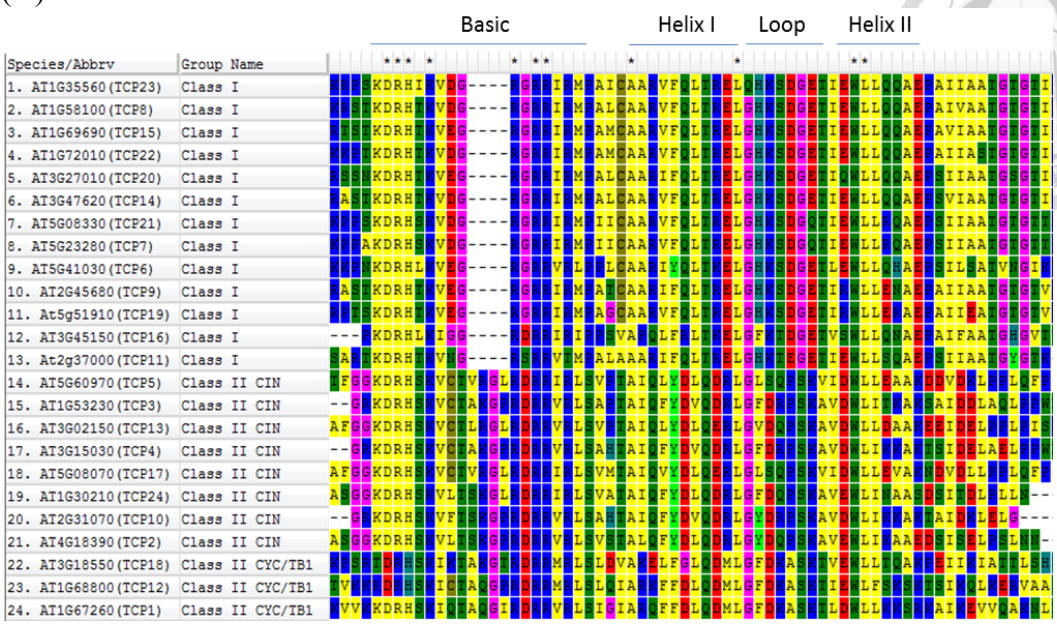


Figure 1. Phylogenic tree of TCP transcription factors. The phylogenic tree was drawn by MEGA6, a tool can be used to compare two or more sequence similarity by DNA or amino acid sequence.

(A)



(B)

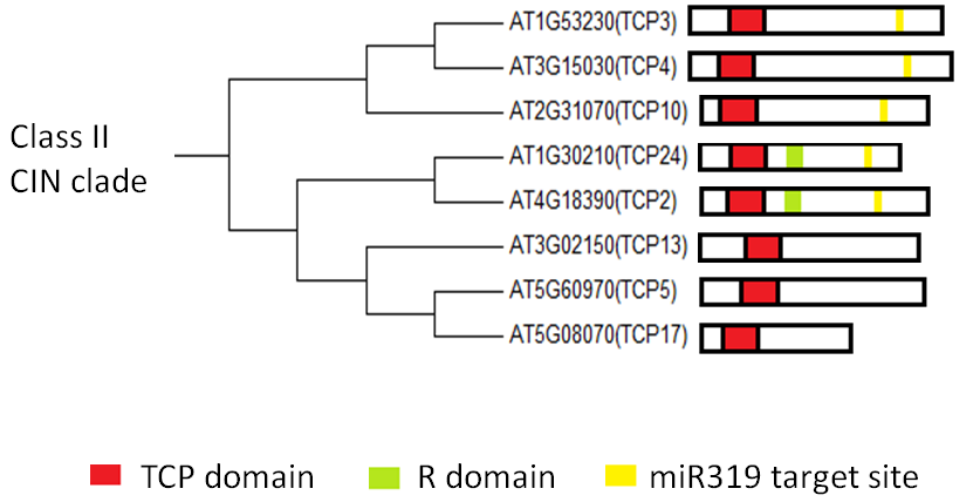
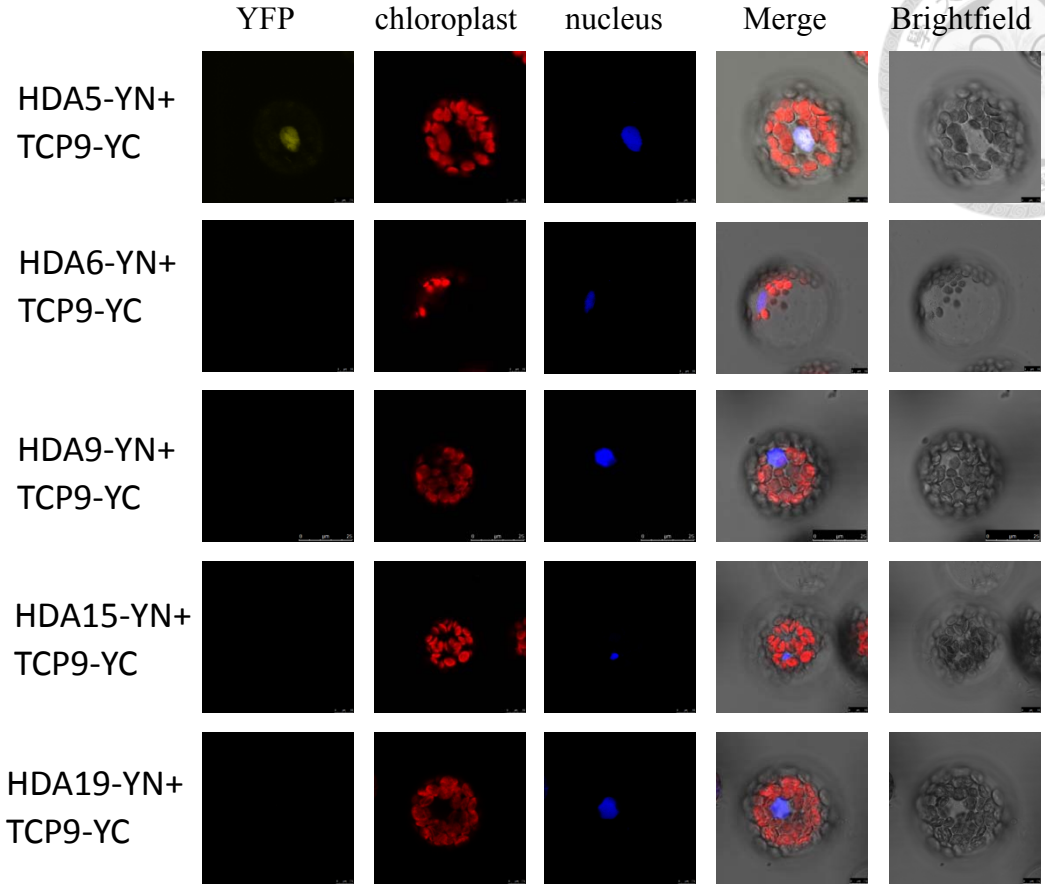


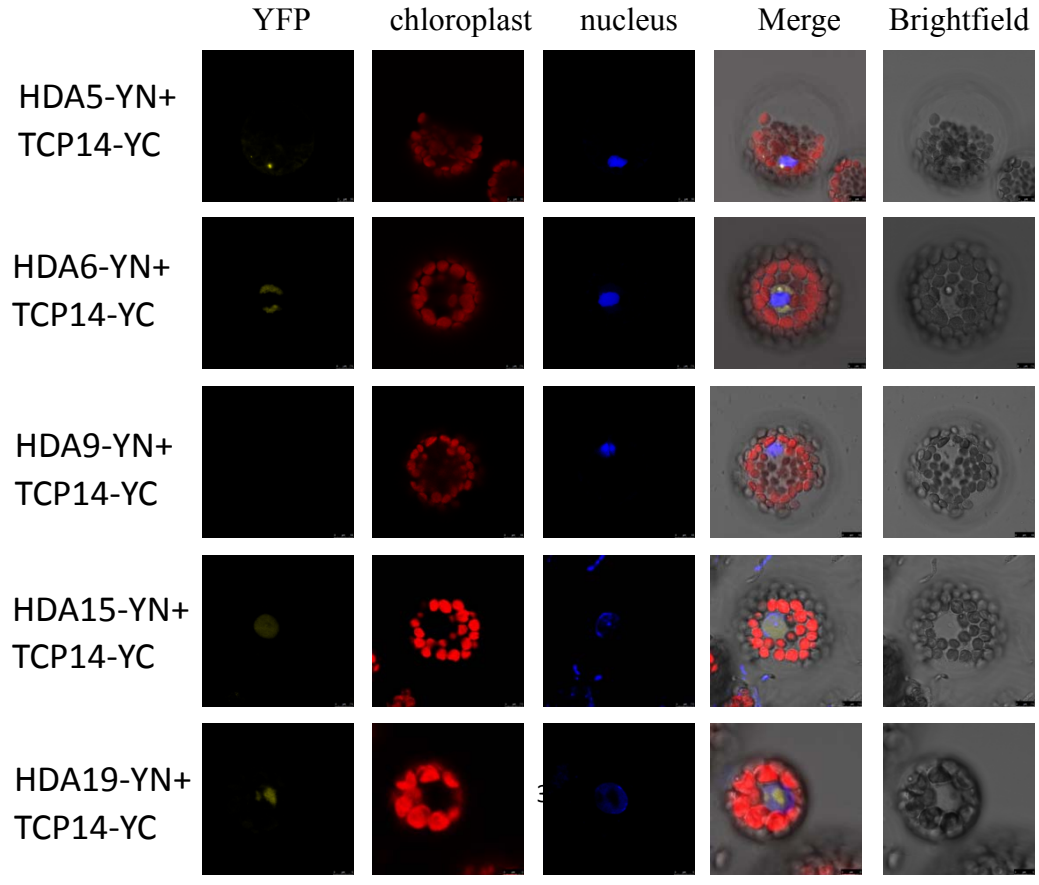
Figure 2. Alignment of the TCP domain of TCP transcription factors.

(A) Conserve basic helix–loop–helix (bHLH) motif of TCP transcription factors. The difference between class I and class II proteins is a four-amino acid deletion in the TCP domain (draw by MEGA6). (B) Domain structure of Class II CIN clade TCP transcription factors.

