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ORIGINAL PAPER

Heterologous expression of *Chrysanthemum nankingense TCP13* suppresses leaf development in *Arabidopsis thaliana*

Xiangyu Qi[1] · Yixin Qu[1] · Jiafu Jiang[1] · Yunxiao Guan[1] · Aiping Song[1] · Peipei Cao[1] · Zhiyong Guan[1] · Weimin Fang[1] · Sumei Chen[1] · Fadi Chen[1] · Haibin Wang[1]

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Abstract

TCP genes encode plant-specific transcription factors in various species that they play important roles in plant growth and development. In the present study, we cloned the TCP transcription factor, *TCP13*, from *Chrysanthemum nankingense* (*CnTCP13*). *CnTCP13* belongs to the class II CIN subfamily of the TCP family and harbors an atypical basic-helix-loop-helix motif, that was preferentially expressed in the leaf. *CnTCP13* transcription was significantly inhibited 24 h after exogenous application of 6-BA. The CnTCP13 protein was localized in the nuclei of transformed onion epidermal cells and did not exhibit transcriptional activation. Heterologous expression of *CnTCP13* in *Arabidopsis thaliana* reduced leaf size. qRT-PCR analysis revealed that the transcription levels of cell division-related genes were altered in transgenic *A. thaliana* containing *CnTCP13*. CnTCP2 and CnF-box were identified as putative interaction proteins of CnTCP13 by a yeast two-hybrid assay and bimolecular fluorescence complementation. *CnF-box* belongs to the F-box family and is abundantly expressed in roots. The CnF-box protein was localized in the nucleus and had no transcriptional activation. In *A. thaliana*, *CnF-box* overexpression led to strong crinkling of leaves. Taken together, *CnTCP13* is involved in leaf development through the regulation of cell division-related genes, and likely by its interaction with CnTCP2 and CnF-box.

Keywords TCP transcription factor · Transcription profiling · Cell proliferation · Yeast two-hybrid assay · BiFC

Introduction

Leaves provide the basis for plant growth through photosynthesis. In plants, leaves develop from a mass of pluripotent cells at the shoot apical meristem, then display marginal meristem activity, and finally expand to form a mature leaf (Bar and Ori 2014). Leaf growth is maintained by cell proliferation and cell expansion. Various phases of the cell cycle depend on a number of proteins, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (Breuer et al. 2010). Leaf development is a complex molecular network regulated by intercellular signaling molecules, including plant hormones, sugars and transcription factors (Kalve et al. 2014).

TEOSINTE-LIKE1, *CYCLOIDEA* and *PROLIFERATING CELL FACTOR* 1 (TCP) transcription factors constitute a small family of plant-specific transcription factors whose members share functions in plant development (Martín-Trillo and Cubas 2010). The TCP domain harbors a 59 amino acid basic helix-loop-helix (bHLH) motif that is involved in DNA binding and protein-protein interactions (Martín-Trillo and Cubas 2010). *Populus euphratica*, *Brassica rapa* and *Medicago truncatula* have 33, 39 and 21 *TCP* genes, respectively (Ma et al. 2016; Du et al. 2017; Wang et al. 2018). Based on the motif, members of the TCP family are classified into class I (PCF or TCP-P class) and class II (TCP-C class) subfamilies (Navaud et al. 2007). Class I and II *TCP* genes act antagonistically in modulating plant cell proliferation and expansion by competing for similar targets or partners. As such, while class I *TCP* genes promote cell proliferation, class II *TCP* genes prevent cell proliferation

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✉ Haibin Wang
hb@njau.edu.cn

[1] State Key Laboratory of Crop Genetics and Germplasm Enhancement, Key Laboratory of Landscaping, Ministry of Agriculture and Rural Affairs. Key Laboratory of Biology of Ornamental Plants in East China, National Forestry and Grassland Administration, College of Horticulture, Nanjing Agricultural University, Weigang No. 1, Nanjing 210095, Jiangsu, China

(Martín-Trillo and Cubas 2010). Inhibition of the class I gene *AtTCP11* at the C-terminal domain resulted in curly rosette leaves with margins tending to fold upwards (Viola et al. 2011). *Arabidopsis TCP14* and *TCP15* regulate trichromes development on stems and leaves, and increase cuticle permeability (Camoirano et al. 2020). The cell cycle genes, *CYCA2;3* and *CYCB1;1*, are known to be the targets of *AtTCP14* (Daviere et al. 2014). The class I gene, *TCP9*, acts with *TCP20* to positively regulate leaf senescence through jasmonic acid signaling pathway (Danisman et al. 2012). The class II gene, *AtTCP4*, directly activates *MIR396b*, which encodes a miRNA that prevents cell proliferation in leaves (Schommer et al. 2014). *BrrTCP2* overexpression in wild-type *A. thaliana* reduces leaf size and also restores the leaf phenotype of the triple mutants of *tcp2/4/10* to wild-type (Du et al. 2017). In Chinese cabbage, *BrTCP3*, *BrTCP4a* and *BrTCP24a* are highly expressed at the rosette stage (Liu et al. 2018). AtTCP13 inhibits leaf growth by repressing *Arabidopsis thaliana homeobox 12* (*ATHB12*) expression, which promotes leaf growth mainly during the cell expansion phase (Hur et al. 2019). *CIN* is expressed in the lamina, proximal to and, perhaps, overlapping the cell-cycle arrest front, and serves as a suppressor of cell proliferation in leaves (Nath et al. 2003).

F-box proteins (FBPs) constitute one of the largest protein families in the plant kingdom. FBPs harbor a roughly conserved F-box motif composed of 40–60 amino acid residues with few invariant positions (Abd-Hamid et al. 2020). Based on the C-terminal region, FBPs are classified into several subfamilies, including leucine-rich repeats, WD-40, Kelch repeats, F-box associated and TUBBY. FBPs are involved in various plant biological processes, including plant development and morphogenesis, cell signaling, circadian clock, cell cycle, phytohormone signaling, biotic and abiotic stress responses (Dharmasiri et al. 2005; Cao et al. 2008; Chen et al. 2014). The FBP STERILE APETALA gene positively regulates cell proliferation through degradation of the plant-specific factors PEAPOD1 and PEAPOD2 in *A. thaliana* (Wang et al. 2016). Also in *Arabidopsis*, FBXL negatively regulates vein pattern formation (Cui et al. 2016), and FBX92 suppresses leaf growth by negatively regulating several cell cycle genes (Baute et al. 2017).

