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The N-terminal region located upstream of the TCP domain is responsible for the antagonistic action of the *Arabidopsis thaliana* TCP8 and TCP23 transcription factors on flowering time

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Abstract

TCP proteins (TCPs) are plant-exclusive transcription factors that exert effects on multiple aspects of plant development, from germination to flower and fruit formation. TCPs are divided into two main classes, I and II. In this study, we found that the *Arabidopsis thaliana* class I TCP transcription factor TCP8 is a positive regulator of flowering time. *TCP8* mutation and constitutive expression delayed and accelerated flowering, respectively. Accordingly, *TCP8* mutant plants showed a delay in the maximum expression of *FT* and reduced *SOC1* transcript levels, while plants overexpressing *TCP8* presented increased transcript levels of both genes. Notably, the related class I protein TCP23 showed the opposite behavior, since *TCP23* mutation and overexpression accelerated and retarded flowering, respectively. To elucidate the molecular basis of these differences, we analyzed TCP8 and TCP23 comparatively. We found that both proteins are able to physically interact and bind class I TCP motifs, but only TCP8 shows transcriptional activation activity when expressed in plants, which is negatively affected by TCP23. From the analysis of plants expressing different chimeras between the TCPs, we found that the N-terminal region located upstream of the TCP domain is responsible for the opposite effect that TCP8 and TCP23 exert over flowering time and regulation of *FT* and *SOC1* expression. These results suggest that structural features outside the TCP domain modulate the specificity of action of class I TCPs.

1. Introduction

TEOSINTE-BRANCHED1/CYCLOIDEA/PCF (TCP) proteins constitute a plant-specific family of transcription factors essential for the regulation of processes related to growth and development (Danisman, 2016). These proteins exert various roles in several aspects of plant development by interacting with endogenous (circadian rhythm and hormones) and environmental (light, temperature, pathogens, and drought stress) factors and through the formation of complexes with proteins belonging to other families, thereby affecting the expression of downstream genes and regulating plant development (Martín-Trillo and Cubas, 2010; Uberti Manassero et al., 2013; Danisman, 2016). Their name is due to a highly conserved domain of about 60 amino acids, called TCP, which mediates the binding to specific DNA sequences and the formation of homo- and heterodimers (Cubas et al., 1999; Kosugi and Ohashi, 2002). Based on characteristics present both inside and outside this domain, TCPs are grouped into two classes: I and II. In addition, class II is divided into two lineages: CIN and CYC/TB1 (Cubas et al., 1999; Kosugi and Ohashi, 2002). The crystal structure of the class II TCP domain of rice OsPCF6 revealed that it adopts a particular bHLH structure with three consecutive short β-strands followed by a helix-loop-helix, termed the ribbon-helix-helix (RHH) motif (Sun et al., 2020). Class I and class II TCP proteins recognize DNA sequences containing the core GGGNCC (Kosugi and Ohashi, 2002; Viola et al., 2011; Zhou et al., 2018; Ferrero et al., 2019; Camoirano et al., 2020; Li et al., 2021; Lan et al., 2021; Alem et al., 2022). Differences between class I and class II binding preferences are dependent on the presence of glycine or aspartic acid at positions 11 or 15 of the TCP domain, respectively (Viola et al., 2012). Interestingly, class I and class II consensus binding site sequences are not mutually exclusive, and a subset of genes were reported as targets of both class I and II TCP proteins (Uberti Manassero et al., 2013; Danisman et al., 2016; Dong et al., 2019; Ferrero et al., 2019; Alem et al., 2022).

TCP family members have been identified in various species including rice, *Arabidopsis thaliana*, *Antirrhinum*, tomato, tobacco, maize, cotton, potato, and grapevine (Gübitz et al., 2003, Navaud et al., 2007; Hao et al., 2012; Li, 2015; Chen et al. 2016; Jiu et al., 2019; Bao et al., 2019), among several others, but mainly characterized in *Arabidopsis*. The *Arabidopsis* genome encodes 13 class I and 11 class II TCP proteins (Martín-Trillo and Cubas, 2010). Class II TCP members were found to act redundantly in the control of several processes, as leaf morphogenesis, bud dormancy, shoot growth, thermomorphogenesis, and photomorphogenesis (Koyama et

al., 2007; Efroni et al., 2008; Schommer et al., 2008; Koyama et al., 2010; Koyama et al., 2017; Bresso et al., 2018; Reddy et al., 2019; Van Es et al., 2019; Zhou et al., 2019; Han et al., 2019; Dong et al., 2019). In addition, two class I TCP members, TCP14 and TCP15, act redundantly in the control of several aspects of plant development including germination (Resentini et al., 2015), response to high temperature (Ferrero et al., 2019; 2021), cuticle and trichome development (Camoirano et al., 2020; 2021), stem growth (Kieffer et al., 2011), flowering (Lucero et al., 2017), cotyledon opening and expansion (Alem et al., 2022), and anthocyanin biosynthesis (Viola et al., 2016). Along with the phylogenetically related class I member TCP8, TCP14 and TCP15 were also redundantly implicated in the regulation of plant immunity (Kim et al., 2014, Li et al., 2018, Spears et al., 2019) and cell-cycle control (Zhang et al., 2018), while analysis of pentuple and septuple mutants in several class I *TCP* genes revealed a redundant function in leaf development (Aguilar-Martinez and Sinha, 2013; Zhang et al., 2019). In addition, DELLA proteins negatively regulate the expression of cell-cycle genes in inflorescence shoot apices by repressing the transcriptional activity of TCP8, TCP14, TCP15, and TCP22 (Davière et al., 2014), indicating that TCP transcription factors act in a semi-redundant fashion in many facets of plant growth and development. Certain processes, as hypocotyl elongation, cotyledon opening, trichome development, and flowering, are also controlled by class II members, mostly acting at different levels of the respective molecular pathways than class I members (Lucero et al., 2017; Liu et al., 2017; Kubota et al., 2017; Li et al., 2019; Zhou et al., 2019; Han et al., 2019; Vadde et al., 2018; Dong et al., 2019; Ferrero et al., 2019; Camoirano et al., 2020; 2021; Alem et al., 2022; Li et al., 2021). For example, several class II CIN TCPs promote flowering through the induction of *CONSTANS* (*CO*) transcription (Liu et al., 2017; Kubota et al., 2017), while others interact with FD to directly induce the expression of the floral meristem identity gene *AP1* (Li et al., 2019). Instead, the class I TCPs TCP15 and TCP7 promote flowering through direct induction of the flowering integrator *SOC1* (Lucero et al., 2017; Li et al., 2021). In addition, TCP15 regulates TCP4 and other CIN TCPs through SOC1-mediated repression of *MIR319* genes (Lucero et al., 2017). Interestingly, other *Arabidopsis* TCP proteins from both classes were reported as negative regulators of flowering. For example, the class II TCP BRC1/TCP18 delays floral transition in axillary meristems and its ectopic expression has a similar effect also in the shoot apical meristem (Niwa et al., 2013). TCP20 and TCP22, class I, interact with clock proteins LWD1 and LWD2 and delay flowering by regulating the expression

of the clock gene *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) (Wu et al., 2016). Overexpression of the class I genes *TCP8* and *TCP23* was also reported to delay flowering (Wang et al., 2019; Balsemao-Pires et al., 2013). In summary, TCP family members from both classes adopted different roles during floral transition and modulate flowering time in different ways, but the cause of the opposite behavior of related TCP proteins during this process is not known. In the course of our studies, we found that *TCP8* mutant plants showed delayed flowering and reduced expression of the flowering time integrators *SOC1* and *FT*, whereas the opposite was observed in *TCP8* overexpressing plants, thus suggesting that TCP8 acts as a positive regulator of flowering. On the contrary, *TCP23* mutant and overexpressing plants exhibited accelerated and delayed flowering, respectively. Structure-function analysis indicated that the N-terminal region located upstream of the conserved TCP domain is responsible for the antagonistic function of TCP8 and TCP23. In a general way, our findings indicate that regions other than the TCP domain can influence the activity of TCP transcription factors and provide diversification of their roles in plant growth and development.