(A)


YFP
chloroplast
nucleus
Merge
Brightfield
HDA5-YN+ TCP9-YC
HDA6-YN+ TCP9-YC
HDA9-YN+ TCP9-YC
HDA15-YN+ TCP9-YC
HDA19-YN+ TCP9-YC

(B)


YFP
chloroplast
nucleus
Merge
Brightfield
HDA5-YN+ TCP14-YC
HDA6-YN+ TCP14-YC
HDA9-YN+ TCP14-YC
HDA15-YN+ TCP14-YC
HDA19-YN+ TCP14-YC

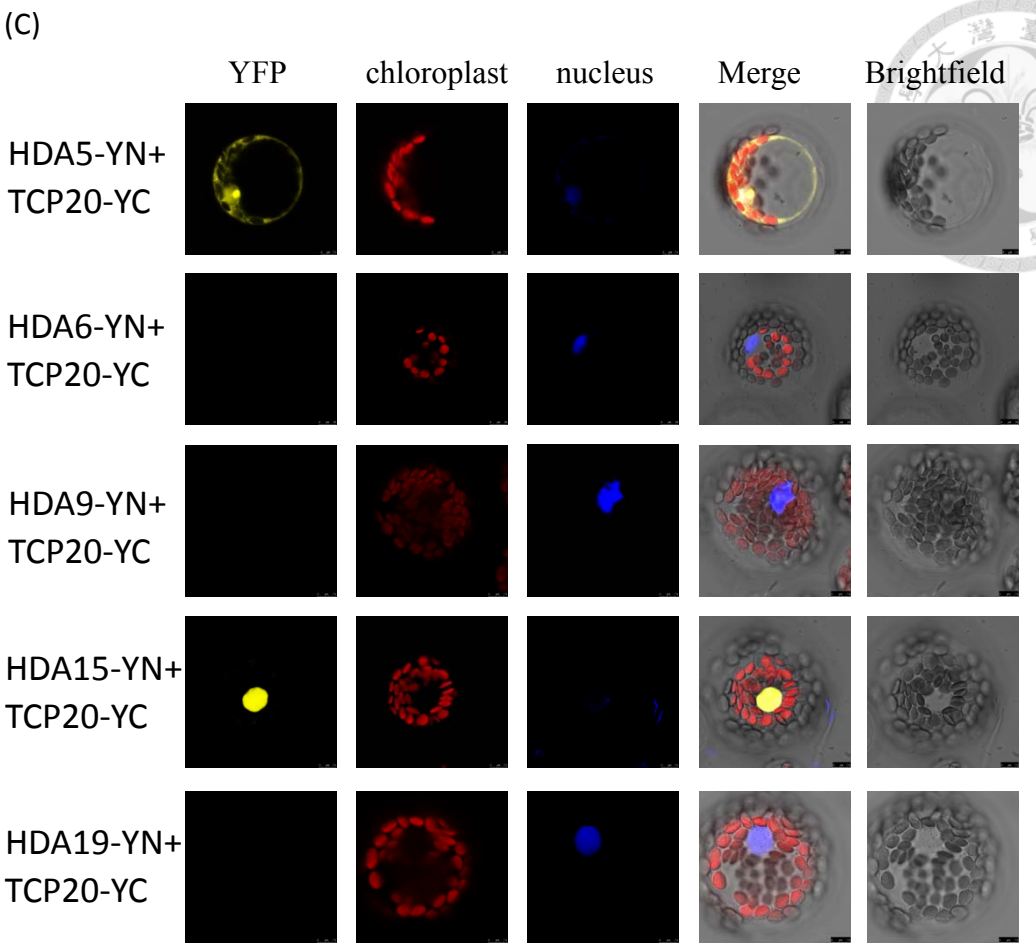
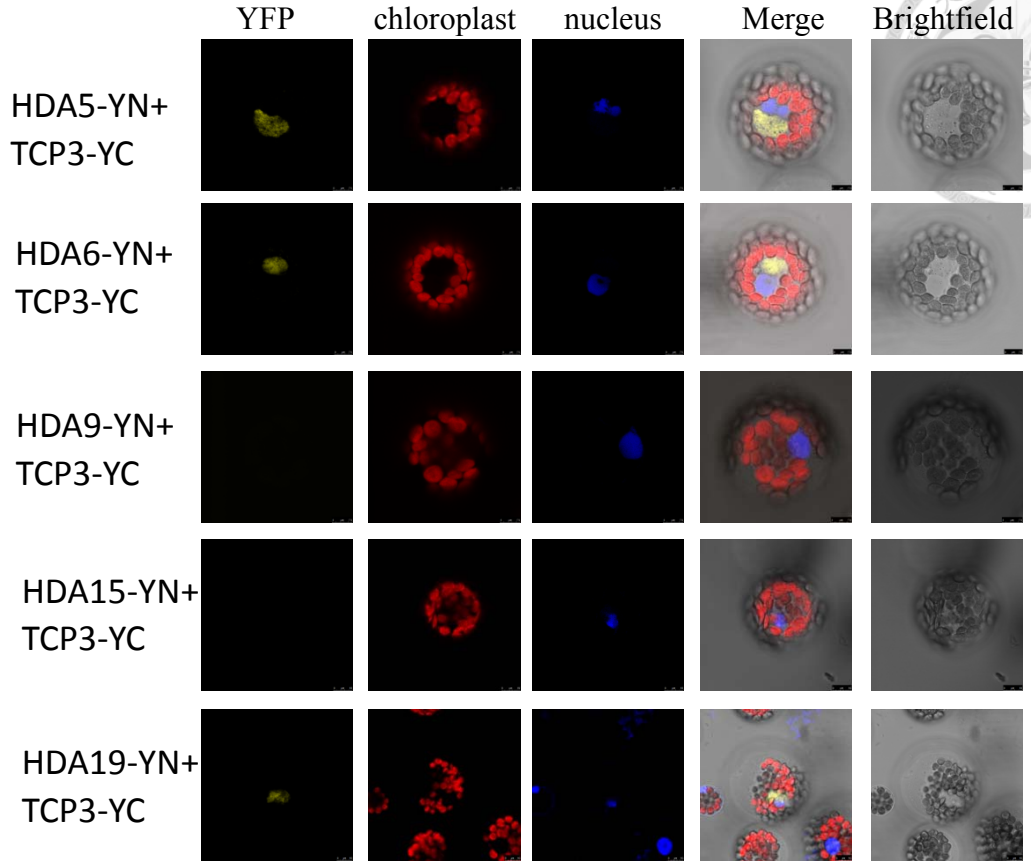


Figure 3. BiFC assays of HDACs interacting with class I TCPs in *Arabidopsis* protoplasts. HDAs (HDA5, HDA6, HDA9, HDA15 and HDA19) fused with the N terminus (YN) of YFP and TCPs (TCP9, TCP14 and TCP20) fused with the C terminus (YC) of YFP were cotransfected into *Arabidopsis* protoplasts and visualized using confocal microscope.

(A)


YFP
chloroplast
nucleus
Merge
Brightfield
HDA5-YN+
TCP3-YC
HDA6-YN+
TCP3-YC
HDA9-YN+
TCP3-YC
HDA15-YN+
TCP3-YC
HDA19-YN+
TCP3-YC

(B)

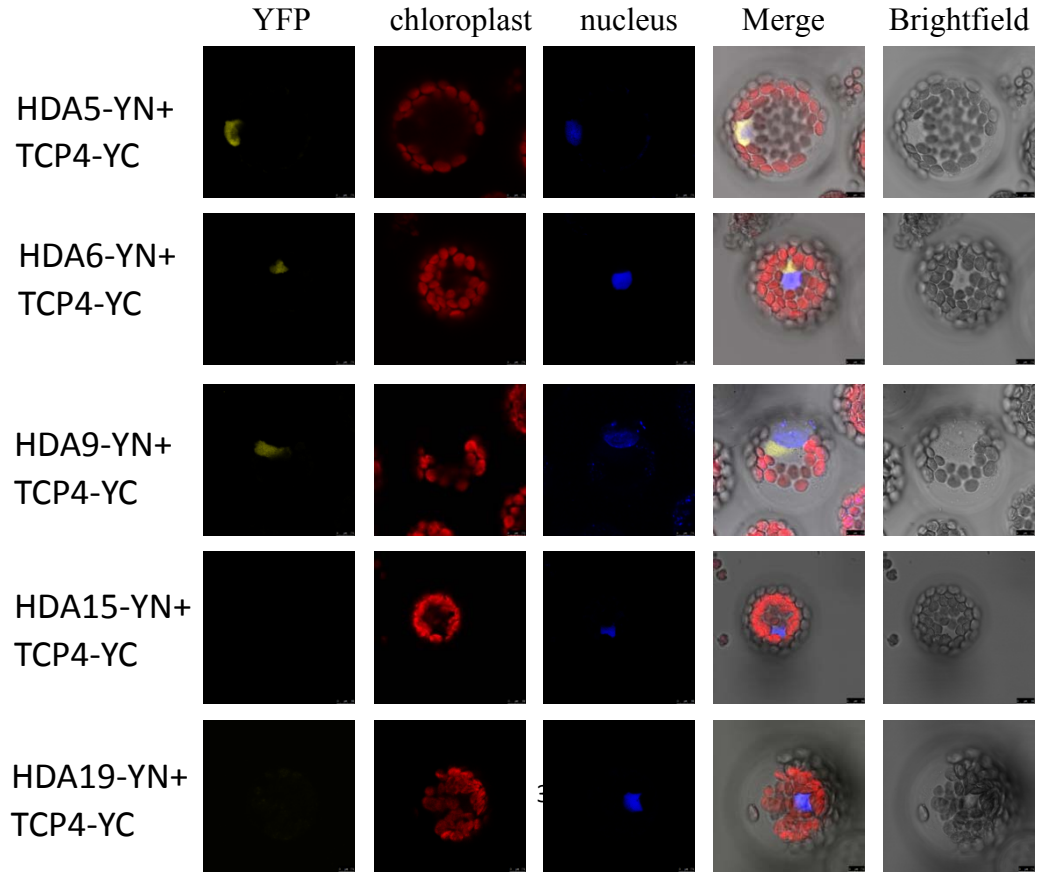

YFP
chloroplast
nucleus
Merge
Brightfield
HDA5-YN+
TCP4-YC
HDA6-YN+
TCP4-YC
HDA9-YN+
TCP4-YC
HDA15-YN+
TCP4-YC
HDA19-YN+
TCP4-YC

Figure 4. BiFC assays of HDACs interacting with class II TCPs in *Arabidopsis* protoplasts. HDAs (HDA5, HDA6, HDA9, HDA15 and HDA19) fused with the N terminus (YN) of YFP and TCPs (TCP3 and TCP4) fused with the C terminus (YC) of YFP were co-transfected into *Arabidopsis* protoplasts and visualized using confocal microscope.