Chrysanthemum nankingense Hand.-Mazz. (Anthemideae, Asteraceae) is a diploid species native to China and is closely related to the important ornamental species *C. morifolium* (Song et al. 2015). Among the *C. morifolium TCP* genes, *BRC1* inhibits lateral branching (Chen et al. 2013), *CmCYC* regulates the ray floret growth (Huang et al. 2016), and *CmTCP14* reduces organ size (Zhang et al. 2017). However, little is known regarding *TCP* genes in *C. nankingense*. Therefore, in this study, we cloned the *TCP* transcription factor *TCP13* from *C. nankingense* (*CnTCP13*), revealed the transcriptional behavior of *CnTCP13*, and studied the phenotypic effect of heterologously expressing it in *A. Thaliana*.

Materials and methods

Plant materials

The accession of *C. nankingense* used was obtained from the Chrysanthemum Germplasm Resource Preserving Center, Nanjing Agricultural University, China. Rooted cuttings were grown in a greenhouse (23 °C during the day and 15 °C at night; 70–75 % relative humidity; natural light). *A. thaliana* (ecotype Col-0) plants were grown in a 1:1:1 (*v*/*v*/*v*) mixture of soil, perlite, and vermiculite under a 16 h photoperiod (80–110 μmol m^{-2} s^{-1} illumination) at a day/night temperature of 21 °C/18°C.

Isolation and sequencing of *CnTCP13* cDNA

Total RNA was isolated from the *C. nankingense* leaves using the RNAiso reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The first-strand cDNA was synthesized from 1 μg of total RNA using an M-MLV RTase cDNA Synthesis Kit (TaKaRa), following the manufacturer's instructions. The full-length *CnTCP13* cDNA was amplified using the specific primer pair CnTCP13-F/R (Table S1) that are based on the sequence identified from the *C. nankingense* transcriptome database (Wang et al. 2013). The amplified products were purified using the Axy-Prep DNA Gel Extraction Kit (Axygen, Shanghai, China) and then inserted into the plasmid pMD19-T (TaKaRa) for sequencing.

Phylogenetic analysis

From the Arabidopsis Tair Database (https://www.arabidopsis.org/), *A. thaliana* TCP amino acid sequences were downloaded. The AtTCP sequences were combined with the obtained CnTCP13 and used to conduct a multiple sequence alignment using ClustalW (Larkin et al. 2007). The phylogenetic analysis was performed using the neighbor-joining method and a graphical representation was drawn using MEGA V6 (Tamura et al. 2013). Bootstrap values were estimated using 1000 bootstrap replicates.

Transcription profiling of *CnTCP13* using qRT-PCR

Total RNA was extracted from various tissues of *C. nankingense* using the RNAiso reagent (Takara) according to the manufacturer's instructions. Transcription profiling was performed using qRT-PCR with the primer pair

CnTCP13-QF/QR (Table S1). *CnEF1α* was used as the reference (Table S1) (Gao et al. 2015). Three biological replicates and three technical replicates were used for qRT-PCR. The cycling conditions were as follows: initial denaturing at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 20 s. Relative expression levels were measured by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2012).

Phytohormone treatment

Young plants (6–8 leaf stage) were used to evaluate *CnTCP13* transcription in response to cytokinin treatment as previously described (Gao et al. 2015). The cytokinin treatment involved spraying the leaves with 5.0 μM 6-benzyladenine (6-BA) (Wang et al. 2017; Qi et al. 2019). Control plants were treated with distilled water. Leaves were sampled before and 1, 2, 4, 8, 12, and 24 h after phytohormone application. The 6-BA was applied to three plants at each time point, and three biological replicates were used for each time point. The 6-BA treatment experiment was replicated three times (Li et al. 2015). The sampled second true leaves were frozen in liquid nitrogen immediately and stored at -80 °C for further usage (Gao et al. 2015).

Subcellular localization of CnTCP13

The primer pair CnTCP13-pENTR1A-F/R (Table S1) was used to amplified the *CnTCP13* ORF without the stop codon using the Phusion High-Fidelity PCR Kit (New England Biolabs, MA, USA). Both the pENTR1A vector (Invitrogen, Carlsbad, CA, USA) and amplicon were cleaved with the restriction enzymes *Sal*I and *Not*I, and the products were ligated with T4 DNA ligase (New England Biolabs). The pENTR1A-*CnTCP13* fusion product was subsequently sequenced for validation. pMDC43 was recombined with pENTR1A-*CnTCP13* to generate the fusion vector *p35S::GFP-CnTCP13* using the LR Clonase™ II Enzyme Mix (Invitrogen). The empty pMDC43 vector and *p35S::GFP-CnTCP13* were transformed into onion epidermal cells using the He-driven particle accelerator PDS-1000 (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. After bombardment, the onion peels were incubated at 22 °C for 16 h on MS medium in the dark (Li et al. 2015). Green fluorescence was observed using a confocal laser microscope (Leica SP2, Germany).

Transactivation activity

A yeast one-hybrid assay was used to test the transactivation activity of CnTCP13 (Li et al. 2015). pENTR1A-*CnTCP13* was recombined with pGBKT7 to produce pGBKT7-*CnTCP13* using the LR Clonase™ II Enzyme Mix (Invitrogen). The pGBKT7-*CnTCP13* construct, pCL1 (positive control), and pGBKT7 (negative control) were individually introduced into Y2H Gold yeast cells (Clontech, Mountain View, CA, USA), following the manufacturer's protocol. Transformants carrying pGBKT7-*CnTCP13* or pGBKT7 were selected by culturing on SD/-Trp medium, and the pCL1 transformants were selected by culturing on SD/-Leu medium. All three transformed cell lines were then plated on SD/-His-Ade + 20 mg/mL X-α-gal medium and incubated at 30 °C to observe cell growth.

Arabidopsis thaliana transformation and expression level of cell cycle marker genes

Arabidopsis thaliana (Col-0) was transformed with *Agrobacterium tumefaciens* (strain EHA105) carrying *p35S::GFP-CnTCP13* using the floral dip method (Clough and Bent 1998). The transformed progenies were selected by plating on 1/2 MS + 20 μg/mL hygromycin agar medium, and the T3 generation was obtained by self-pollination. Transgene zygosity was identified by RT-PCR using the primer pair CnTCP13-ORF-F/R (Table S1). The cell size of the seventh leaves at 35 days was observed using phase contrast microscopy (Olympus BX41, Tokyo, Japan). Leaves and cell sizes were measured using Image J software (version 1.8.0_172). All measurements represent the mean of 10 replications. Total RNA was extracted from the seventh leaves of 4-week-old Col-0 and *35 S::CnTCP13* transgenic plants using the RNAiso reagent (Takara) according to the manufacturer's instructions. Changes in the expression levels of cell cycle marker genes were detected using qRT-PCR. All primer pairs are listed in Table S1, and *AtActin* was used as the reference (Table S1).