2. Material and Methods

2.1. Plant materials and growth conditions

All experiments were performed in *Arabidopsis thaliana* accession Col-0 background. Mutant lines *tcp8-1* and *tcp23-1*, *tcp14-4 tcp15-3*, *tcp8-1 tcp14-5 tcp15-3*, and the reporter line *pTCP23::GUS* were previously described (Aguilar-Martinez and Sinha, 2013; Kieffer et al., 2011). The double mutant *tcp8-1 tcp23-1* was generated by crossing *tcp8-1* and *tcp23-1* single mutants, followed by selection based on the appropriate resistance marker genes and genotyping. Expression levels were evaluated by RT-qPCR. Primers used for genotyping are listed in Table S1.

Plants were grown on soil or in plates containing 0.5X Murashige and Skoog (MS) medium and 0.8% agar at 23°C under long-day conditions (16 h light/8 h dark) at a light intensity of 100 µmol m^{-2} s^{-1}, except in Fig. S2, where plants were grown under short-day conditions (8 h light/16 h dark). All seeds were surface-sterilized and stratified at 4°C for 4 days in the dark to synchronize germination.

2.2. Gene Cloning and Plant Transformation

To generate the *TCP8* promoter-driven GUS expression (*pTCP8::GUS*) construct, a 1.5-kbp region located upstream of the translation start codon of *TCP8* was amplified by PCR and cloned into the binary vector pBi101.3. To generate *TCP8* and *TCP23* overexpressing plants, the coding regions of each *TCP* gene were amplified and cloned in the binary vector pBI121 under the control of the *CaMV 35S* promoter. Clones to express protein chimeras between TCP8 and TCP23 (Ch-1-Ch-4, N_{TCP23}-Ch-3 and $Ch3_{G32Q}$) were constructed by overlap extension mutagenesis (Silver et al., 1995) using complementary oligonucleotides with the desired chimeric sequences or mutation (Table S1) and cloned in the binary vector pBI121 under the control of the *CaMV 35S* promoter. All constructs were checked by DNA sequencing and introduced into *Agrobacterium tumefaciens* strain LB4404. Arabidopsis plants were transformed by the floral dip procedure (Clough and Bent, 1998). Transformed plants were selected on the basis of kanamycin resistance and genotyping. RNA expression levels were measured by RT-qPCR. The primers used for PCR amplifications are listed in Table S1. T3 homozygous lines generated from T1 individuals were analyzed.

2.3. One- and two-hybrid analysis in yeast

Fragments encoding full-length TCP8 and TCP23 were cloned in vectors pGBKT7 or pGADT7 (Clontech), to allow expression as fusions to the GAL4 BD and AD, respectively. The primers used for PCR amplifications are listed in Table S1. For one-hybrid assays, yeast strains carrying the class I TCP binding sequence (TCP-BS, GTGGGACC) or mutated TCP binding sequence (TCP-BS_{mut}, GTAGGCCC) inserted into the genome upstream of the minimal promoter of the *Saccharomyces cerevisiae CYC1* gene fused to the *E. coli LacZ* reporter gene (Viola et al., 2011; 2012) were transformed with constructs that express fusions of TCP proteins to the GAL4 AD. In one- and two-hybrid experiments, DNA was introduced into yeast using the lithium acetate transformation method (Gietz et al., 1992). β-Galactosidase activity was assayed as described in Ausubel et al. (2010) using *o*-nitrophenylgalactoside as substrate.

2.4. Phenotypic analysis

For flowering time analysis, stratified seeds were sown directly on soil. Flowering time was measured by counting the number of rosette leaves and the number of days after sowing when the main stem reached 0.5 cm. The experiments were repeated at least three times with 12 individual plants per genotype.

2.5. β-Glucuronidase assay

β-Glucuronidase (GUS) activity was analyzed by histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as described by Vitha (2012). Plants were immersed in a 0.5 μg/μL X-gluc solution in 50 mM citrate-HCl buffer, pH 7.0, and 0.05% Triton X-100. Vacuum was applied for 5 min and reactions were incubated in darkness at 37°C during 16 hours. Chlorophyll was removed by incubating samples in 70% ethanol.

2.6. RNA isolation and analysis

RNA extractions were performed using Trizol reagent (Invitrogen) followed by LiCl precipitation. Quantification of transcript levels was carried out by RT-qPCR. The cDNA was obtained by reverse transcription using 1.5-2.0 μg of RNA with oligodTv primer and MMLV reverse transcriptase (Promega). The qPCR reactions were performed in StepOne™ Real Time PCR System or StepOnePlus™ Real Time PCR System thermocyclers (LifeTechnologies™). The reactions were carried out in a final volume of 20 μL with reaction buffer provided by the manufacturer (10x), 3 mM $MgCl_2$, 0.15 U of *Taq* Pegasus DNA polymerase (PBL, Argentina), 62.5 μM dNTPs, 1 μL SYBR Green, 0.4 μL of specific oligonucleotides (the concentration was optimized for each pair of primers) and 10 μL of a cDNA dilution. A comparative C_t method was used to calculate relative transcript levels, with *ACT2* and *ACT8* actin genes as normalizers (Charrier et al. 2002). Primers used for RT-qPCR are listed in Table S1.

2.7. Transcriptional activity assays in plants

To generate the synthetic promoter-driven *GUS* expression construct, *p(TCP-BS)x6::GUS*, six tandem copies of oligonucleotides with the class I binding sequence (GTGGGACC) from the *p(TCP-BS)x6::LacZ* plasmid used in yeast one-hybrid analysis (Viola et al., 2012) were fused to the minimal *CaMV 35S* promoter and cloned in front of the *GUS* reporter gene in the binary vector pBi101.3. For transcriptional activity assays in *Nicotiana benthamiana*, leaves of 2-week-old plants were co-infiltrated with *Agrobacterium tumefaciens* cells carrying the *p(TCP-BS)x6::GUS* reporter and the effector constructs *35S::TCP8* and *35S::TCP23* as described by de Felippes & Weigel (2010). Co-infiltration with the *35S::GFP* construct was used as negative control. The ratio of cells with reporter and effector/control constructs was 1:1. For transcriptional assays in *Arabidopsis thaliana*, 12-day-old *pSOC1::GUS* seedlings (Hepworth et al.,

2002) were infiltrated with combinations of the effector constructs *35S::TCP8* and *35S::TCP23* and the control *35S::GFP* essentially as described by Viola et al. (2013). The transformed plants were kept in the greenhouse under long-day conditions at 23°C. Transcriptional activity of the promoters was determined by analysis of *GUS* transcript levels 48 h after infiltration.