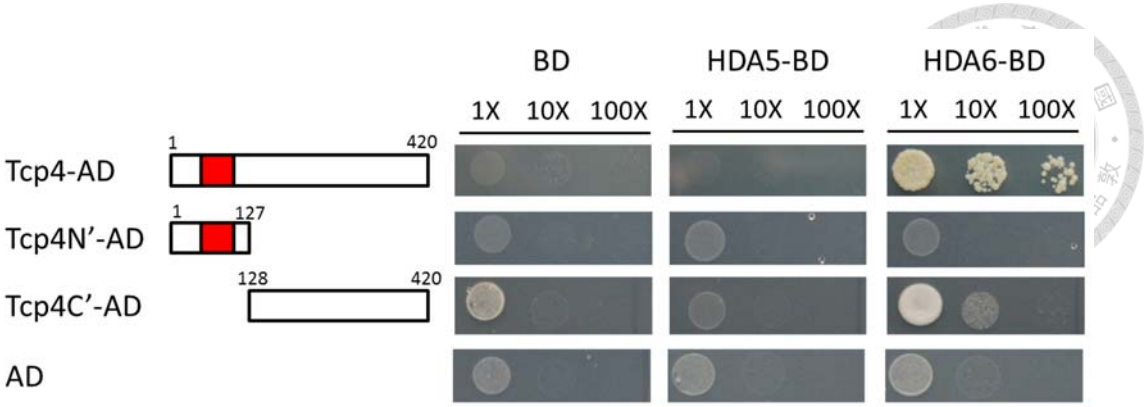
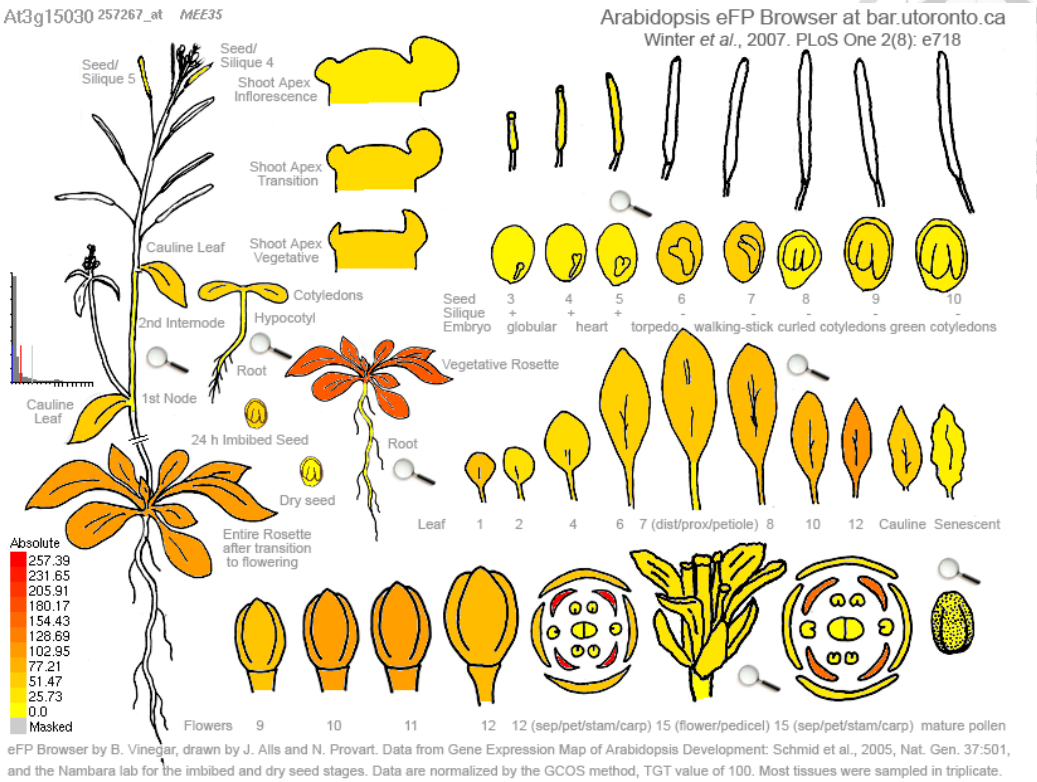


Figure 5. The interaction of TCP4 with HDA5 and HDA6 in yeast two-hybrid assays. Full-length and deletions of TCP4 fused with the Gal4 activation domain (AD) and full-length HDA5 and HDA6 fused with the Gal4 binding domain (BD) were co-transformed into the yeast strain AH109. Yeast cells were grown under the –Leu/-Trp/-His plate. To prevent self-activation, 5 mM 3-AT (3-Amino-1,2,4-triazole) was added to the amino acid deficient plate. Red box represents the TCP domain.

(A)



(B)

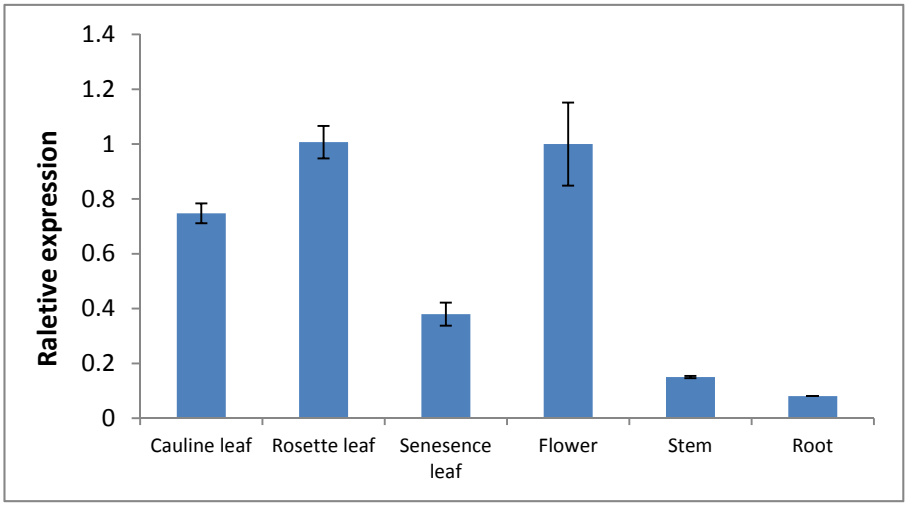


Figure 6. Analysis of the *TCP4* expression in different tissues of *Arabidopsis*. (A) The gene expression map of *TCP4* during different development stages in *Arabidopsis* (predicted by eFP Browser). *TCP4* was expressed at a high level

in rosette leaves and flowers. (B) The expression of *TCP4* in different tissues of *Arabidopsis* was analyzed by qRT-PCR. The expression of *TCP4* was normalized using *UBQ10* as an internal control.

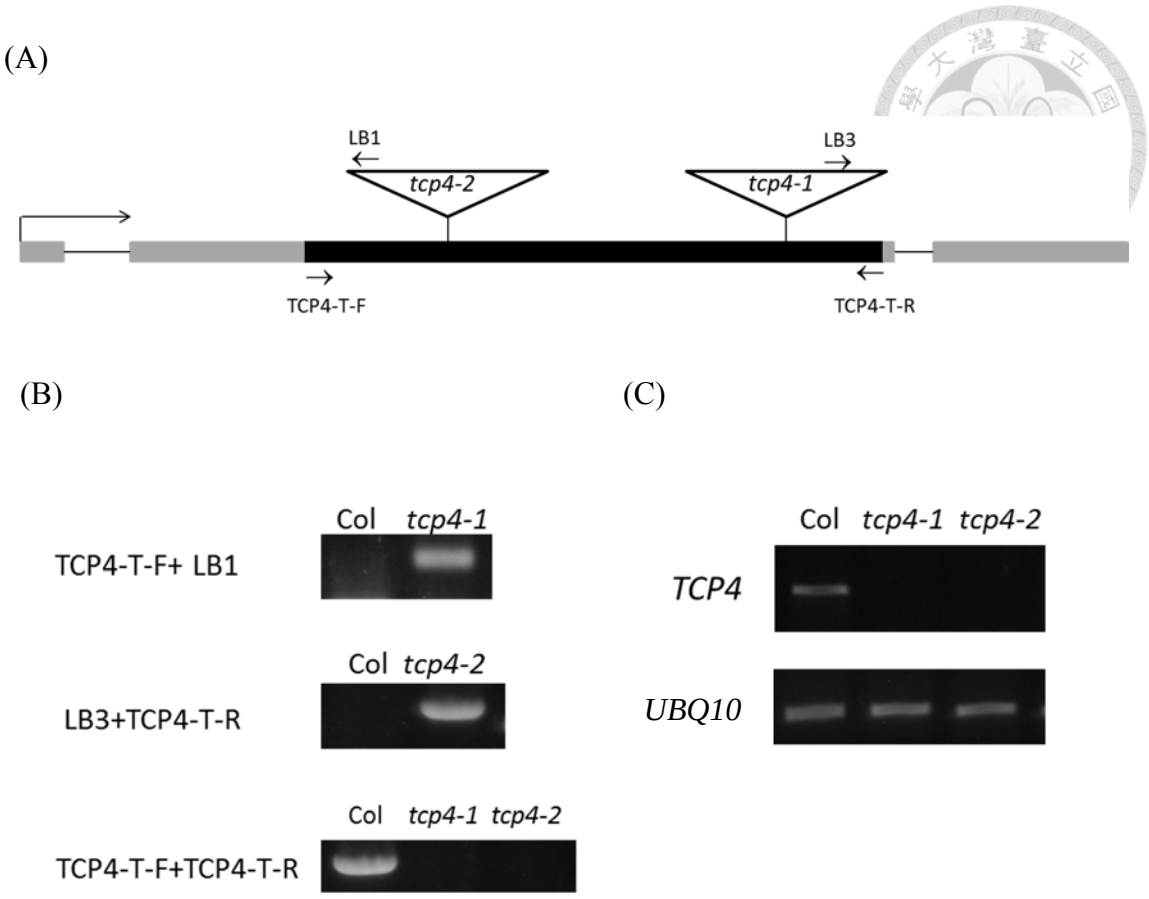
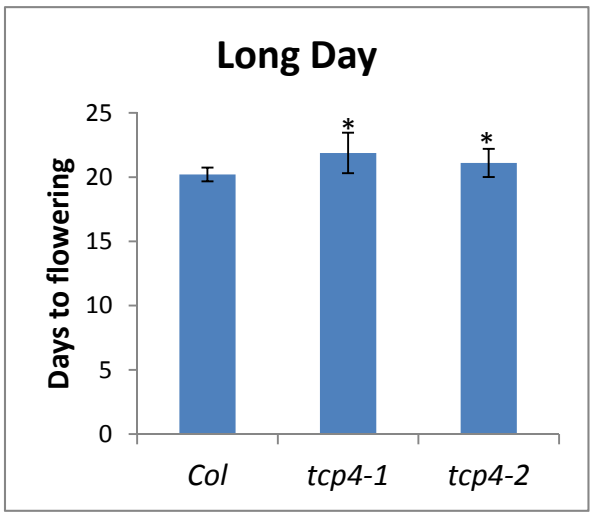


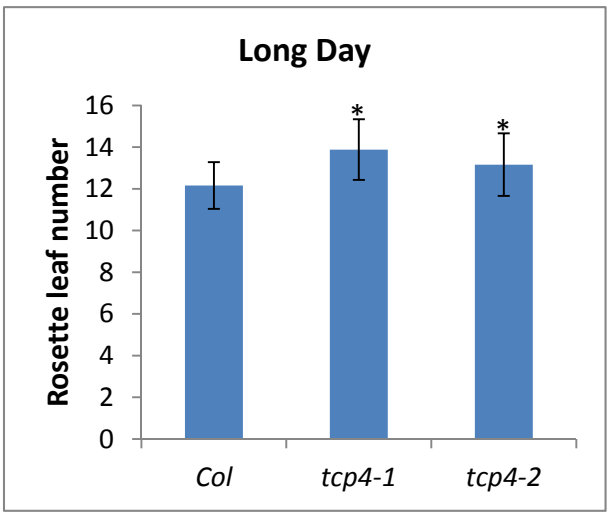
Figure 7. Identification of the *tcp4-1* and *tcp4-2* T-DNA insertion mutants. (A) Schematic representation of *tcp4-1* and *tcp4-2* alleles. Arrow represents transcription starting site, black box represents exon, gray boxes represent UTRs, lines represent introns; and triangles indicate T-DNA insertions. (B) Genotype assay of *tcp4-1* and *tcp4-2* using PCR. (C) The *TCP4* transcript levels in Col-0, *tcp4-1*, and *tcp4-2* plants.