Yeast two-hybrid assay

The bait pGBKT7-*CnTCP13* construct was transformed into yeast strain AH109 (Clontech) to screen the prey *Chrysanthemum* yeast cDNA library according to the manufacturer's protocol, followed by the selection of interacting partners on SD/-Trp-Leu-His-Ade medium and incubation at 30 °C for 3 days. All clones were selected and cultured on SD/-Trp-Leu-His-Ade and SD/-Trp-Leu-His-Ade + 20 mg/mL X-α-gal medium and incubated at 30 °C for 3 days to observe cell growth. Positive clones with blue color were amplified using Mighty Amp® DNA Polymerase PCR (TaKaRa) based on the pGADT7 plasmid primer pair AD-F/R (Table S1). Clones containing only one prey were sequenced. Sequence analysis was performed using BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A gene-specific primer pair (CnF-box-ORF-F/R, Table S1) was designed to amplify the ORF sequence of *CnF-box*. The amplified ORF sequence was cloned into the

pENTR1A vector to generate pENTR1A-*CnF-box* (CnF-box-pENTR1A-F/R, Table S1). pENTR1A-*CnF-box* was then recombined with both pGBKT7 and pGADT7 plasmids. The transactivation activity of CnF-box was tested by growing on SD/-His-Ade medium.

The interactions between CnTCP13, CnTCP2, and CnF-box were identified by testing combinations of CnTCP13-bait and CnTCP2-prey, and CnTCP13-bait and CnF-box-prey constructs co-expressed in the yeast strain AH109 (Clontech) following the manufacturer's instructions. The pGADT7-T vector was combined with the pGBKT7-53 vector as the positive interaction control and with the pGBKT7-Lam vector as the negative interaction control. Interactions were identified by culturing on SD/-Trp-Leu-His-Ade and SD/-Trp-Leu-His-Ade + 20 mg/mL X-α-gal medium.

BiFC assay

In the bimolecular fluorescence complementation (BiFC) constructs, the ORF of *CnTCP13* was cloned as the C-terminal fusion of the fluorescent protein fragments in the pSAT4A-cEYFP-N1 and pSPYCE vectors at the *Kpn*I/*Sma*I and *Xho*I/*Kpn*I cloning sites using the primer pairs CnTCP13-BiFC-F/R and CnTCP13-BiFC-f/r, respectively (Table S1). The coding region of *CnTCP2* was cloned as the N-terminal fusion in the pSAT4A-nEYFP-N1 and pSPYNE vectors at the *Kpn*I/*Sma*I and *Bam*HI/*Kpn*I cloning sites using the primer pairs CnTCP2-BiFC-F/R and CnTCP2-BiFC-f/r, respectively (Table S1). The full-length cDNA encoding the CnF-box protein was cloned as the N-terminal fusion in the pSAT4A-nEYFP-N1 and pSPYNE vectors at the *Kpn*I/*Sma*I and *Bam*HI/*Kpn*I cloning sites using the primer pairs CnF-box-BiFC-F/R and CnF-box-BiFC-f/r, respectively (Table S1).

Transient gene expression in onion epidermal cells was performed using the PDS-1000 biolistic transformation system (Bio-Rad) according to the manufacturer's instructions. After bombardment, the onion peels were incubated at 22 °C for 16 h on MS medium in the dark (Song et al. 2013). YFP fluorescence was tested using a confocal laser scanning microscope (Leica SP2). An *in planta* transient assay was used to investigate whether CnTCP13 interact with CnTCP2 or CnF-box in *N. benthamiana*. *A. tumefaciens* strain GV3101 cells harboring pSPYCE-CnTCP13, pSPYNE-CnTCP2, pSPYNE-CnF-box, pSPYCE or pSPYNE were suspended in infiltration buffer (2 mM NaH_2PO_4, 100 μM acetosyringone, 50 mM MES and 0.5 % Glc) to obtain an OD_{600} of 0.2, and then spot-infiltrated into the leaves of 7-week-old *N. benthamiana* plants, following the protocol described by Wang et al. (2014). At 48–96 h after infiltration, YFP fluorescence was observed using a confocal laser scanning microscope (Leica SP2).

Statistical analysis

Data were analyzed by analysis of variance and for significance with means separated by Duncan's Multiple Range tests at $p = 0.05$ using SPSS V17.0 software (SPSS Inc., Chicago, IL, USA). Results are presented as means ± standard errors (SE) of three biological replicates.

Results

Identification and characterization of *CnTCP13*

A *TCP* transcript with the full-length of 1,240 nt and a 1,032 nt ORF, which encodes a 343 amino acids, was isolated from the *C. nankingense* transcriptome database (SRS591679) (Wang et al. 2013). Homology BLAST showed that *CnTCP13* belonged to the class II CIN subfamily of the TCP family and was the most similar to *AtTCP13* (Fig. 1a). The CnTCP13 protein harbored an atypical bHLH motif (Fig. 1b).

qRT-PCR revealed that *CnTCP13* was highly expressed in the leaf, and its expression was the lowest in roots (Fig. 2a). *CnTCP13* transcription was significantly inhibited (two-fold) 24 h after exogenous application of 6-BA (Fig. 2b). To