3. Results

3.1. TCP8 positively regulates flowering time in Arabidopsis

To gain insight into the function of TCP8 in *Arabidopsis* development, we analyzed the phenotype of the described *tcp8-1* mutant, that contains a T-DNA inserted within the open reading frame of *TCP8* and presents low levels of *TCP8* transcripts in comparison with wild-type plants (Aguilar-Martinez and Sinha, 2013). The transition to flowering was delayed in *tcp8-1* plants considering both the number of days required to flowering and the number of rosette leaves at bolting (Fig. 1A-C). As a result, the *tcp8-1* mutant showed more rosette leaves in comparison with wild-type plants after flowering (Fig. 1D). To further investigate this, we generated plants expressing *TCP8* under the control of the *CaMV 35S* promoter (*35S::TCP8*). In contrast to *tcp8* mutant plants, we observed an early flowering phenotype in about 15 *35S::TCP8* independent lines during the T1 generation. To confirm this result, we selected three representative lines which were reproduced to homozygosis and analyzed (Fig. S1A). Flowering was accelerated in the three independent lines of *35S::TCP8* plants (Fig. 1A-C, Fig. S1). As a consequence, *35S::TCP8* plants showed fewer rosette leaves than wild-type plants after flowering (Fig. 1D). We also analyzed the effect of *TCP8* mutation on flowering of plants impaired in the class I TCPs TCP14 and TCP15, two reported positive regulators of flowering (Lucero et al., 2017). The triple mutant line *tcp8-1 tcp14-5 tcp15-3* showed a further increase in both the time to bolting and rosette leaf number at bolting relative to the *tcp14-4 tcp15-3* double mutant (Fig. 1E-G), suggesting that TCP8 acts additively with TCP14 and TCP15. Flowering time of *TCP8* mutant and overexpressing plants was also assessed under short day conditions. A clear delay in flowering was observed in *tcp8-1* plants while *35S::TCP8* plants flowered earlier than wild-type plants under this condition (Fig. S2). This suggests that TCP8 is a positive regulator of flowering in *Arabidopsis* regardless of photoperiod.

Although the transition to flowering was affected in *tcp8-1* and *35S::TCP8* plants, no differences were observed in the rate of elongation of the primary inflorescence in relation to wild-type plants (Fig. S3), suggesting that the observed differences in stem height at a given time are related to changes in flowering time.

3.2. Expression of *FT* and *SOC1* is affected in *tcp8-1* and *35S::TCP8* plants

To further characterize the molecular effect of TCP8 on flowering time, we analyzed the expression levels of the major floral integrator *FT* in *TCP8* mutant and overexpressing plants at different days after sowing. In wild-type plants, we observed a peak of *FT* transcripts at day 10, while maximum expression of *FT* was reached two days later, at day 12, in *tcp8-1* plants (Fig. 2A). This delay in the time of *FT* maximum expression could explain the late flowering phenotype of *tcp8-1* mutant plants. Consistently, transcript levels of *SOC1*, a floral integrator regulated by FT (Lee and Lee, 2010), were reduced in *tcp8-1* mutant plants relative to wild-type at day 12 (Fig. 2B). In agreement with their early flowering phenotype, *35S::TCP8* plants consistently showed higher *FT* transcript levels than wild-type plants with a pronounced peak at day 10 (Fig. 2A). *SOC1* was also more highly expressed in *35S::TCP8* plants as compared with wild-type plants (Fig. 2B). Because the transcriptional expression of *FT* is known to be regulated by the circadian clock, we wondered whether the daily rhythm of *FT* transcripts was affected by *TCP8* overexpression. To address this, *FT* expression was analyzed over a 24-h period under long day conditions at day 10. The results showed that the daily rhythm of *FT* transcript levels was not affected by TCP8, but the amplitude of the response was (Fig. 2C). Altogether, the results support the participation of TCP8 in the regulation of flowering time upstream of *FT* and *SOC1*.

3.3. TCP8 and TCP23 antagonistically regulate flowering time

Different from our results, TCP8 was proposed as a negative regulator of flowering by Wang et al. (2019). Notably, TCP23, which is similar to TCP8, was also suggested as a negative regulator of flowering by Balsemão-Pires et al. (2013), who observed that *tcp23-1* mutant plants flower earlier than wild-type plants, while plants that overexpress *TCP23* show a delayed flowering phenotype under long- and short-day conditions. We then generated plants constitutively expressing *TCP23* under the control of the *CaMV 35S* promoter using the same system used to express *TCP8*. In agreement with the results reported by Balsemão-Pires et al. (2013), *35S::TCP23* plants showed delayed

flowering (Fig. S4). In addition, maximum expression of *FT* was delayed and *SOC1* transcript levels were significantly reduced in *35S::TCP23* plants (Fig. 2D,E), while *tcp23-1* mutant plants showed significantly increased levels of *FT* and *SOC1* transcripts in comparison with wild-type plants (Fig. 2D,E). Flowering time of the *tcp8-1 tcp23-1* double mutant was similar to wild-type plants (Fig. 2F), suggesting that TCP8 and TCP23 regulate flowering antagonistically.

3.4. Analysis of the expression patterns of *TCP8* and *TCP23*

To analyze the basis of the different effects observed for TCP8 and TCP23 on flowering, we first analyzed the expression of the corresponding genes between days 8 and 12 post-germination, the period when induction of flowering genes was observed. There were no significant changes in transcript levels of the two TCPs during these days (Fig. 3A). When analyzing the expression throughout day 10, we observed that the expression of both *TCP* genes was higher during the day and decreased at night (Fig. 3B). Next, we decided to compare the spatial pattern of *TCP8* and *TCP23* expression. To do this, we obtained plants that express the *uidA* (*GUS*) reporter gene under the control of a 1.5-kbp region located upstream of the translation start codon of *TCP8* (*pTCP8::GUS*) and analyzed *pTCP23::GUS* plants (Aguilar-Martinez and Sinha, 2013) that contain a 2.5-kbp region located upstream of the start codon of *TCP23* fused to *GUS*. In 6-d-old *pTCP8::GUS* seedlings, GUS activity was detected in the shoot apical meristem, hypocotyl, root, and the vasculature of cotyledons and leaf primordia (Fig. 3C). GUS was also expressed in trichomes and the vasculature of fully expanded rosette leaves. In mature flowers, GUS expression was observed in petals, stamen filaments, and anthers (Fig. 3C). In siliques, GUS was detected in the replum (Fig. 3C). In *pTCP23::GUS* plants, GUS expression was restricted to the hypocotyl, shoot apical meristem and emerging leaves (Fig. 3C). In adult plants, GUS expression was detected in the emerging flower buds and at the base of the fertilized flowers (Fig. 3C). This pattern of expression, although different from the one observed for *pTCP8::GUS* plants, is similar with regard to meristematic tissues. Altogether, our expression studies suggest that the action of TCP8 and TCP23 on flowering would not be related to a different spatial or daily transcriptional expression. This is consistent with the fact that expression of these proteins under the control of a constitutive promoter, like *CaMV 35S*, causes opposite changes in flowering time.

3.5. TCP8 and TCP23 bind a class I TCP target sequence *in vivo* in yeast

The phenotypic analysis and the expression studies of *TCP8* and *TCP23* overexpressing plants suggested that their different effects on flowering would depend on the properties of the encoded proteins. Accordingly, we first studied the ability of TCP8 and TCP23 to bind the class I TCP target sequence GTGGGACC (TCP-BS) using a yeast one-hybrid assay with a strain containing a construct in which six TCP-BS tandem copies are located upstream of the minimal promoter of the *S. cerevisiae CYC1* gene fused to the *E. coli LacZ* reporter (Viola et al., 2012). Expression of TCP8 and TCP23 fused to the GAL4 activation domain (AD) in this yeast strain caused a considerable increase in β-galactosidase activity relative to cells that express the AD alone (Fig. 4A). Conversely, we did not observe any induction of the reporter gene when a strain containing a similar construct with a mutated version of TCP-BS that is not bound by class I TCPs (TCP-BS_{mut}, GTAGGCCC Viola and col., 2011) was used (Fig. 4A). These results suggest that both TCPs are able to specifically bind class I TCP target sequences.