(A)


Col
tcp4-1
tcp4-2

(B)


Long Day
Days to flowering
25
20
15
10
5
0
*
*
Col
tcp4-1
tcp4-2


Long Day
Rosette leaf number
16
14
12
10
8
6
4
2
0
*
*
Col
tcp4-1
tcp4-2

(C)

Col *tcp4-1* *tcp4-2*

(D)

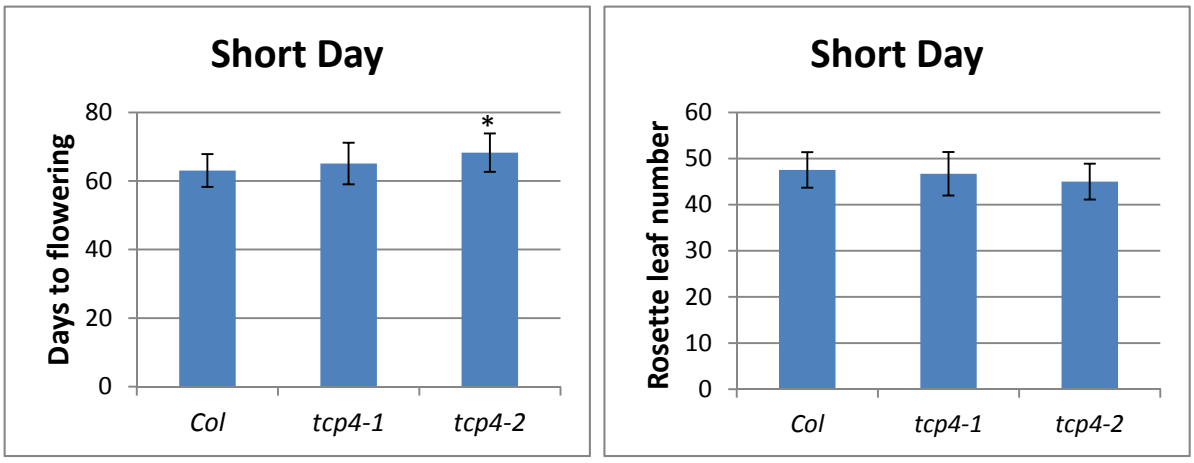


Figure 8. *tcp4-1* and *tcp4-2* show a delayed flowering phenotype under long day conditions. Rosette leaf numbers and bolting time of Col, *tcp4-1*, and *tcp4-2* plants grown under long day (LD) (A-B) and short day (SD) (C-D) conditions are shown. At least 20 plants were scored for each line. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

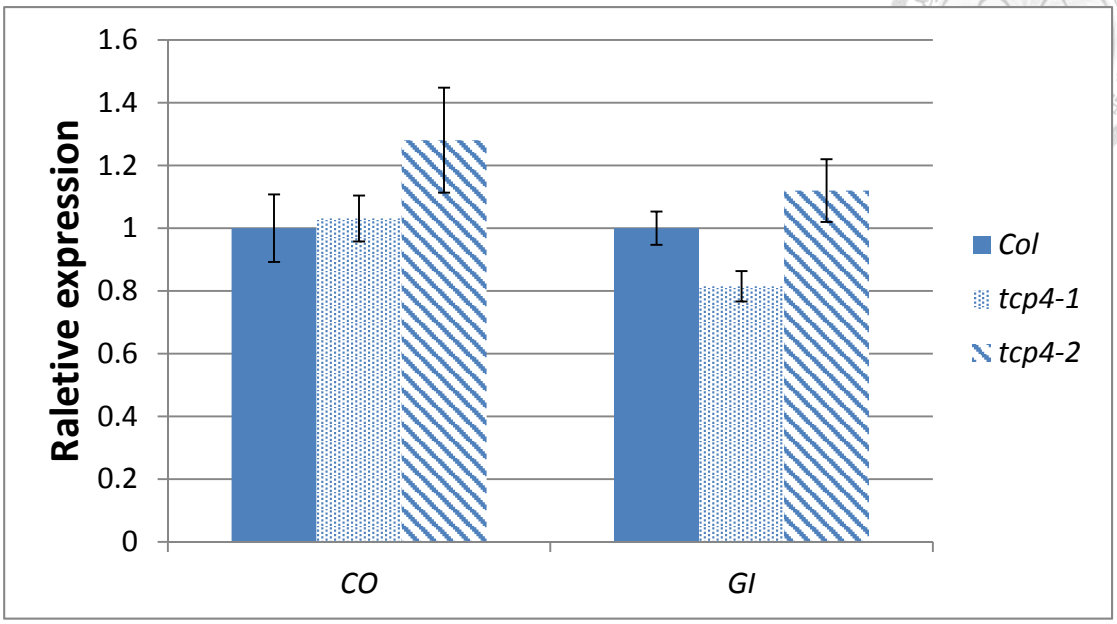


Figure 9. The expression of *GI* and *CO* in *tcp4* mutants. qRT-PCR analyses of the *COSTANT (CO)* and *GIGANTEA (GI)* transcripts. Plants were grown in 1/2 MS plates under long day conditions for 20 days.

(A)

Col *tcp4-1* *tcp4-2* *axe1-5* *tcp4-2/axe1-5*

(B)

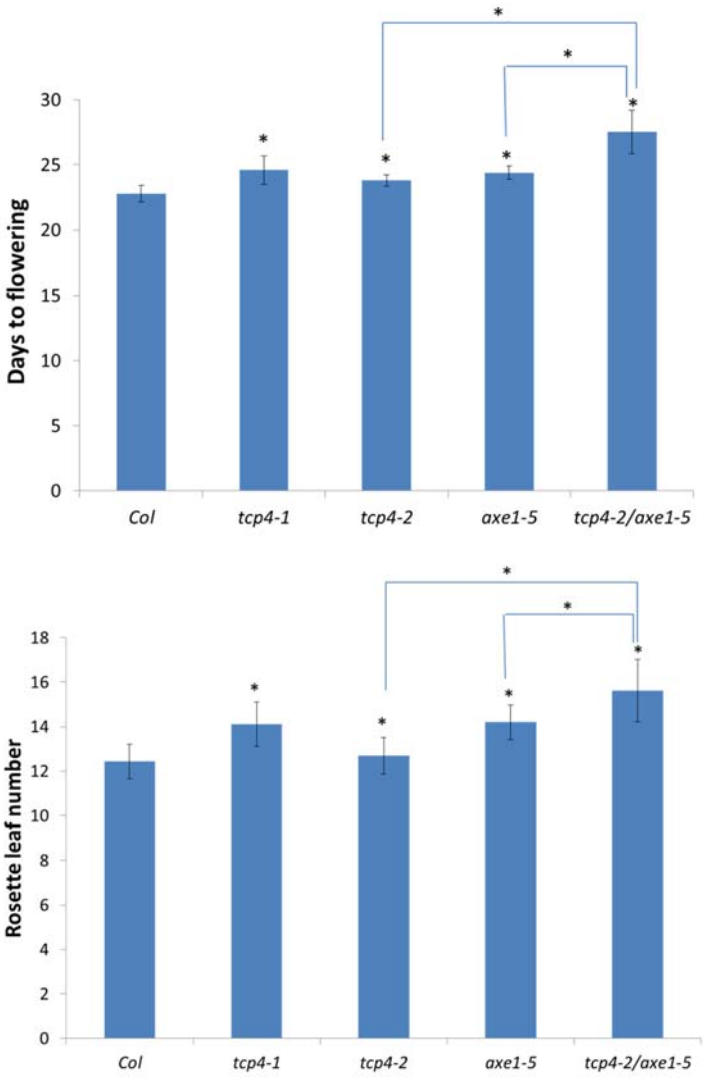


Figure 10. The *tcp4-2/axe1-5* double mutant shows a delayed flowering phenotype under long day conditions. Rosette leaf numbers and bolting time of Col, *tcp4-1*, *tcp4-2*, *axe1-5* and *tcp4-2/axe1-5* plants grown under LD conditions are shown. At least 20 plants were scored for each line. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