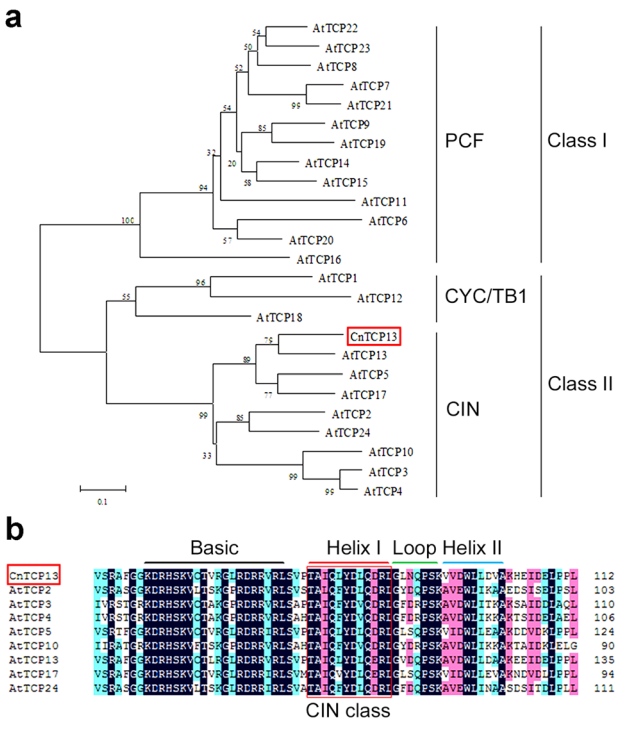

Fig. 1 Characterization of the CnTCP13. **a** Phylogeny of *Arabidopsis* TCP family and CnTCP13. **b** Amino acid comparison of CnTCP13 and *Arabidopsis* CIN-TCPs. The bar (0.1) indicates branch length

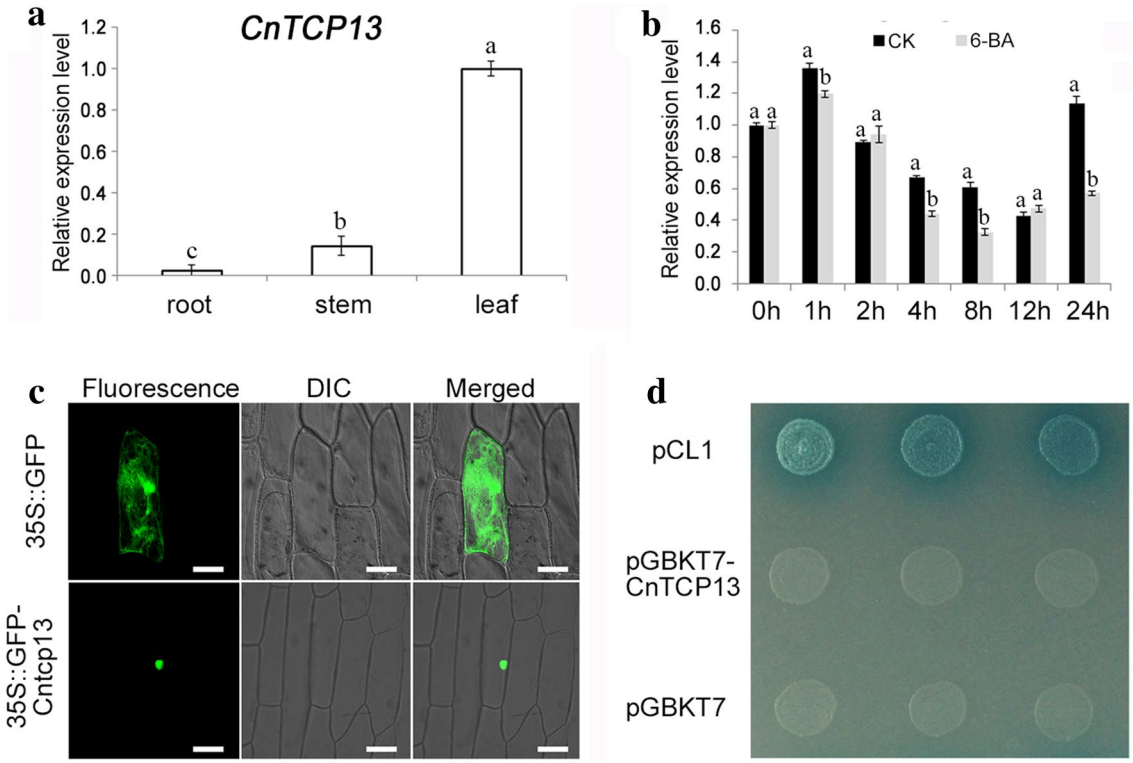

Fig. 2 Expression patterns of *CnTCP13*. **a** Differential expression patterns of *CnTCP13* in various tissues of *C. nankingense*. **b** Effects of 5.0 μM 6-BA on *CnTCP13* transcription. Columns headed by a different letter indicate significantly different transcript abundances ($p < 0.05$) compared with the control (CK). The *x*-axis indicates the time point of the assay following the spray treatment. **c** GFP activity generated by the p35S::*GFP-CnTCP13* transgene introduced into onion epidermal peels. Fluorescence: images obtained in the green fluorescence channel; DIC: images obtained in bright light; Merge: overlay plots. Bar: 50 μm. **d** Yeast one-hybrid assay was used to detect the transcriptional activation of CnTCP13. In **a** and **b**, *CnEF1α* was used as the reference gene, and values are presented as mean ± SE ($n = 3$)

determine the subcellular localization of CnTCP13, a transient assay involving the bombardment of the *p35S::GFP-CnTCP13* fusion construct into onion epidermal cells was used. The CnTCP13-GFP fusion protein was localized in the nucleus (Fig. 2c). CnTCP13 transcriptional activity was tested using a yeast one-hybrid assay. Yeast cells harboring the positive control pCL1 were able to grow on SD/-His-Ade + 20 mg/mL X-α-gal medium, whereas the cells harboring the negative control pGBKT7 and pGBKT7-CnTCP13 failed to grow (Fig. 2d). These results indicated that CnTCP13 exhibits no transcriptional activity.

Phenotype of *A. thaliana* plants heterologously expressing *CnTCP13*

To investigate the function of *CnTCP13*, two independent overexpression lines, OX-1 and OX-2, from the T_3 generation were used for subsequent analysis (Fig. 3a). The transgenic plants showed crinkled middle-layer leaves (Fig. 3b). The growth of transgenic plants was greatly restricted at the vegetative stage (Fig. 3c), although they had a large number of rosette leaves (Fig. 3d). While, their leaf length (Fig. 3e) and width (Fig. 3f) were significantly reduced. The size of the lower epidermal cells was larger in the transgenic lines than in the wild-type plants (Fig. 3g and h). These results indicated that overexpression of *CnTCP13* reduced leaf size in *A. thaliana*.