3.6. TCP8 and TCP23 form protein-protein complexes with each other

TCP proteins act as homodimers but are also capable of forming heterodimers with other TCP members of the same class (Danisman et al., 2013). The fact that TCP8 and TCP23 play opposite roles in the control of flowering prompted us to explore whether these proteins could interact with each other. To do this, a yeast two-hybrid assay in which these TCP proteins were expressed fused to the GAL4 AD or DNA binding domain (BD) in a yeast strain carrying the *LacZ* reporter gene under the control of a promoter containing GAL4 binding sites was performed. The results indicated that TCP8 is able to form homodimers, while no activation of the reporter gene was observed when TCP23 fusions to the AD and BD were co-expressed (Fig. 4B). In addition, AD and BD fusions of TCP23 showed activation of the reporter when co-expressed with TCP8-BD and TCP8-AD, respectively (Fig. 4B). These results suggest that TCP8 is able to interact with TCP23 and that TCP23 may have a tendency to form heterodimers with TCP8 rather than homodimers. However, the fact that TCP23-AD is able to activate a reporter gene based on TCP-BS (Fig. 4A) suggests that TCP23 also forms homodimers.

TCP8, but not TCP23, is a transcriptional activator

Since we observed that TCP8 and TCP23 can bind class I TCP binding motifs *in vivo* in yeast cells, we decided to use a similar synthetic promoter to analyze the transcriptional

activity of these proteins *in vivo* in plants. Then, we generated a synthetic promoter containing six copies of the class I TCP binding motif fused to a minimal *CaMV 35S* promoter upstream of the *GUS* reporter gene (Fig. 5A). When *N. benthamiana* leaves were transiently co-transformed with the reporter and the *35S::TCP8* construct, *GUS* expression was significantly increased relative to a control in which GFP was expressed instead of TCP8 (Fig. 5A,B), indicating that TCP8 acts as a transcriptional activator. However, TCP23 was not able to modify the transcription of the synthetic promoter by itself (Fig. 5A,B) although it would be able to bind it, according to the yeast one-hybrid results (Fig. 4A). This suggests that TCP23 lacks a transcriptional activation activity and may require additional factors to modulate transcription. Next, we wondered about the transcriptional function of TCP8 and TCP23 in the context of a target gene. Since *SOC1* is directly regulated by class I TCPs through binding to TCP binding motifs present in its promoter (Lucero et al., 2017; Li et al., 2021), we analyzed the effect of TCP8 and TCP23 on *SOC1* promoter activity. For this, *Arabidopsis* seedlings harboring the *SOC1* promoter fused to the *GUS* reporter gene were transiently transformed with *35S::TCP8* and *35S::TCP23* constructs (Fig. 5A). Transformation with *35S::GFP* was used as a negative control. We observed that TCP8 significantly activated the expression of the reporter gene (Fig. 5C). However, no effect on the transcriptional activity of the *SOC1* promoter was observed for TCP23 (Fig. 5C) suggesting that TCP23 may regulate *SOC1* expression indirectly, possibly through additional factors. Indeed, the ability of TCP8 to activate the *SOC1* promoter decreased when TCP23 was co-expressed (Fig. 5C). This may indicate that TCP23 regulates *SOC1* expression in part through competition with TCP8, and perhaps other activating TCPs.

3.7. The N-terminal portion located upstream of the TCP domain is responsible for the differential effect of TCP8 and TCP23 on flowering time

The fact that ectopic expression of *TCP8* and *TCP23* cause opposite effects on flowering suggests that their differential action depends on the characteristics of the encoded proteins. To date, no motifs or conserved sequences other than the TCP domain have been described for class I TCP proteins, making it difficult to find variations in TCP8 and TCP23 that might be responsible for the opposite regulation of flowering. By sequence analysis, we identified two conserved regions in TCP8 and TCP23: a region rich in serine residues next to the TCP domain, that we named “SRR” (serine-rich

region) (Fig. 6A), and another region towards the C-terminus that contains hydrophobic and aromatic amino acids that we called FWMLPV (Fig. 6A). To identify the protein regions responsible for the functional differences between TCP8 and TCP23, chimeric proteins combining regions of these two transcription factors were designed using sequences located proximal to these conserved regions as splice sites to avoid the interruption of amino acid sequences that may be important for protein structure or function. The two conserved regions allowed proteins to be divided into thirds and joined in reciprocal combinations (Fig. 6B). All the chimeras were expressed in *Arabidopsis* under the control of the *CaMV 35S* promoter (Fig. S5). Plants expressing the Ch-1 chimeric protein, consisting of the N-terminal two-thirds (up to region FWMLPV) of TCP8 fused to the C-terminal third of TCP23 (Fig. 6B) reached the reproductive stage before wild-type plants (Fig. 6C-E). In turn, *35S::Ch-2* plants, that expressed the reciprocal construct, exhibited a late flowering phenotype (Fig. 6C-E). Flowering was also accelerated in *35S::Ch-3* plants, consisting of the N-terminal third (up to the SRR) of TCP8 fused to the C-terminal two-thirds of TCP23 (Fig. 6B-E), while *35S::Ch-4* plants, which express a chimera of the N-terminal third of TCP23 fused to the rest of TCP8, showed a late-flowering phenotype (Fig. 6C-E). Altogether, the results suggest that the N-terminal third of the proteins, consisting of the TCP domain and a variable N-terminal arm, contains the features that determine the opposite roles of TCP8 and TCP23 in flowering time regulation.

The TCP domain is highly conserved in TCP family members (Uberti Manassero et al., 2013) and the sequence identity of the TCP8 and TCP23 TCP domains is 94.4%. Comparison of 119 class I TCP domain sequences from various species showed the presence of a conserved Gly at position 32 (92.6%) which is occupied by a Gln residue in TCP23 (Fig. S6, Fig. 6A). Based on the structure reported by Sun et al. (2020), Gly32 is located at the beginning of the loop that connects helices α1 and α2 of the HLH motif of the TCP domain. We reasoned that the change from a non-polar, helix-breaking small residue (Gly32) to a polar and largest one (Gln32) could have effects on the structure of the HLH motif of the TCP domain. To analyze whether residue 32 is responsible for the opposite functions of TCP23 and TCP8 in flowering control, we mutagenized Gly32 to Gln in the Ch-3 chimera and generated the Ch-3_{G32Q} mutant (Fig. 7A). In addition, to evaluate the effect of the N-terminal region located upstream of the TCP domain, we constructed a chimeric gene in which the N-terminal coding sequence of *TCP8* was replaced with that of *TCP23* in the Ch-3 chimera (N_{TCP23}-Ch-3, Fig. 7A). The Ch-3_{G32Q}

mutant and the N_{TCP23}-Ch-3 chimera were expressed under the *CaMV 35S* promoter in *Arabidopsis* and T3 homozygous lines with transcript levels of the transgenes similar or higher than those of Ch-3 lines were selected (Fig. S5B). Like *35S::Ch-3* plants, *35S::Ch-3$_{G32Q}$* lines exhibited an early flowering phenotype compared with wild-type plants (Fig. 7B-D, Fig. S7). Like *35S::TCP8*, *35S::Ch-1* and *35S::Ch-3* lines, *35S::Ch-3$_{G32Q}$* plants showed significantly increased *FT* and *SOC1* transcript levels compared to wild-type plants (Fig. 7E,F). These results indicate that the identity of residue 32 of the TCP domain does not play a significant role in the regulation of flowering time by the TCPs. On the other hand, constitutive expression of N_{TCP23}-Ch-3 caused a delay in flowering compared to wild-type plants (Fig. 7B-D), which is opposite to what is observed with Ch-3. In agreement, expression of the flowering integrators *FT* and *SOC1* was lower in N_{TCP23}-Ch-3 expressing plants than in wild-type plants (Fig. 7E,F). Since Ch-3 and N_{TCP23}-Ch-3 only differ in the N-terminal region located upstream of the TCP domain, it can be postulated that this region is mainly responsible for the differential effect of TCP8 and TCP23 on flowering. In fact, all analyzed proteins that contain the TCP8 N-terminal region accelerate flowering while those containing the TCP23 N-terminal region show the opposite behavior.