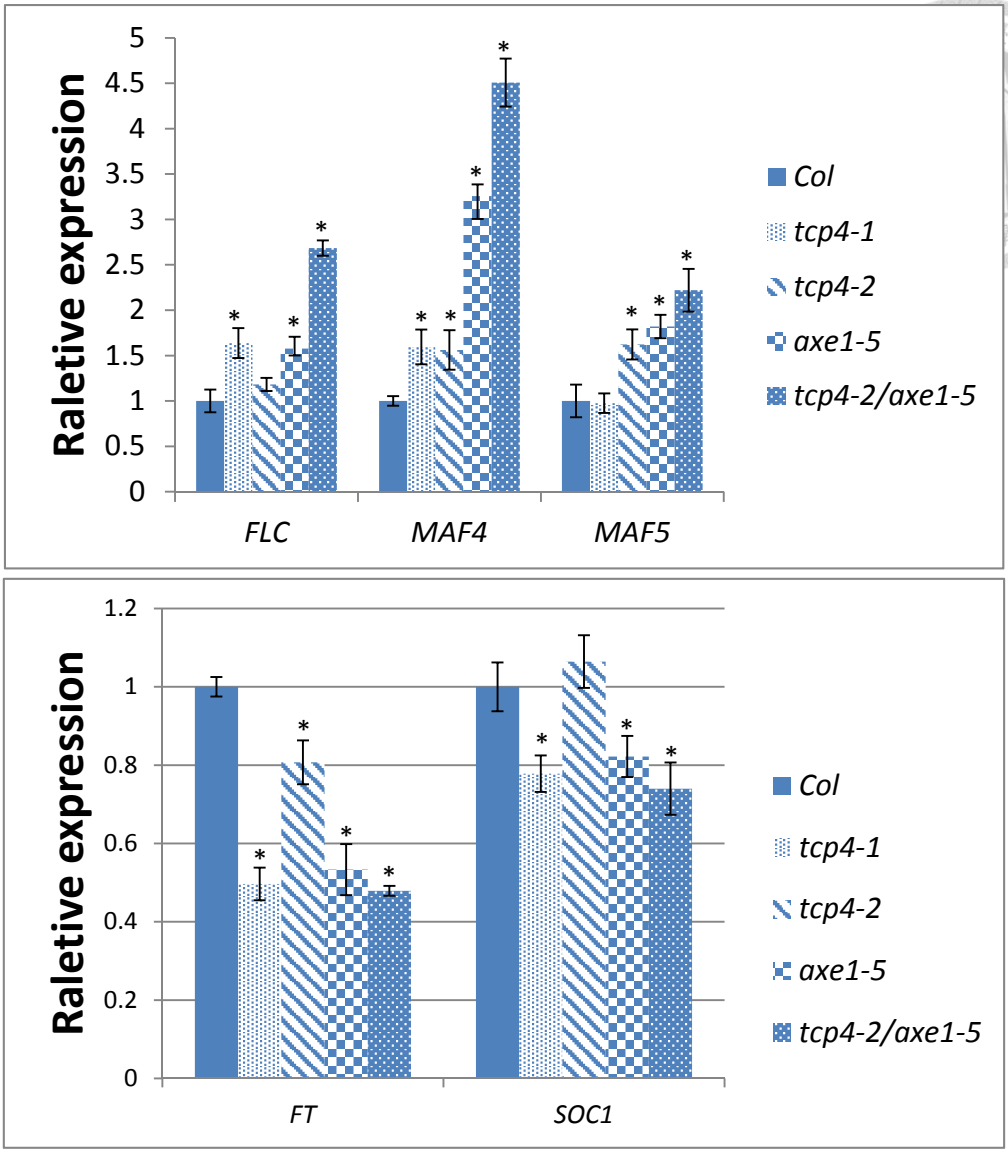
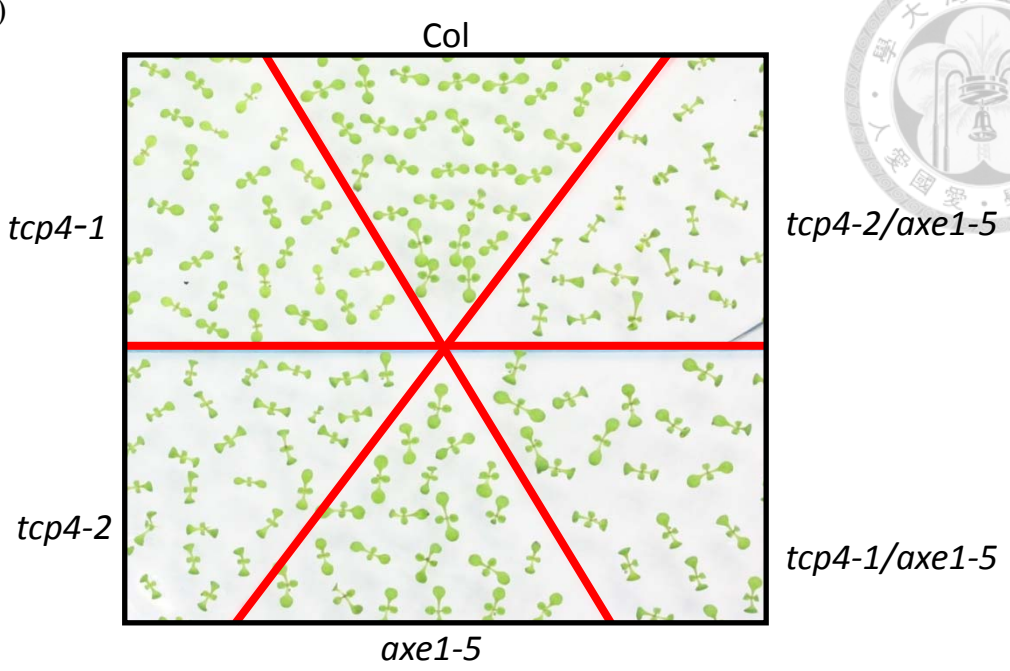
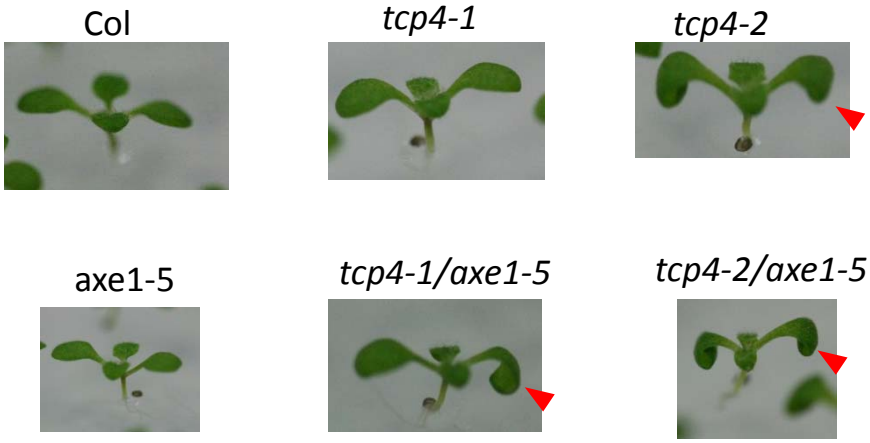


Figure 11. The expression of flowering-related genes in *tcp4-2/axe1-5* double mutants. qRT-PCR analyses of *FLC*, *MAF4*, *MAF5*, *FT* and *SOC1* transcript in Col, *tcp4-1, tcp4-2, axe1-5,* and *tcp4-2/axe1-5*. Plants were grown in soil under long day conditions for 20 days. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

(A)


Col
tcp4-1
tcp4-2/axe1-5
tcp4-2
tcp4-1/axe1-5
axe1-5

(B)


Col
tcp4-1
tcp4-2
axe1-5
tcp4-1/axe1-5
tcp4-2/axe1-5

(C)

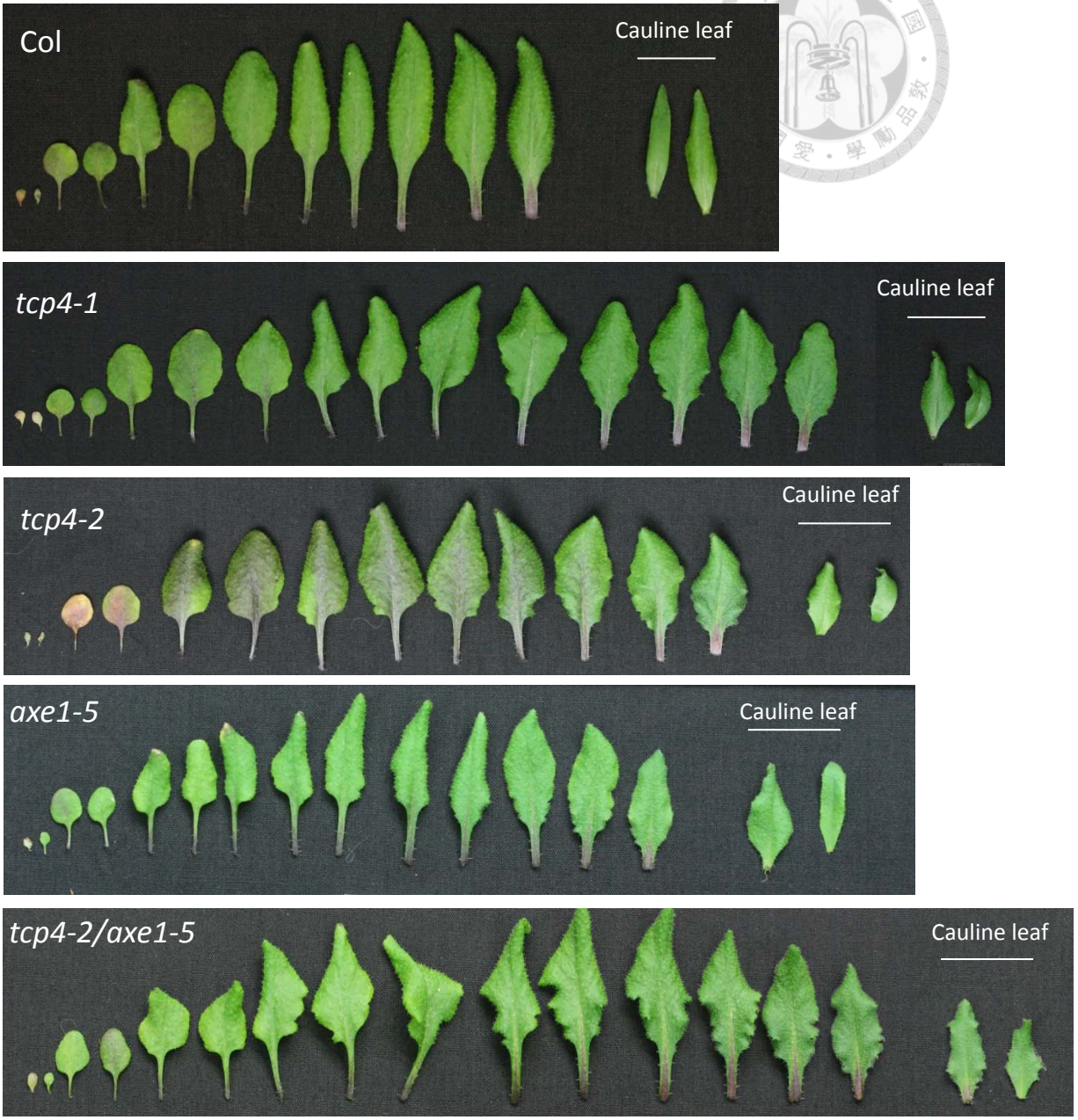


Figure 12. Leaf phenotypes of *tcp4-1*, *tcp4-2*, *axe1-5* and *tcp4-2/axe1-5*.

(A-B) 7 day old seedlings of *tcp4-1*, *tcp4-2*, *axe1-5* and *tcp4-2/ axe1-5*. (C) Compared with *tcp4-2* and *axe1-5*, *tcp4-2/ axe1-5* displayed enhanced severe serration and curling leaf phenotype. Plants were grown under LD conditions.