It reported that AtTCP4 protein blocks cell cycle of budding yeast, specifically at G1→S transition, by regulating G1 checkpoint control pathway, and the expression of a number of cell cycle genes is altered (Aggarwal et al. 2011). To further reveal the role of *CnTCP13* in regulating *A. thaliana* leaf development, the expression of 12 cell division-related genes was analyzed (Fig. 4). Transcription levels of the positive regulators of cell cycle, including *AtCYCA1;1*, *AtCYCA3;1*, *AtCYCB2;4*, *AtCDKB1;2*, *AtCDKB2;2*, *AtCDKD;2* and *AtCDKD;3* were downregulated in the transgenic lines. The transcription level of *AtCYCA3;2* remained unchanged. Transcription levels of the negative regulators of cell cycle, including *AtCDKC;1*, *AtCDKG;2*, *AtKRP5* and *AtE2Fc* were upregulated in the transgenic lines. The above

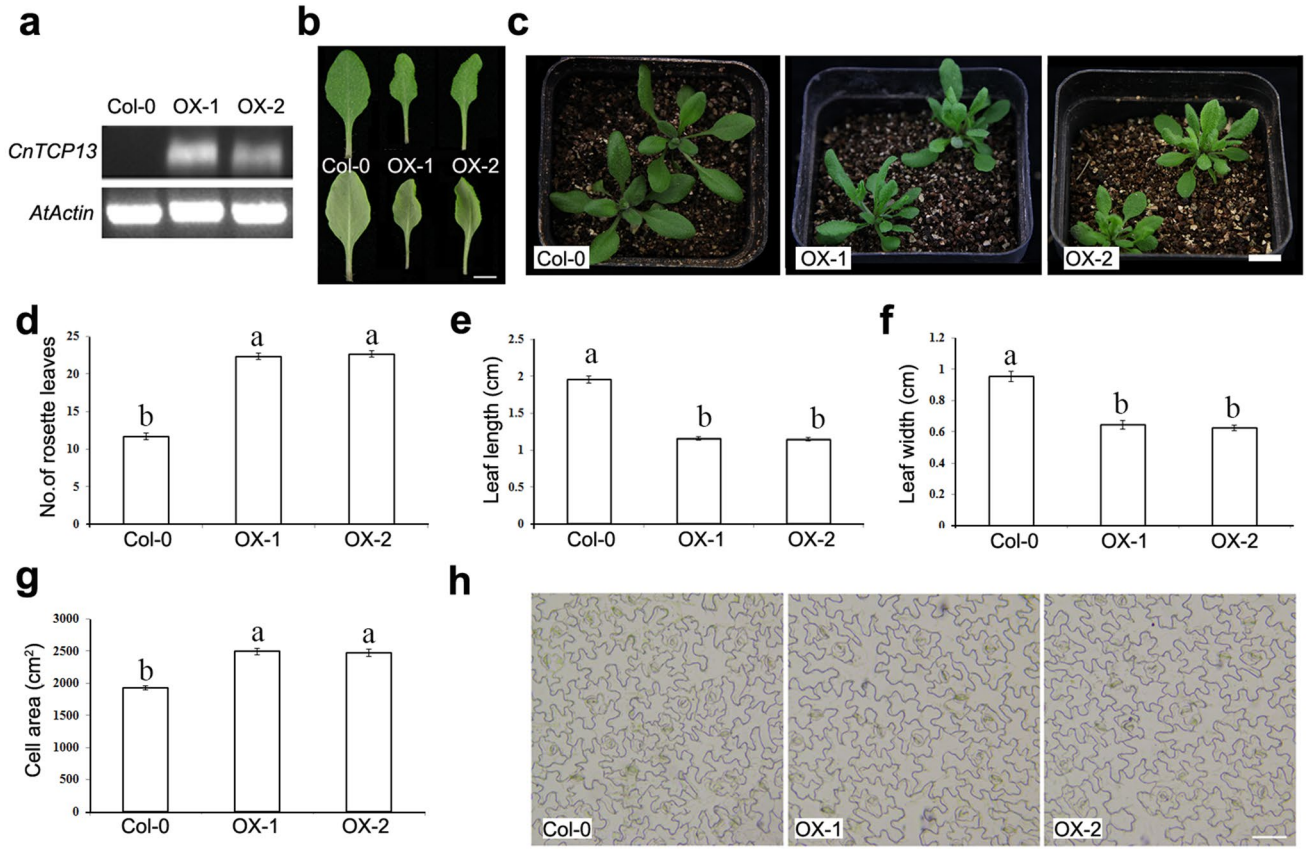

Fig. 3 Phenotype of *A. thaliana* constitutively expressing *CnTCP13*. **a** RT-PCR-based identification of the transgenic *A. thaliana* lines OX-1 and OX-2. **b** The appearance of seventh rosette leaves. Bar: 0.5 cm. **c** he appearance of 35-day-old wild-type Col-0, OX-1, and OX-2 plants. Bar: 1 cm. Quantification of leaf growth. Bar: 1 cm. **d** Number of rosette leaves. **e** Leaf length of the seventh leaf. **f** Leaf width of the seventh leaf. **g** Epidermal cell size of the seventh leaf. Values in (**d**) through (**g**) are presented as mean ± SE ($n = 10$). **h** Appearance of epidermal cells sampled from the seventh leaf. Bar: 50 µm. Col-0: wild-type plants; OX-1, OX-2: transgenic lines

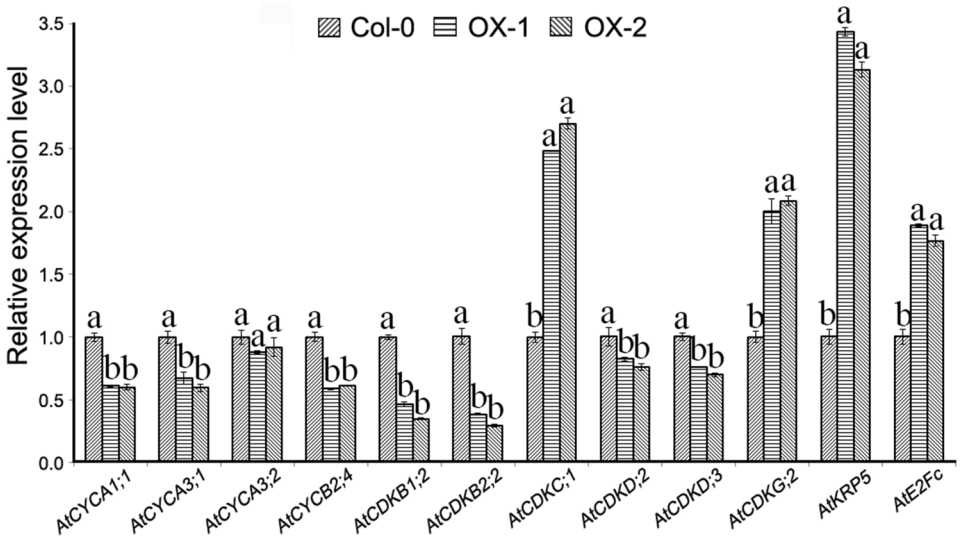

Fig. 4 Effects of constitutive *CnTCP13* expression on the transcription of cell cycle marker genes in *A. thaliana*. Col-0: wild-type plants; OX-1, OX-2: transgenic lines. Values are presented as mean ± SE ($n = 3$). Columns headed by different letters indicate significantly different transcript abundances ($p < 0.05$) compared with the wide-type

results suggested heterologous expression of *CnTCP13* in *A. thaliana* alters the transcription levels of cell division-related genes.