4. Discussion

In this study, we found that the class I TCP protein TCP8 from *Arabidopsis* acts as an inducer of the transition to the reproductive stage. *TCP8* single mutant plants show delayed flowering and a delay in maximal *FT* expression. *TCP8* promoter activity was detected in the vascular tissues of leaves, the sites where *FT* is induced (Takada and Goto, 2003). FT activates the expression of *SOC1*, which also shows reduced expression in *TCP8* mutant plants and increased expression in *TCP8* overexpressing plants. This behavior is similar that of TCP15 and TCP7, other class I TCPs that promote flowering directly inducing the expression of *SOC1* by binding to a TCP box present in its promoter (Lucero et al., 2017; Li et al., 2021). Since *TCP8* is expressed in the shoot apical meristem and the vasculature, it is possible that TCP8 regulates *SOC1* through *FT* and, as observed here, also directly. Moreover, the *tcp8 tcp14 tcp15* triple mutant shows delayed flowering relative to the *tcp14 tcp15* double mutant, reinforcing the idea that these three class I TCPs additively participate in the induction of flowering. Consistent with this, Lucero et al. (2017) observed that *tcp14 tcp15* mutant plants

expressing an artificial miRNA that reduces *TCP8* and *TCP22* expression show a delay in flowering relative to the *tcp14 tcp15* mutant. Different from our results, Wang et al. (2019) observed a delay in flowering upon *TCP8* overexpression in *Arabidopsis*. The reason for these different observations is not obvious and deserve further analysis. Nevertheless, the fact that *TCP8* mutation in wild-type, *tcp14 tcp15* and *tcp23* backgrounds delays flowering is consistent with TCP8 acting as a flowering activator. In addition, using the same vector system, we observed a negative effect on flowering after expression of TCP23 and several chimeric proteins, ruling out unspecific effects due to extra sequences present in the constructs used for transformation. In addition, the fact that the flowering phenotype of the *tcp8 tcp23* double mutant is similar to that of wild-type plants confirms the antagonistic action of TCP8 and TCP23 and suggests that these proteins exert their effects probably acting on the same group of genes. In agreement with this idea, *TCP23* overexpressing and mutant plants also showed altered expression of *FT* and *SOC1*. Even if *TCP23* has a more restricted spatial expression pattern than *TCP8*, both gene promoters are active in the apical meristem and young leaves. In addition, since expression of *TCP8* and *TCP23* from the *CaMV 35S* promoter causes opposite flowering phenotypes, it can be speculated that their differential role in the control of flowering is mainly due to different properties of the encoded proteins rather than to differences in the expression patterns of the genes. In agreement with this, there would be differences in the mechanisms used by these proteins to regulate the transcription of *SOC1*. TCP8 seems to directly activate the transcription of *SOC1*, similar to the positive regulators of flowering TCP7 and TCP15 (Lucero et al., 2017; Li et al., 2021). In contrast, TCP23 would regulate the expression of *SOC1* by inhibiting the DNA binding or transcriptional activity of an activator of *SOC1* (such as TCP8 and related TCPs), or facilitating DNA binding or activity of a *SOC1* repressor. This is reminiscent of the mechanism used by the flowering repressors TCP20 and TCP22, that bind TCP boxes in the promoter of *CCA1* but activate transcription only in complex with the co-activator LWD1 (Wu et al., 2016). In this scenario, the final effect on *SOC1* expression, and flowering time, will be determined by the relative levels of the individual TCP proteins.

Detailed phylogenetic studies have not been reported for class I TCPs and in several cases alignments were performed only with the sequence of the TCP domain, omitting the contribution of other regions of the proteins, which are highly divergent. Although

various authors grouped *Arabidopsis* class I TCPs differently, TCP14 and TCP15 appear as closely related proteins and are generally clustered together with TCP8, TCP22 and TCP23 (Aggarwal et al., 2010; Aguilar-Martinez and Sinha, 2013; Danisman et al., 2013; Uberti Manassero et al., 2013; Li, 2015). However, TCP22 and TCP23 control flowering in an opposite way than TCP14 and TCP15 (Balsemao-Pires et al., 2013; Wu et al., 2016; Lucero et al., 2017). Single mutants in the class I TCPs *TCP20* and *TCP7* have also been reported to show early and late flowering phenotypes, respectively (Wu et al., 2016; Li et al., 2021). This suggests that, although a certain degree of functional redundancy has been proposed for class I TCPs (Cubas et al., 1999; Danisman et al., 2013), different members of this class show antagonistic functions in the control of flowering. Considering the similarities in the structure of the respective TCP domains, it is logical to assume that these different functions are conferred by differences in other portions of the proteins. In agreement with this, we found that chimeric proteins between TCP8 and TCP23 containing the N-terminal region of TCP8 induce flowering and the expression of *FT* and *SOC1* when they are expressed in plants, while the expression of proteins containing the N-terminal region of TCP23 show the opposite behavior. Thus, our results show that the identity of the N-terminal arm is the main determinant of the positive or negative effect on this process. Recently, it has been reported that the N-terminal domain of TEN, a class II TCP protein from cucumber (*Cucumis sativus L.*), is involved in the modulation of the chromatin state (Yang et al., 2020). In this protein, the C-terminal region binds to DNA sequences different from the TCP box, while the N-terminal region acetylates histones to modulate gene expression. However, this N-terminal region (and consequently the ability to acetylate histones) is not present in all class II TCPs of the CYC/TB1 clade or in class I TCPs, making it unlikely that the differences between TCP8 and TCP23 are related to this. Rather, as discussed above, the differences between these two class I TCPs may reside in their different activation capacity and their ability to interact among themselves and probably with other proteins, which would require further experimentation. Altogether, different observations indicate that, in addition to the TCP domain, the N-terminal regions of both class I and class II TCP proteins play an important role in the specificity of action of these transcription factors, increasing the range of their possible functions.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplemental Fig. 1. Flowering phenotype of *TCP8* overexpressing lines grown under long-day conditions

Supplemental Fig. 2. Flowering phenotype of *TCP8* overexpressing lines grown under short-day conditions

Supplemental Fig. 3. Inflorescence stem length of *TCP8* mutant and overexpressing plants

Supplemental Fig. 4. Flowering time phenotype of *35S::TCP23* plants

Supplemental Fig. 5. Expression levels of chimeric constructs in *Arabidopsis* transformed plants

Supplemental Fig. 6. Protein sequence alignment of class I TCP domains

Supplemental Fig. 7. Flowering time phenotype of *35S::Ch3*$_{G32Q}$ and *35S::N*$_{TCP23}$*-Ch3* plants

Supplemental Fig. 8. Expression levels of *TCP8* and *TCP23* in transcriptional activity assays.