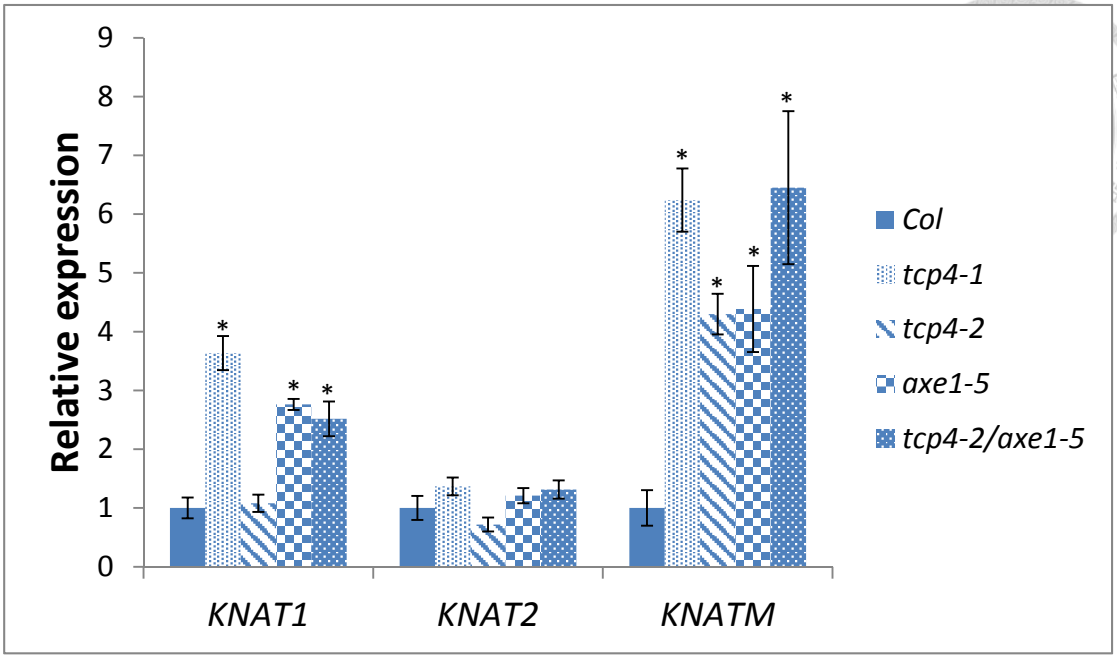


Figure 13. The expression of *KNAT1*, *KNAT2* and *KNATM* in *tcp4-2/axe1-5* double mutants. qRT-PCR analyses of the HDA6 regulated genes *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA* (*KNAT1*), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*) and *KNOX ARABIDOPSIS THALIANA MEINOX (KNATM)* transcripts in Col, *tcp4-1*, *tcp4-2*, *axe1-5*, and *tcp4-2/axe1-5*. Plants were grown in soil under long day conditions for 30 days. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

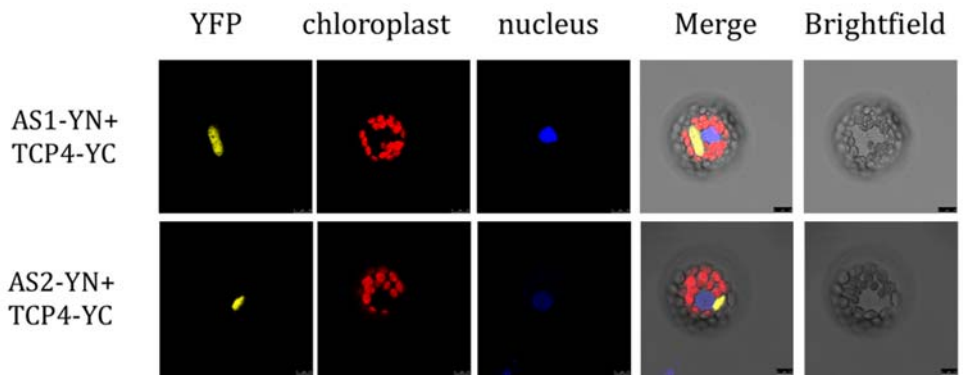


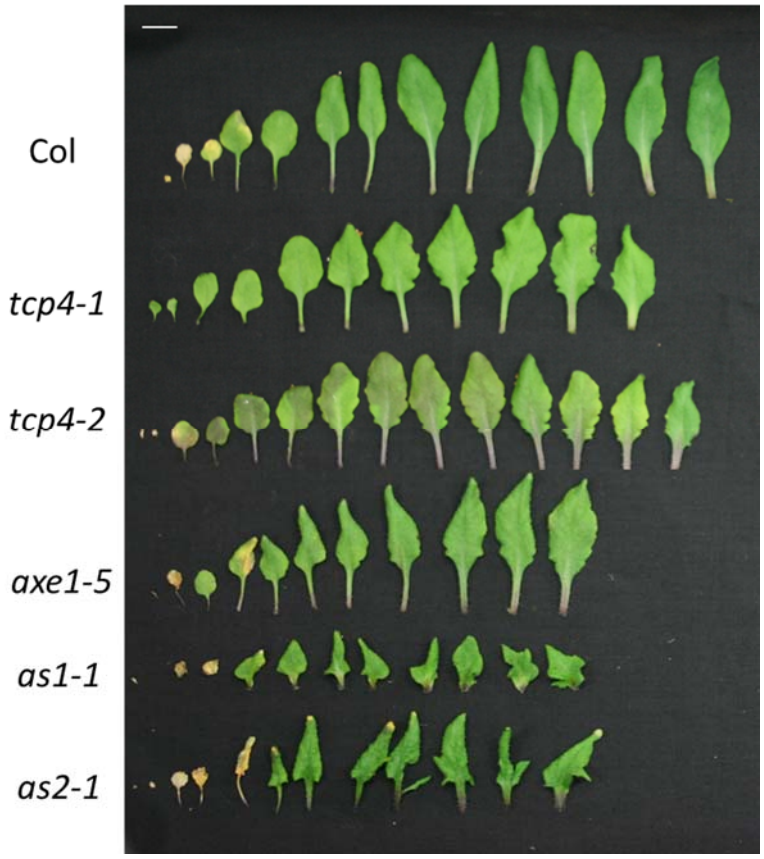
Figure 14. TCP4 interacts with AS1 and AS2 in *Arabidopsis* protoplasts. AS1 or AS2 fused with N terminus (YN) of YFP and TCP4 fused with C terminus (YC) of YFP were co-transfected into *Arabidopsis* protoplasts and visualized using confocal microscope.

(A)

Col　*tcp4-1*　*tcp4-2*　*axe1-5*　*as1-1*　*as2-1*　*tcp4-1/ axe1-5*　*tcp4-2/ axe1-5*

as1-1/ axe1-5　*tcp4-1/ as1-1/ axe1-5*　*tcp4-2/ as1-1/ axe1-5*　*as2-1/ axe1-5*　*tcp4-1/ as2-1/ axe1-5*　*tcp4-2/ as2-1/ axe1-5*

(B)