Putative interaction proteins of CnTCP13

Yeast two-hybrid assay was used to identify putative interaction proteins of CnTCP13. The *CnTCP13* gene was fused to the DNA-binding domain of the bait plasmid pGBKT7. A *Chrysanthemum*-yeast cDNA library was used as the prey. Sixty-four positive clones were amplified (Fig. S1), and 44 clones containing only one prey were sequenced. Twenty-four positive clones were identified as candidate interaction protein of CnTCP13 using NCBI BLASTX (Table 1). Taking the number of clones, their predicted subcellular location and proteins that typically interact with the bait in this system into consideration, two candidates, CnTCP2 and CnF-box, were considered as putative interaction proteins of CnTCP13. Interactions were validated using yeast two-hybrid assay again. *CnTCP2* and *CnF-box* genes were cloned into the pGADT7 plasmid. pGADT7-T co-transformed with the pGBKT7-53 vector, as the positive interaction control, was able to grow on SD/-Trp-Le-His-Ade medium and show X-α-galactosidase activity, whereas the negative interaction control failed to grow on this medium or show X-α-galactosidase activity (Fig. 5a); CnTCP2 and CnF-box showed significantly growth when co-transformed with CnTCP13 and exhibited X-α-galactosidase activity. The interactions among CnTCP13, CnTCP2 and CnF-box were further confirmed by BiFC assays in onion and *N. benthamiana*. YFP fluorescence signals were observed in the onion epidermal cells co-expressing nEYFP-CnTCP2 and cEYFP-CnTCP13 or nEYFP-CnF-box and cEYFP-CnTCP13, but not in cells co-expressing nEYFP and cEYFP-CnTCP13, nEYFP-CnTCP2 and cEYFP, nEYFP-CnF-box and cEYFP or nEYFP and cEYFP (controls) (Fig. 5b). In addition, YFP fluorescence signals were observed in *N. benthamiana* leaves co-expressing pSPYNE-CnTCP2 and pSPYCE-CnTCP13 or pSPYNE-CnF-box and pSPYCE-CnTCP13, but not in cells co-expressing pSPYNE and pSPYCE-CnTCP13, pSPYNE-CnTCP2 and pSPYCE, pSPYNE-CnF-box and pSPYCE or pSPYNE and pSPYCE (controls) (Fig. 5c).

Characterization of the *CnF-box* sequence

CnF-box harbored an 897 nt ORF predicted to encode a 298 amino acid residue product. *CnF-box* belongs to the FBP family and harbored an F-box motif (Fig. S2a). It was the most closely related to *CcF-box* (Fig. S2b). *CnF-box* was abundantly expressed in the root (Fig. S3). It was localized in the nucleus (Fig. S4a), and showed no transcriptional activity (Fig. S4b).

To explore the function of *CnF-box*, two independent overexpression lines, namely OX-1 and OX-2, from the T_3 generation were selected (Fig. 6a). The growth of transgenic plants was slightly restricted at the vegetative stage (Fig. 6b). *CnF-box* overexpression plants bore the same number of rosette leaves as Col-0 plants, but the middle-layer leaves were strongly crinkled (Fig. 6c).

Discussion

The TCP family comprises plant-specific transcription factors that are involved in multiple processes during plant growth and development, such as leaf (Aguilar-Martínez

Table 1 Result of candidate positive cloning interaction with CnTCP13 by BLASTX in NCBI

Prediction protein	No. of cloning
Cytochrome b6-f complex Fe-S subunit [*Cynara cardunculus* var. scolymus]	6
TB1-like TCP family transcription factor, partial [*Helianthus tuberosus*]	**4**
F-box domain, cyclin-like protein [*Cynara cardunculus* var. scolymus]	**2**
K Homology domain-containing protein [*Cynara cardunculus* var. scolymus]	2
Ribosomal protein S6 [*Cynara cardunculus* var. scolymus]	1
Ribosomal protein S2 [*Cynara cardunculus* var. scolymus]	1
Ribosomal protein L18/L5 [*Cynara cardunculus* var. scolymus]	1
ribosome-inactivating protein [Binary vector pGV4945]	1
1-deoxy-D-xylulose-5-phosphate synthase [*Artemisia annua*]	1
5'-AMP-activated protein kinase subunit beta-1 [*Theobroma cacao*]	1
26 S proteasome subunit P45 [*Cynara cardunculus* var. scolymus]	1
DNA-directed RNA polymerase, 14-18 kDa subunit, conserved site-containing protein [*Cynara cardunculus* var. scolymus]	1
tubulin beta-2/beta-3 chain [*Arabidopsis thaliana*]	1
Alpha/beta hydrolase fold-3 [*Cynara cardunculus* var. scolymus]	1