Table S1. Oligonucleotides used in this study

Table S2. Gene names of selected sequences used in sequence alignment of class I TCP domains

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Figure legends

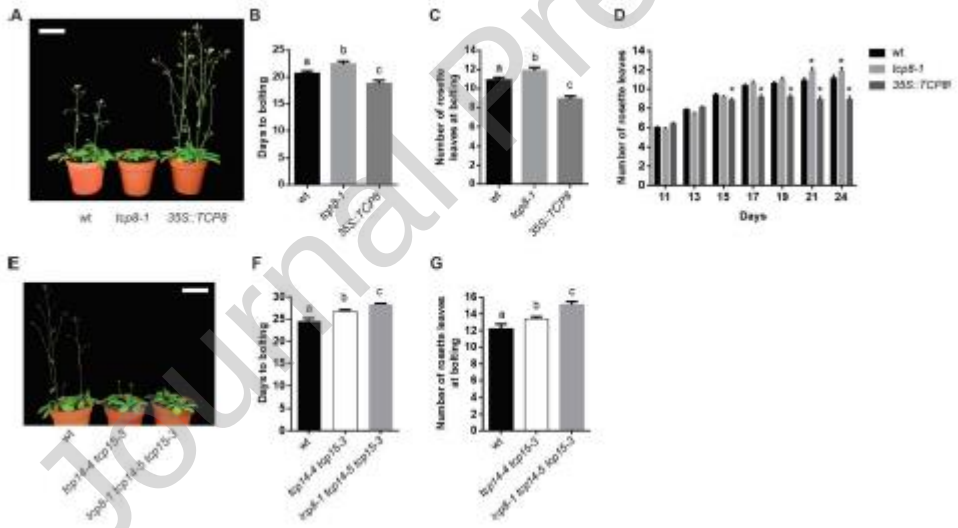

Fig. 1. TCP8 is a positive regulator of flowering time. (A) Representative image of three-week-old wild-type (wt), *tcp8-1* and *35S::TCP8* plants. Scale bar: 5 cm. (B, C) Flowering time phenotype measured as number of days required for flowering (B) and number of rosette leaves at bolting (C) of the genotypes indicated in (A). (D) Number of rosette leaves in wild-type (wt), *tcp8-1* and *35S:TCP8* plants during the transition to flowering. (E) Representative image of four-week-old wild-type (wt), *tcp14-4 tcp15-3* and *tcp8-1 tcp14-5 tcp15-3* plants. Scale bar: 5 cm. (F, G) Flowering time phenotype of the genotypes indicated in (E) measured as number of days required for flowering (F) and number of rosette leaves at bolting (G). Bars indicate the mean±SD of 12 plants per

genotype. In (B, C, F, G), different letters denote statistically significant differences (P <0.05; ANOVA). In (D), asterisks indicate statistically significant differences relative to wild-type (P <0.05; ANOVA). The experiments were repeated three times with similar results.

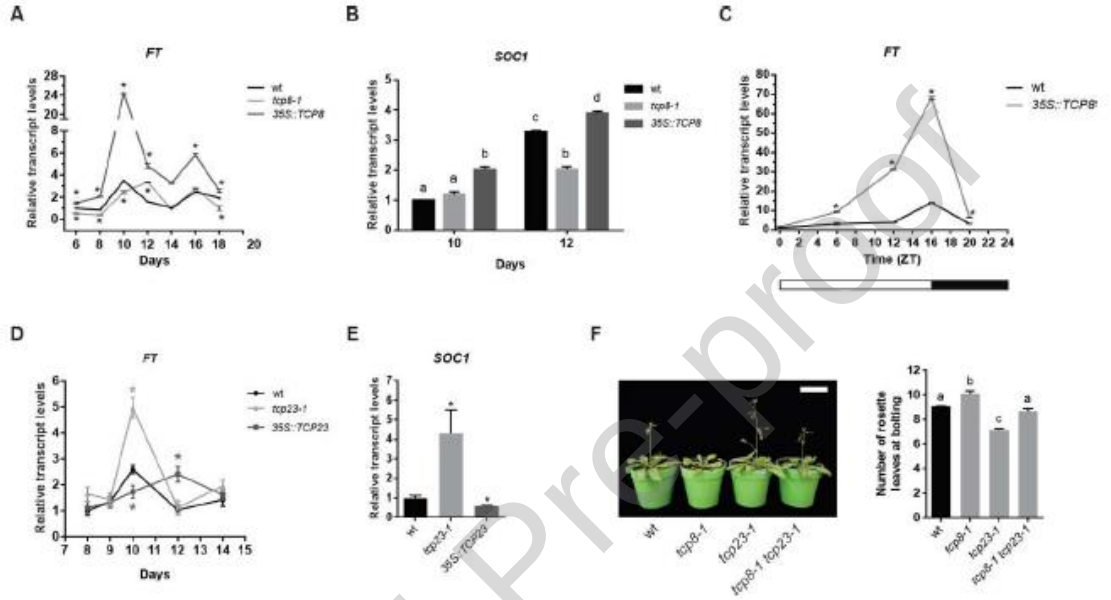

Fig. 2. TCP8 and TCP23 antagonistically regulate the expression of flowering genes. (A) Quantitative analysis of *FT* transcript levels in wild-type (wt), *tcp8-1* and *35S::TCP8* seedlings at different times after sowing. Samples were collected 1 h before the end of the illumination period (ZT15). (B) *SOC1* transcript levels at ZT15 in seedlings of the indicated genotypes. (C) Diurnal expression pattern of *FT* in 10-day-old seedlings of wild-type (wt) and *35S::TCP8* plants. White and black bars represent the light and dark periods, respectively. (D) Quantitative analysis of *FT* transcript levels in wild-type (wt), *tcp23-1* and *35S::TCP23* seedlings at different times after sowing. Samples were collected at ZT15. (E) *SOC1* transcript levels at ZT15 in 12-day-old seedlings of the indicated genotypes. (F) Flowering time phenotype of wild-type (wt), *tcp8-1*, *tcp23-1* and *tcp8-1 tcp23-1* plants measured as number of rosette leaves at bolting. Representative images are shown on the left (Scale bar: 5 cm). (A-E) Values are expressed relative to wild-type and correspond to the mean±SD of three biological replicates. (F) Bars indicate the mean±SEM of 12 plants per genotype. (A, C-E) Asterisks indicate significant differences compared with the wild-type (P <0.05;

Student's t-test). (B, F) Different letters denote statistically significant differences (P <0.05; ANOVA). The experiments were repeated three times with similar results.