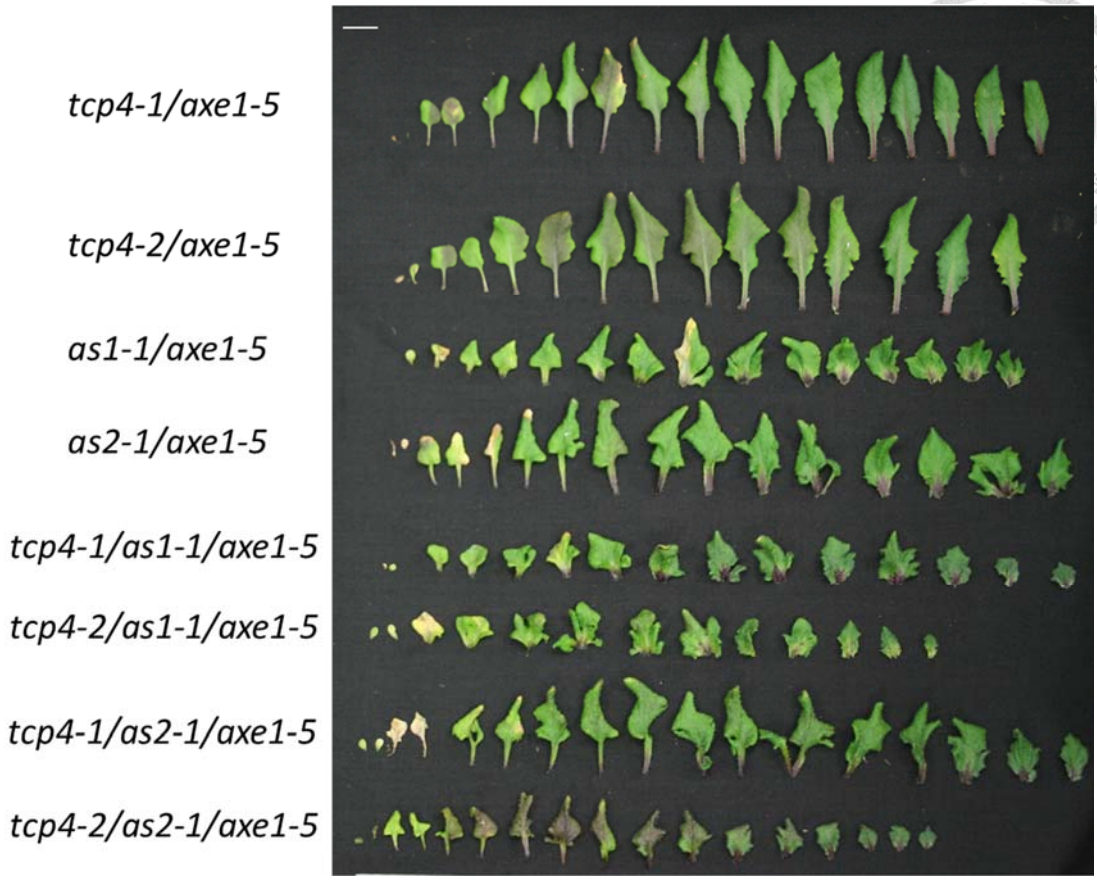


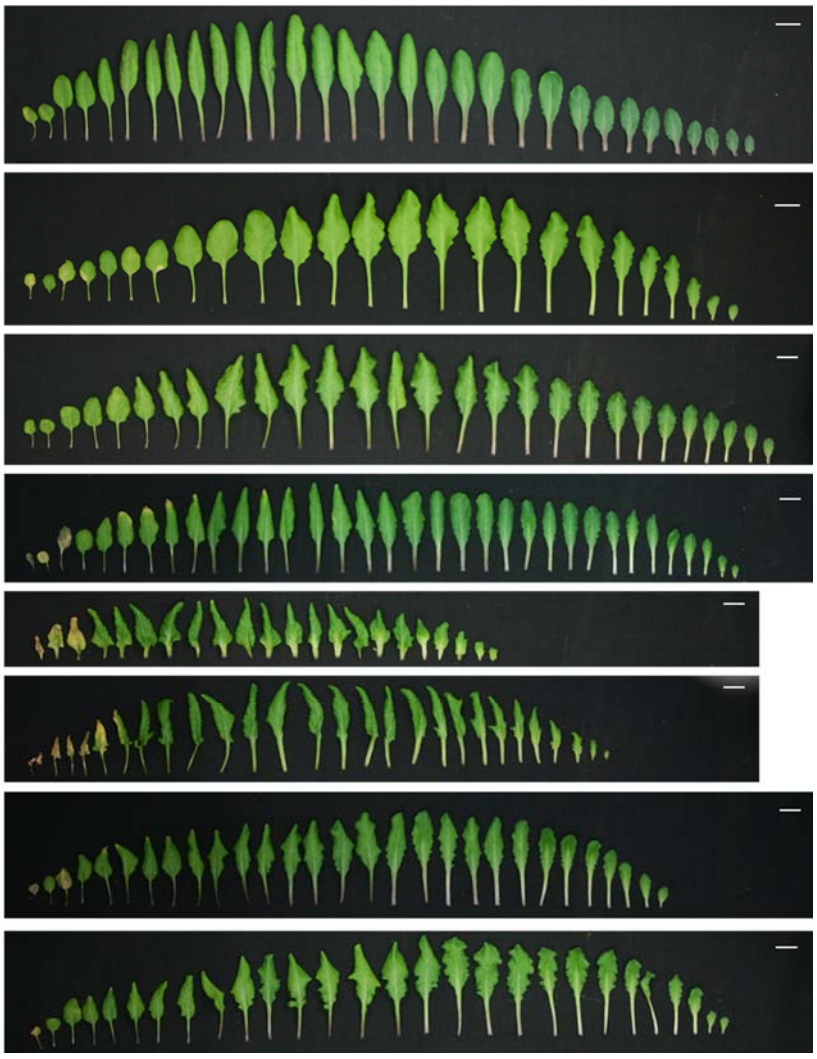
Figure 15. Leaf phenotype of *tcp4, axe1-5, as1-1* and *as2-1* triple mutants under LD conditions. *tcp4/as1-1/axe1-5* and *tcp4/as2-1/axe1-5* triple mutants displayed smaller plant sizes, shorter petioles and enhanced severe phenotypes in leaf serration and curing compared with the single and double mutants, t Plants were grown under LD conditions.

(A)

Col axe1-5 tcp4-1 tcp4-2 as1-1 as2-1 tcp4-1/ axe1-5 tcp4-2/ axe1-5

as1-1/ axe1-5 tcp4-1/ as1-1/ axe1-5 tcp4-2/ as1-1/ axe1-5 as2-1/ axe1-5 tcp4-1/ as2-1/ axe1-5 tcp4-2/ as2-1/ axe1-5

(B)



Col

tcp4-1

tcp4-2

axe1-5

as1-1

as2-1

tcp4-1/axe1-5

tcp4-2/axe1-5

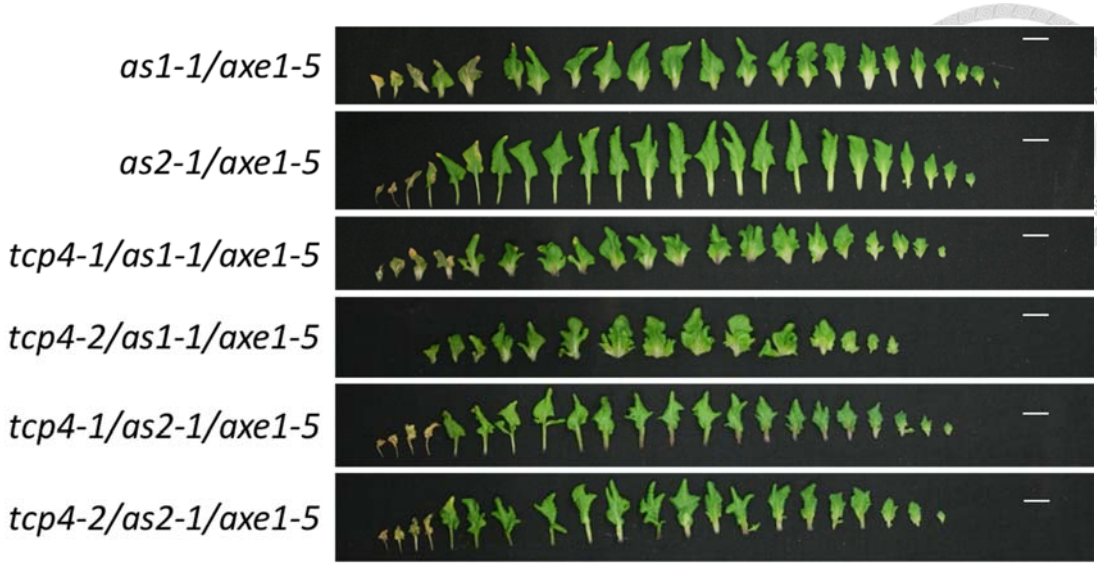
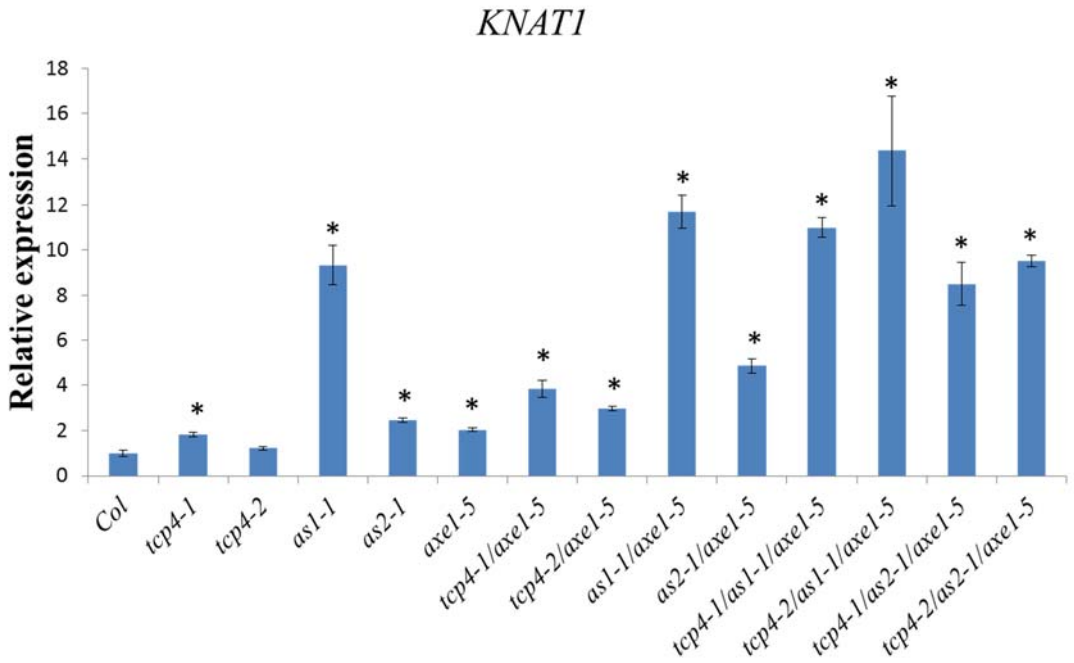


Figure 16. Leaf phenotype of *tcp4, axe1-5, as1-1* and *as2-1* triple mutants under SD conditions. *tcp4/as1-1/axe1-5* and *tcp4/as2-1/axe1-5* triple mutants displayed smaller plant sizes, shorter petioles and enhanced severe phenotypes in leaf serration and curing compared with the single and double mutants, Plants were grown under SD conditions.

(A)



(B)

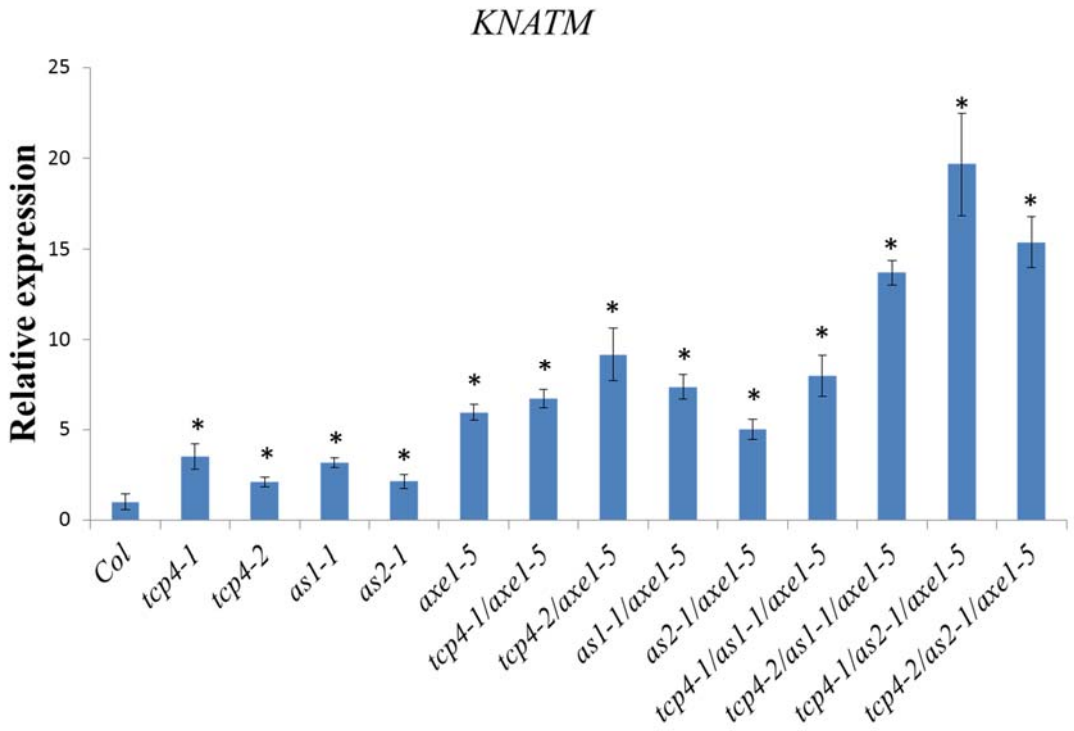


Figure 17. The expression of *KNAT1* and *KNATM* in *tcp4/as1-1/axe1-5* and *tcp4/as2-1/axe1-5* triple mutants. qRT-PCR analyses of *KNAT1* and

KNATM transcripts in Col, *tcp4-1*, *tcp4-2*, *as1-1*, *as2-1*, *axe1-5*, *tcp4/axe1-5*, *as1-1/axe1-5*, *as2-1/axe1-5*, *tcp4/as1-1/axe1-5* and *tcp4/as2-1/axe1-5*. Plants were grown in soil under long day conditions for 30 days. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