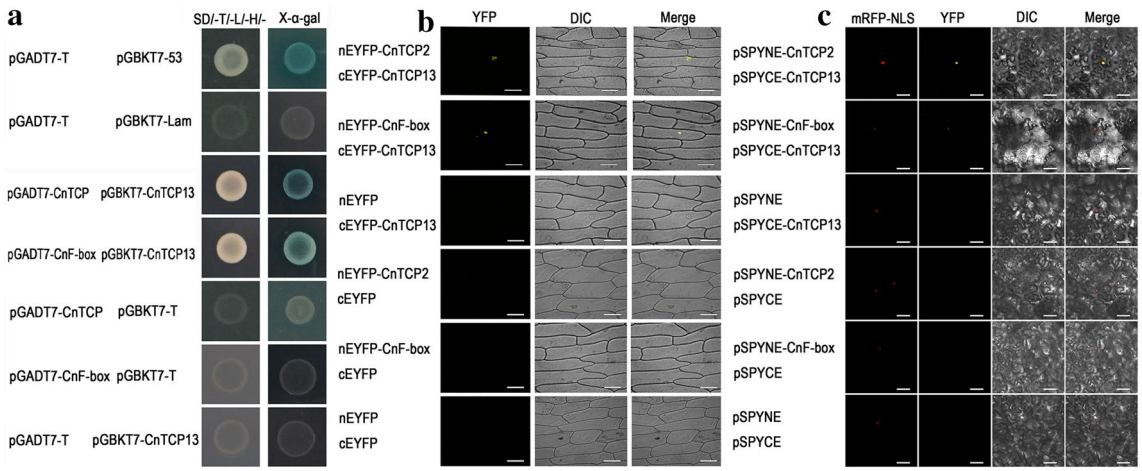

Fig. 5 Verification of CnTCP13 interaction with CnTCP2 and CnF-box. (**a**) Yeast two-hybrid assay verifying the interactions among CnTCP13, CnTCP2, and CnF-box. The left panel shows the selection of yeast colonies on SD/-T/-L/-H/-A medium; the right panel shows the selection of yeast colonies on SD/-T/-L/-H/-A medium containing X-α-gal. SD/-T/-L/-H/-A: SD/-Trp/-Leu/-His/-Ade; X-α-gal: SD/-T/-L/-H/-A + X-α-gal. (**b**) BiFC assay verifying the interactions among CnTCP13, CnTCP2, and CnF-box in transiently transfected onion cells. YFP: images obtained in the yellow fluorescence channel; DIC: images obtained in bright light; Merge: overlay plots. Bar: 50 μm. (**c**) BiFC assay verifying the interactions among CnTCP13, CnTCP2, and CnF-box in transiently transfected *N. benthamiana* leaves. mRFP-NLS: nuclear location shown by RFP activity; YFP: images obtained in the yellow fluorescence channel; DIC: images obtained in bright light; Merge: overlay plots. Ba: 50 μm

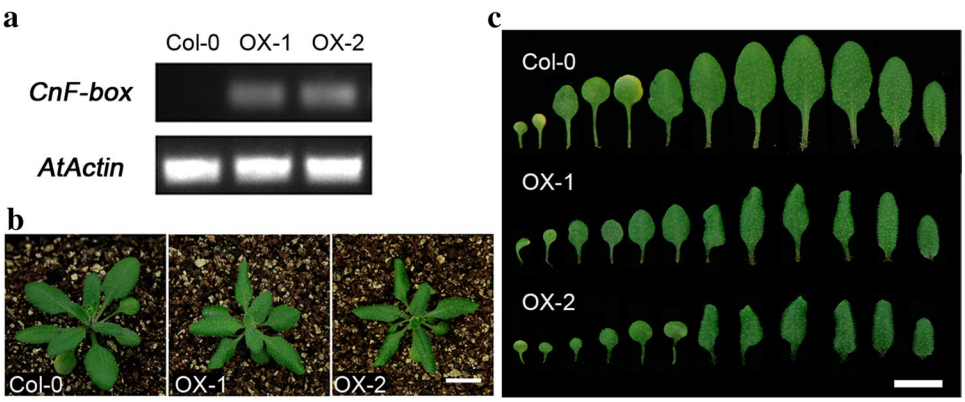

Fig. 6 Phenotype of *A. thaliana* constitutively expressing *CnF-box*. **a** RT-PCR-based identification of the transgenic *A. thaliana* lines OX-1 and OX-2. **b** The appearance of 35-day-old wild-type Col-0, OX-1, and OX-2 lines. Bar: 1 cm. **c** Appearance of rosette leaves. Bar: 1 cm. Col-0: wild-type plants; OX-1, OX-2: transgenic lines

and Sinha 2013; Ma et al. 2016; Bresso et al. 2017), flower (Crawford et al. 2004; Es et al. 2018) and nodule (Wang et al. 2018) development, as well as hormonal pathways (Koyama et al. 2010; Danisman et al. 2012; Es et al. 2018). In the present study, *CnTCP13* in *C. nankingense* was identified. CnTCP13 belongs to the class II subfamily of the TCP family and harbors an atypical bHLH motif. In Antirrhinum *cin* mutants, leaves are larger with an undulating edge because of excessive growth in marginal regions (Crawford et al. 2004). *LA* (a *CIN* ortholog in tomato) activity in young leaf primordia is increased in a *LA* gain-of-function mutant (*La-2*); this mutation confers resistance to miR319, and leads to the formation of small, simple tomato leaves instead of large, compound ones (Ori et al. 2007). In *Brassica rapa*, *Brp-MIR319a2* overexpression inhibites *BrpTCP4* expression, leading to the formation of crinkly leaves (Mao et al. 2014). Compared with wild-type *A. thaliana*, the *tcp2/tcp4* mutant borns enlarged flat leaves, and the *tcp2/tcp3/tcp4/tcp10* plants born strongly crinkled leaves (Bresso et al. 2017). Constitutive *CnTCP4* expression suppresses cell proliferation in fission yeast and reduced the leaf size in *A. thaliana* (Qi et al. 2019). Here, plants with heterologously expressing *CnTCP13* were smaller and bore crinkly middle-layer leaves (Fig. 3), indicating that *CnTCP13* suppresses leaf development.

CIN-like TCP transcription factors regulate plant growth through their involvement in hormone-associated pathways. For instance, *AtTCP3* directly upregulates the expression of the auxin signaling repressor *IAA3/SHY2* and modulates auxin responses (Koyama et al. 2010). In *A. thaliana*, *TCP4*

promotes the expression of *LIPOXYGENASE2* (*LOX2*), a gene involved in jasmonate (JA) biosynthesis, consequently, the increased JA content inhibites cell proliferation (Danisman et al. 2012). *AtTCP4* physically interacts with the SWI/SNF chromatin remodeling ATPase BRAHMA (BRM), and then TCP4-BRM binds to the promoter and activates the CK response inhibitor *ARABIDOPSIS RESPONSE REGULATOR16* (*ARR16*) (Efroni et al. 2013). In the present study, 6-BA treatment inhibited *CnTCP13* expression (Fig. 2b), which was in consistent with previous reports, suggesting the involvement of CnTCP13 in the hormonal regulation of plant growth.