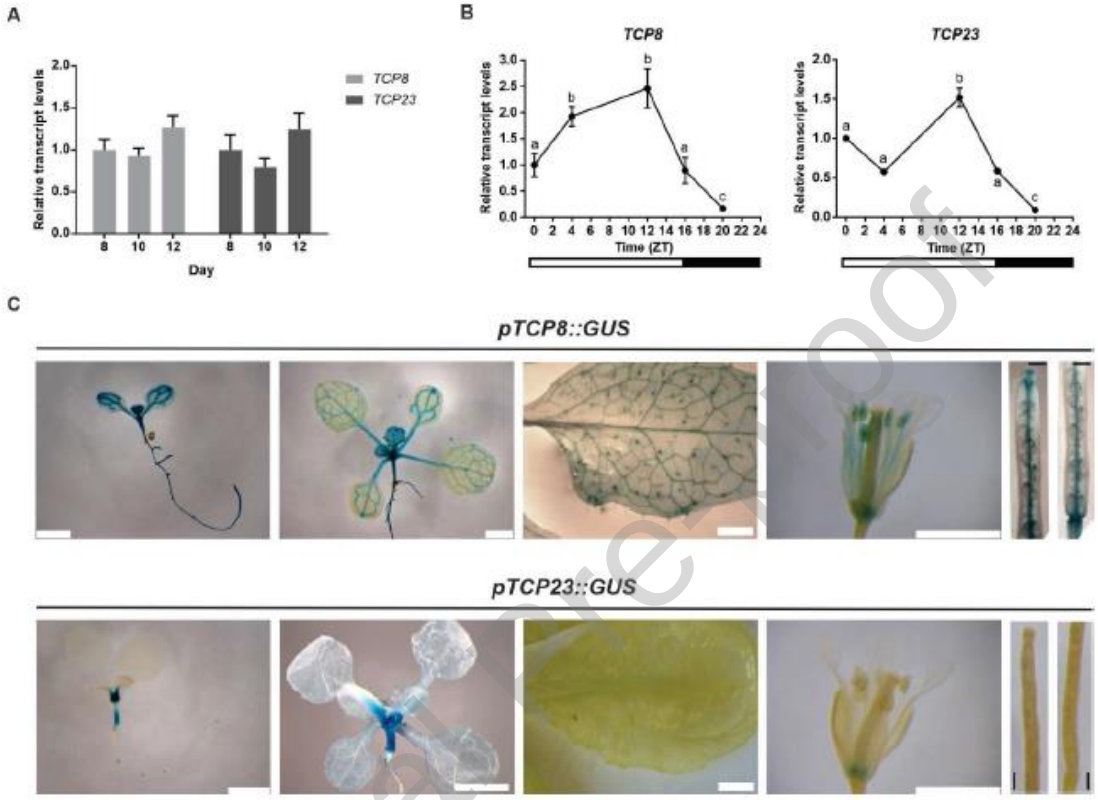

Fig. 3. Analysis of *TCP8* and *TCP23* expression patterns. (A) RT-qPCR analysis of the expression of *TCP8* and *TCP23* in wild-type seedlings at days 8, 10 and 12 after sowing. Samples for RNA extraction were collected at ZT15. The bars indicate the mean±SD of three biological replicates. (B) Diurnal levels of *TCP8* and *TCP23* transcripts in 10-day-old wild-type seedlings. White and black bars represent the light and dark periods, respectively. Values are expressed relative to those at the start of the light period. Different letters denote statistically significant differences (P <0.05; ANOVA). (C) Histochemical GUS staining of plants transformed with a fusion of the *TCP8* (top) or *TCP23* (bottom) promoters fused to *gus*. From left to right: 6-day- and 12-day-old plants, rosette leaf, flower and siliques from a 5-week-old plant. Scale bars: 2 mm (white), 0.5 mm (black). The experiments were repeated three times with similar results.

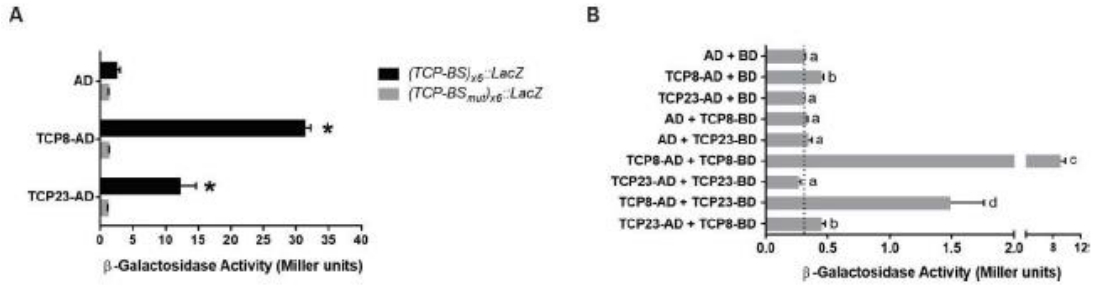

Fig. 4. DNA binding and protein-protein interactions of TCP8 and TCP23. (A) Binding of TCP8 and TCP23 to a class I TCP target sequence *in vivo* in yeast. Specific β-galactosidase activity of yeast cells carrying six tandem copies of the class I TCP binding site (TCP-BS, black) or a mutated version of this site ($TCP\text{-}BS_{mut}$, gray) fused to the *LacZ* gene containing a minimal promoter and transformed with constructs expressing the indicated TCP proteins fused to the GAL4 activation domain (AD). Cells transformed with a construct that expresses only the AD were used as controls. The mean ± SD of three independent measurements is shown. Asterisks indicate statistically significant differences relative to the corresponding AD control ($P < 0.05$; ANOVA). The experiment was repeated three times with similar results. (B) Yeast two-hybrid analysis of the interaction between TCP8 and TCP23. AD and BD indicate fusions to the activation domain or the DNA-binding domain of GAL4, respectively. Constructs expressing only AD or BD were used as controls. Average β-galactosidase activity values obtained in three independent tests are shown. Different letters denote statistically significant differences ($P < 0.05$; ANOVA). The experiment was repeated three times with similar results.

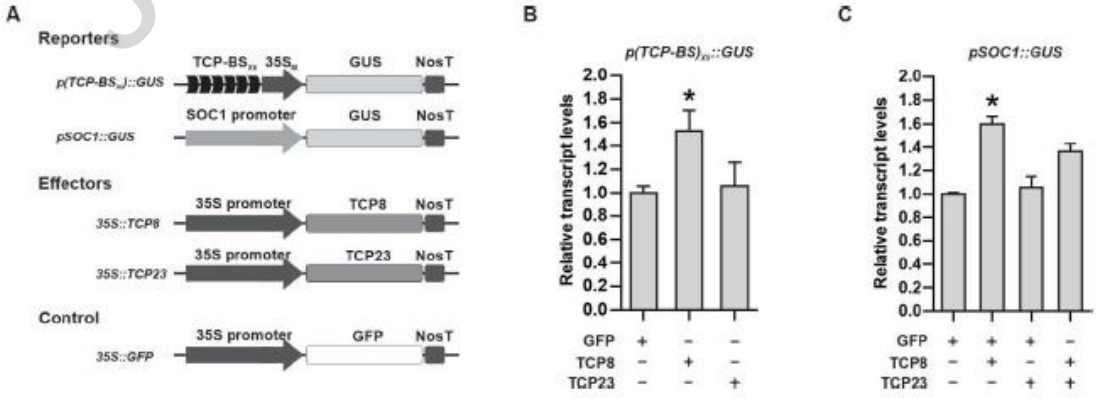

Fig. 5. Transcriptional activity of TCP8 and TCP23 *in vivo* in plants. (A) Schematic diagram of reporters, effectors and control constructs used in transient expression

analysis. (B) *N. benthamiana* leaves were co-infiltrated with the *p(TCP-BS$_{X6}$)::GUS* reporter and the *35S::GFP* (GFP, negative control), *35S::TCP8* (TCP8) and *35S::TCP23* (TCP23) constructs in a ratio of 1:1. (C) *Arabidopsis* seedlings carrying the *pSOC1::GUS* construct were transiently transformed with the indicated combinations of the effectors *35S::TCP8* (TCP8) and *35S::TCP23* (TCP23) and the *35S::GFP* (GFP) control in a ratio of 1:1. (B,C) The transcriptional activity of promoters, determined by analysis of the *GUS* reporter transcript levels, is expressed relative to the *35S::GFP* control and corresponds to the mean±SD of three biological replicates. Asterisks indicate significant differences compared with the control (P <0.05; ANOVA). *TCP8* and *TCP23* transcript levels in transient assays are shown in Fig. S8. The experiments were repeated three times with similar results. 35S$_M$: minimal *CaMV 35S* promoter.