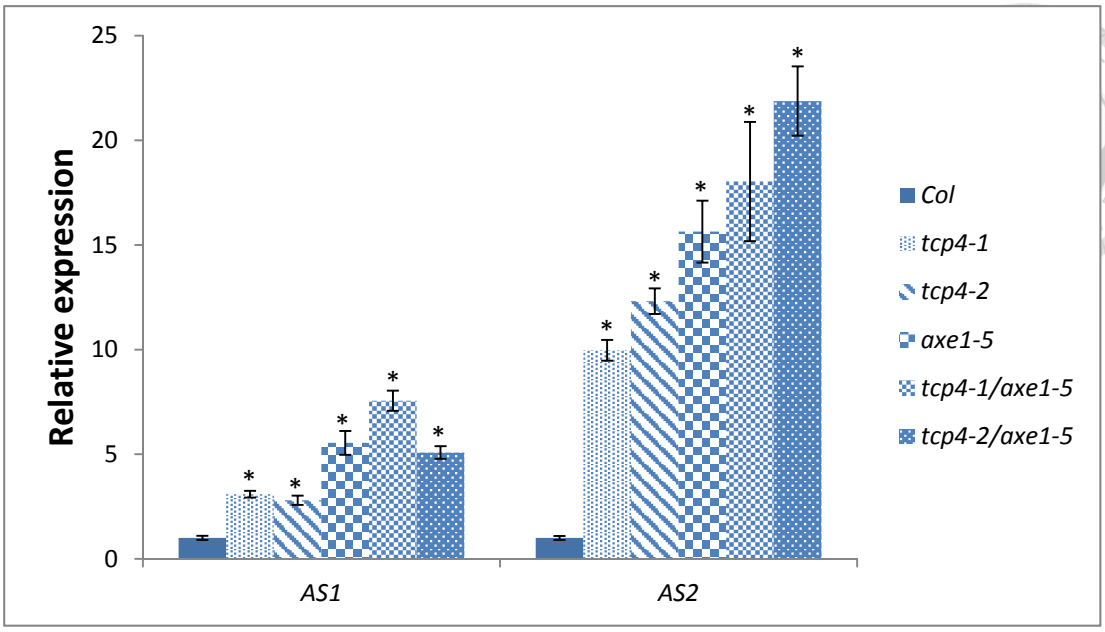
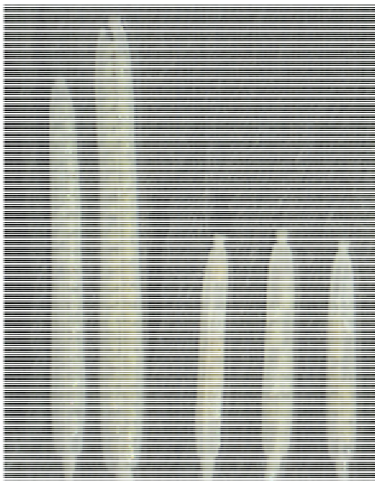


Figure 18. The expression of *AS1* and *AS2* in *tcp4/axe1-5* double mutants. qRT-PCR analyses of *AS1* and *AS2* transcripts in Col, *tcp4-1*, *tcp4-2*, , *axe1-5* and *tcp4/axe1-5*. Plants were grown in soil under long day conditions for 30 days. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

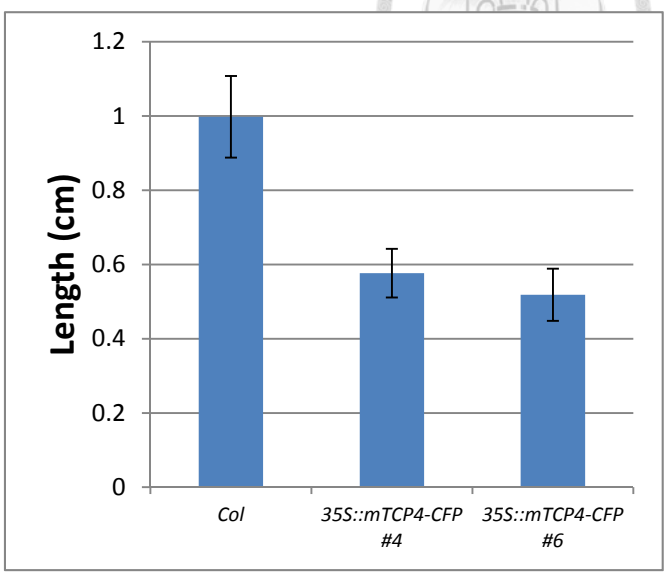
(A)



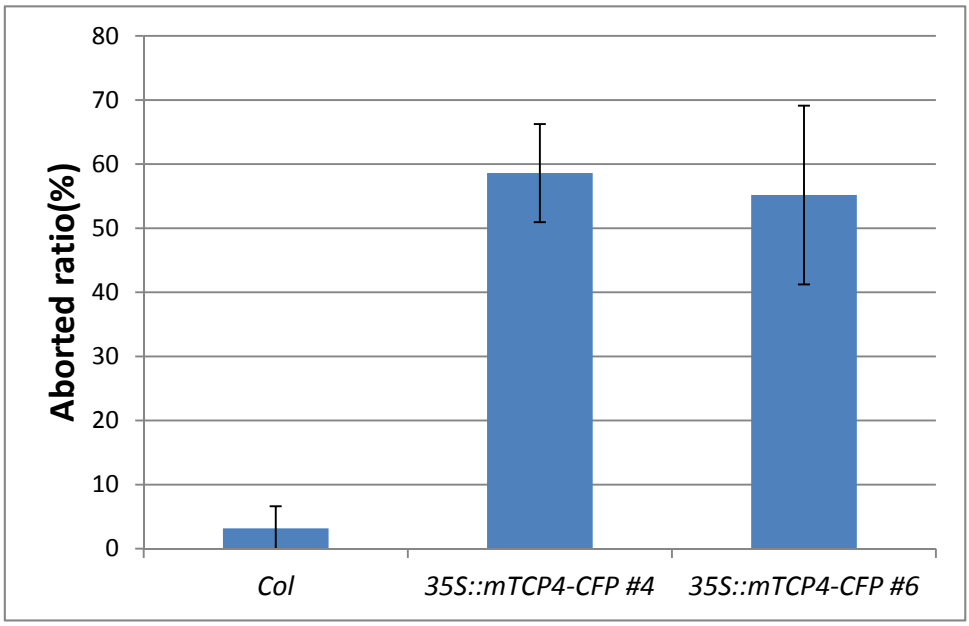
Col

35S::mTCP4-CFP #4

(B)



(C)



(D)

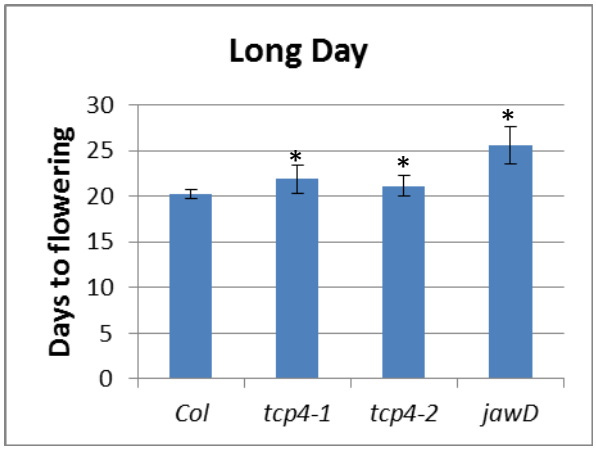
Figure 19. The silique phenotype of *35S::mTCP4-CFP*. (A-B)The silique of *35S::mTCP4-CFP* is shorter than that of Col. Each line was scored for at least 30 siliques. (C) In *35S::mTCP4-CFP* line, the number of aborted seeds is much higher than that of Col. Each line was scored for at least 30 siliques. (D) The valves and septum twisted together in some siliques.

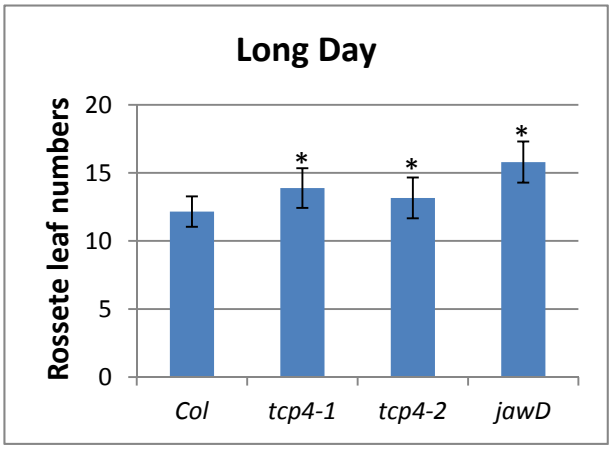
Supplementary Figures

(A)

Col *tcp4-1* *tcp4-2* *jawD*

(B)

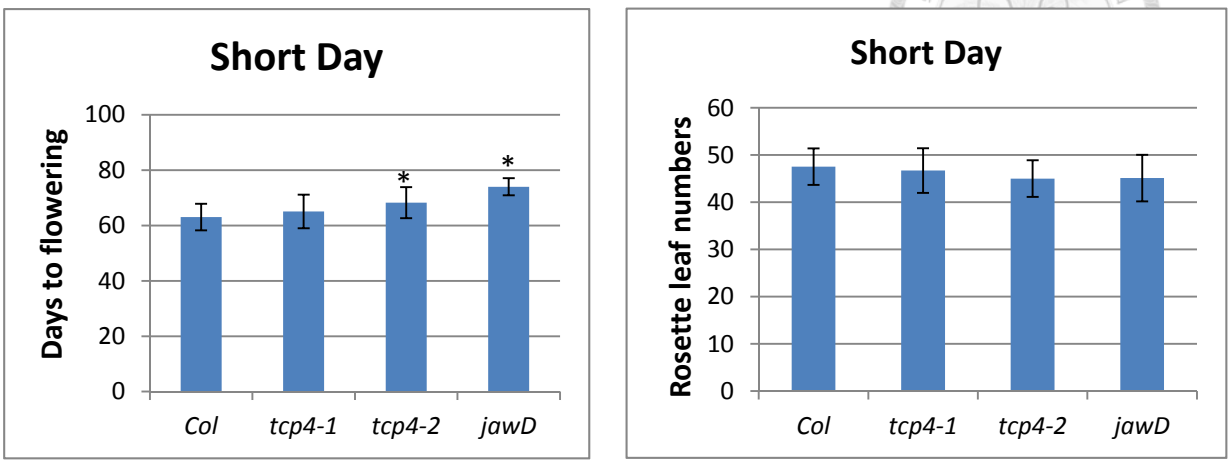




(C)

Col *tcp4-1* *tcp4-2* *jawD*

(D)



Supplementary Figure 1. *TCP* mutants *tcp4-1, tcp4-2* and *jawD* show delayed flowering phenotype under long day conditions (LD). Rosette leaf numbers and bolting time of Col, *tcp4-1*, *tcp4-2* and *jawD* plants grown under LD (A-B) and SD (C-D) conditions are shown. At least 20 plants were scored for each line. Asterisks indicate statistically significant differences between Col and the other lines ($P \leq 0.05$; Student's t-test).

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