AtTCP4 prevents cell proliferation in leaves by positively regulating miR396 and negatively regulating *GROWTH-REGULATING FACTORs* (*GRFs*) (Rodriguez et al. 2010). In *A. thaliana*, plants expressing a hyper-activated form of *TCP4* shows decreased cell proliferation and reduced leaf size with cup-shaped lamina in extreme cases (Sarvepalli and Nath 2011). In the present study, constitutive *CnTCP13* expression in *A. thaliana* reduced leaf size but enlarged epidermal cells on the abaxial leaf surface (Fig. 3c, g). In *A. thaliana tcp20* mutant leaves, there is a significant increase in average epidermal cell size, but no obvious size or shape alterations can be observed, due to a reduction in total number of cells in the leaf (Danisman et al. 2012). Average epidermal cell size is significantly increased in transgenic *CnTCP2/4 A. thaliana* leaves, but there are no obvious leaf size or shape alterations in these transgenic plants as the cell size effect was compensated by a reduction in total cell number in the leaf (Qi et al. 2019). Smaller leaves could be observed in transgenic *A. thaliana* containing *CnTCP13*, implying that the effect of enlarged epidermal cells was compensated for by a decrease of the total cell number in the leaf. These results suggest that *CnTCP13* suppresses cell proliferation but promotes cell expansion.

The development of multicellular organisms is controlled by precise cell proliferation and expansion. The mechanisms regulating plant organ size through the cell cycle are rather complex, as they must suit fluctuating environments (Mizukami 2001). The cell cycle involves a series of phases, and every process is regulated by specific cyclins and CDKs (Breuer et al. 2010). Mitotic A- and B-type cyclins show expression peaks at the G2-to-M boundary, and plant-specific B-type CDKs positively control the entry into and passed through mitosis (Leene et al. 2010). In the quintuple mutant *tcp8/tcp15/tcp21/tcp22/tcp23*, *CYCLINA1;1* (*CYCA1;1*) and *CYCA2;3* are regulated, resulting in larger leaf blades than the wild-type (Aguilar-Martínez and Sinha 2013). *Arabidopsis* expresses four CDK-activating kinases (CDKDs): CDKD;1, CDKD;2, CDKD;3 and CDKF;1. The complex of CDKD;2 or CDKD;3 with CYCH;1 positively regulates the cell cycle by phosphorylating CDKA at the T161 residue (Gutierrez 2009). In the present study, *AtCYCA1;1*, *AtCYCA3;1*, *AtCYCB2;4*, *AtCDKB1;2*, *AtCDKB2;2*, *AtCDKD;2* and *AtCDKD;3* were downregulated in transgenic lines (Fig. 4) likely suppressing cell proliferation, thus leading to the formation of small leaves. CDKCs phosphorylate the C-terminal domain (CTD) of RNA polymerase II. The CDKC-CycT complex negatively regulates the positive transcription elongation factor b (P-TEFb) (Kitsios et al. 2008), whereas the loss of *CDKC;2* promotes cell division in *A. thaliana* (Zhao et al. 2017). There are two CDKGs, namely CDKG;1 and CDKG;2, in *A. thaliana*. CDKG;2 forms a complex with CYCL1, which negatively regulates cell cycle onset (Leene et al. 2010). Kip-related protein 5 (KRP5) prevents cell cycle progression by inhibiting the activity of the CYCD2-CDKB complex kinase and reconstitutes CYCD2-associated kinases at both G1/S and G2/M transitions (Nakai et al. 2006). E2FC inhibites cell division by negatively regulating the entry into G1/S, and *E2FC* overexpression obviously reduces the root meristem cell number (del Pozo et al. 2006). In the present study, *AtCDKC;1*, *AtCDKG;2*, *AtKRP5* and *AtE2Fc* upregulation likely suppressed cell proliferation, resulting in small leaves in transgenic *A. thaliana*. These results indicated that heterologous expression of *CnTCP13* in *A. thaliana* alters the transcription levels of cell division-related genes and reduces the size of leaves.

Some TCP transcription factors are not transcriptional activators *per se*, but require interaction with other proteins to form homo- and heterodimers to control transcription (Martín-Trillo and Cubas 2010). A yeast two-hybrid assay confirms that AtPURα interacts with AtTCP20, suggesting that these two proteins act together at the promoter of ribosomal genes (Trémousaygue et al. 2003). The TCP transcription factor CCA1 HIKIHG EXPEDITION (CHE) interacts with the *CAA1* transcriptional activator TIMING OF CAB EXPRESSION1 (TOC1), which downregulated *CAA1* by binding to its promoter (Pruneda-Paz et al. 2009). CmTCP14 reduces plant stature by interacting with CmDELLA1, CmDELLA2 and CmDELLA3 in the gibberellic acid signaling pathway (Zhang et al. 2017). AtTCP24 interacts with ABAP1 and negatively regulates the transcription of *AtCDT1α* and *AtCDT1b*, thus limiting mitotic DNA replication and repressing cell proliferation in leaves (Masuda et al. 2008). In the present study, we isolated factors interacting with CnTCP13 using yeast two-hybrid system. The interactions between CnTCP13 and CnTCP2 or CnTCP13 and CnF-box were confirmed by a yeast two-hybrid assay and by BiFC (Fig. 5). The F-box protein is part of an SCF complex, and harbors a roughly conserved F-box motif that binds to SKP1, and diverse typical protein-protein interaction domains involved in various aspects of plant biology (Lechner et al. 2006). In *A. thaliana*, *CnF-box* overexpression plants showed strongly crinkled leaves, similar to *CnTCP13* transgenic plants with crinkled middle-layer leaves (Figs. 3b

and 6c). Moreover, in *A. thaliana*, *CnTCP2* overexpression plants shows smaller leaves, similar to *CnTCP13* transgenic plants, which plays negative roles in leaf development by suppressing cell proliferation and promoting cell expansion (Qi et al. 2019). These results indicated that CnTCP13 controls leaf development in *C. nankingense* maybe through its interaction with CnTCP2 and CnF-box.

In conclusion, heterologous *CnTCP13* expression in *A. thaliana* reduced leaf size. *CnTCP13* was involved in leaf development by modulating the expression of cell cycle marker genes, and maybe through its interaction with CnTCP2 and CnF-box, which remains further investigation in more details.

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Author contributions XYQ, HBW and FDC designed the experiment. XYQ, YXQ, APS and PPC performed the experiment. XYQ, ZYG and WMF analyzed the data and wrote the manuscript. XYQ, JFJ, YXG and SMC revised the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare no conflicts of interest.

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