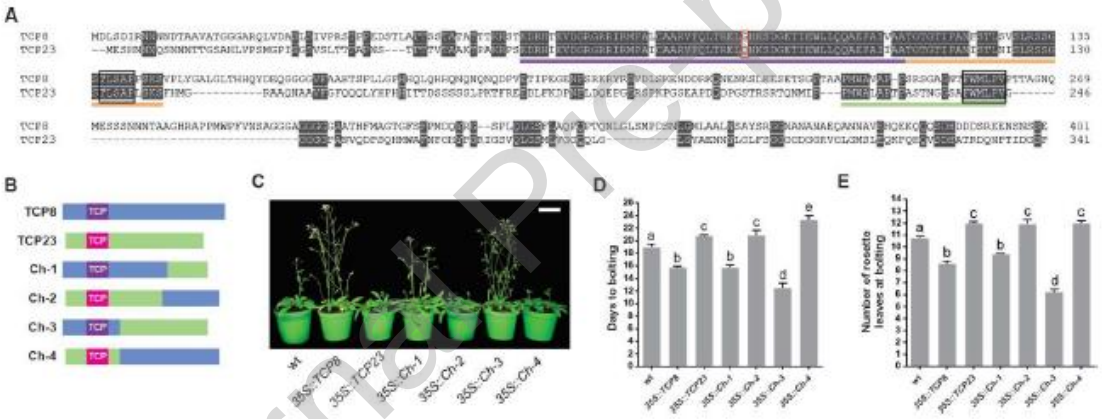

Fig. 6. Analysis of plants expressing chimeras between TCP8 and TCP23. (A) Alignment of TCP8 and TCP23. Conserved regions are shaded. The TCP domain, the serine-rich region (SRR) and the FWMLPV region are underlined with purple, orange and green lines, respectively. The red rectangle indicates the amino acids at position 32 of the TCP domain. Black rectangles show the splice sites used to construct the chimeric proteins. (B) Schematic structure of the chimeric proteins used to analyze their effect on flowering time. Portions from TCP8 or TCP23 are indicated in different colors. The names of the proteins are indicated on the left. TCP, TCP domain. (C) Representative image of 22-day-old wild-type (wt), *35S::TCP8*, *35S::TCP23*, *35S::Ch1*, *35S::Ch2*, *35S::Ch3* and *35S::Ch4* plants grown under long-day conditions. Scale bar: 5 cm. (D, E) Flowering time measured as number of days required for flowering (D) and number of rosette leaves at bolting (E) of the genotypes indicated in (C). Bars indicate the mean ± SD of 12 plants per genotype. Different letters denote statistically significant

differences (P <0.05; ANOVA). The experiments were repeated three times with similar results.

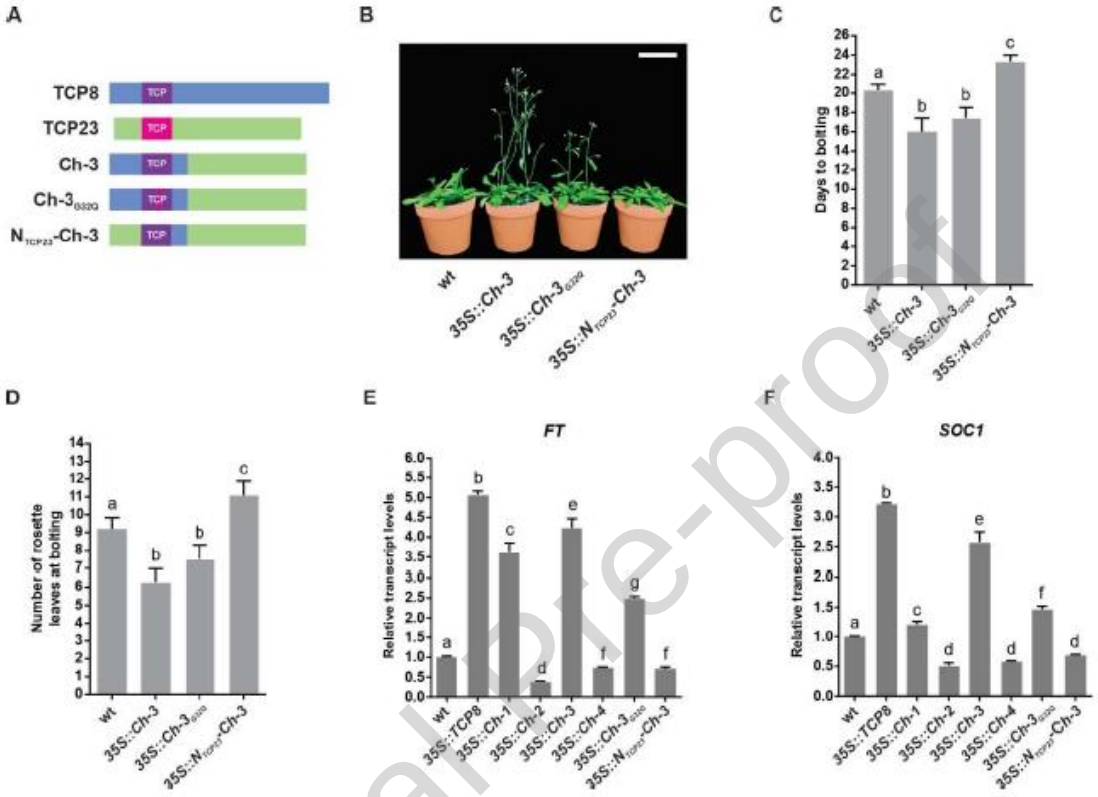

Fig. 7. The N-terminal region located upstream of the TCP domain is responsible for the differential effect of TCP8 and TCP23 on flowering. (A) Schematic structure of the $Ch3_{G32Q}$ and N_{TCP23}-Ch3 proteins. Portions from TCP8 or TCP23 are indicated in different colors. G32Q indicates the presence of glutamine at position 32 of the TCP8 TCP domain. The names of the proteins are indicated on the left. (B) Representative image of 20-day-old wild-type (wt), *35S::Ch3*, *35S::Ch3*$_{G32Q}$ and *35S::N*$_{TCP23}$*-Ch3* plants grown under long-day conditions. Scale bar: 5 cm. (C, D) Flowering time, measured as number of days required for flowering (C) and number of rosette leaves at bolting (D) of the genotypes indicated in (B). Bars indicate the mean ± SD of 12 plants per genotype. (E, F) RT-qPCR analysis of *FT* (E) and *SOC1* (F) transcript levels at ZT15 in 10-day-old wild-type (wt), *35S::TCP8*, *35S::Ch1*, *35S::Ch2*, *35S::Ch3*, *35S::Ch4*, *35S::Ch3*$_{G32Q}$ and *35S::N*$_{TCP23}$*-Ch3* seedlings. Values are expressed relative to wild-type. The bars indicate the mean±SD of three biological replicates. (C-F) Different letters denote statistically significant differences (P <0.05; ANOVA). The experiments were repeated three times with similar results.

Author contributions

I.L.V and D.H.G designed the research; A.C and A.L.A performed the experiments; I.L.V and D.H.G analyzed the data and wrote the manuscript; all authors read and approved the manuscript.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- The *Arabidopsis* class I TCP TCP8 acts as positive regulator of flowering time.
- TCP8 and the related class I TCP TCP23 antagonistically regulate flowering time and *FT* and *SOC1* expression.
- Both TCP8 and TCP23 specifically bind class I TCP target sequences *in vivo* and form heterodimers.
- TCP8 activates transcription and its transcriptional activity is affected in the presence of TCP23.
- The N-terminal portions located upstream of the TCP domain determine the differential effects of TCP8 and TCP23 on flowering time.