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Research

SlTCP24 and *SlTCP29* synergistically regulate compound leaf development through interacting with *SlAS2* and activating transcription of *SlCKX2* in tomato

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Summary

- The complexity of compound leaves results primarily from the leaflet initiation and arrangement during leaf development. However, the molecular mechanism underlying compound leaf development remains a central research question.
- SlTCP24 and SlTCP29, two plant-specific transcription factors with the conserved TCP motif, are shown here to synergistically regulate compound leaf development in tomato. When both of them were knocked out simultaneously, the number of leaflets significantly increased, and the shape of the leaves became more complex. SlTCP24 and SlTCP29 could form both homodimers and heterodimers, and such dimerization was impeded by the leaf polarity regulator SlAS2, which interacted with SlTCP24 and SlTCP29.
- SlTCP24 and SlTCP29 could bind to the TCP-binding *cis*-element of the *SlCKX2* promoter and activate its transcription. Transgenic plants with *SlTCP24* and *SlTCP29* double-gene knockout had a lowered transcript level of *SlCKX2* and an elevated level of cytokinin.
- This work led to the identification of two key regulators of tomato compound leaf development and their targeted genes involved in cytokinin metabolic pathway. A model of regulation of compound leaf development was proposed based on observations of this study.

Introduction

Plant leaves can be either simple or compound in shape. Simple leaves have a single, continuous leaf, whereas compound leaves are made up of multiple leaflets, each of which is similar to a simple leaf (Efroni *et al.*, 2010; Bar & Ori, 2015; Du *et al.*, 2018). On developmental timescales, single leaves differentiate and flatten faster, whereas compound leaves are an intermediate morphology between lateral branches and single leaves in some aspects (Bar & Ori, 2014). Tomato (*Solanum lycopersicum*) is a model plant for studying compound leaf development because it has typical compound leaves ranging from simple to complex in leaf types (Ori, 2012). Plant leave morphogenesis is a complex physiological and biochemical process that is regulated by hormones, functional genes, and environmental factors (Palatnik *et al.*, 2003; Shani *et al.*, 2006; Blein *et al.*, 2010; Bilsborough *et al.*, 2011; Durbak *et al.*, 2012). This process is regulated by different mechanisms, including intracellular regulation, intercellular regulation, and extracellular regulation. The formation and differentiation of leaf primordia and the entire process of leaf proto-maturation are part of plant leaf development.

Different families of transcription factors have been shown to regulate the leaf initiation process (Siegfried *et al.*, 1999; Hay & Tsiantis, 2010; Horstman *et al.*, 2014). Transcription factors of the Class I KNOTTED1 (KN1)-like homeobox (KNOX I) proteins, for example, coordinate the activities of various plant hormones in defining different regions of the shoot apical meristem (SAM), ensuring that the SAM function is maintained at the onset of leaf primordia (Randall *et al.*, 2001; Kelley *et al.*, 2012; Tsuda & Hake, 2015). In tomato, mutation or knockdown of *KNOX I* resulted in a decrease in the number of leaflets formed on each compound leaf (Parnis *et al.*, 1997), while constitutively expressed *KNOX I* in *mouse-ear* and *curl* tomato mutants resulted in significantly increased leaflet number (Janssen *et al.*, 1998). The homeodomain-leucine zipper (HD-ZIP) supergene family plays an essential function in establishing leaf polarity. The three members of this family, *REVOLUTA* (*REV*), *PHAVOLUTA* (*PHV*), and *PHABULOSA* (*PHB*), coordinately regulate the development of the adaxial domains of cells in leaf primordia (McConnell *et al.*, 2001). The WUSCHEL (WUS)-related homeobox (WOX) transcription factors are known to be important in the final leaf elongation and outgrowth along with the mediolateral axis (Dolzblasz *et al.*, 2016; Zhang *et al.*, 2020; Nakayama *et al.*, 2021; Wang *et al.*, 2021, 2022).

The TCP proteins are a class of plant-specific transcription factors that are important regulators of leaf development, regulating

leaf size and morphology (Martin-Trillo & Cubas, 2010). This 'TCP' family of proteins is named based on three different types of transcription factors, TEOSINTE BRANCHED1 (TB1), CYCLOIDEA (CYC), and PROLIFERATING CELL FACTORS 1 and 2 (PCF1 and PCF2), which are involved in the regulation of apical dominance, floral symmetry, and cell division in different plants. TCP proteins can be divided into two main classes (I and II) based on the presence of key amino acids in the conserved TCP domain and their phylogenetic relationships. Class II TCP transcription factors are often functionally redundant (Danisman *et al.*, 2013; Koyama *et al.*, 2017) and can be further divided into the CYC/TB1 clade and the CINCINATA (CIN) clade. The CIN clade genes have been shown to control the transition of leaf development from cell division to the cell expansion phase (Sarvepalli & Nath, 2018). These genes are expressed in a very controlled manner in specific regions of leaves during the organ development (Bresso *et al.*, 2018), and disruption of expression of CIN-like TCPs can cause abnormal leaf morphology in Arabidopsis (*Arabidopsis thaliana*) and tomato (Palatnik *et al.*, 2003; Ori *et al.*, 2007; Koyama *et al.*, 2017).

TCP transcription factor LANCEOLATE (LA) and the MYB transcription factor CLAUSA are involved in leaf-shape formation in tomato. *CLAUSA* promotes differentiation, thereby narrowing the morphogenetic window at the leaf margin (Ori *et al.*, 2007; Kang & Sinha, 2010; Bar *et al.*, 2015, 2016), and *LA* and *CLAUSA* effect similar outcomes in tomato leaf development via likely partially parallel genetic pathways. These genetic pathways converge on the modulation of the CK/GA balance (Israeli *et al.*, 2021). The presence of CK favors maintaining cell activity at the leaf edge. Ectopic expression of the Arabidopsis cytokinin synthesis gene *AtIPT7* in tomato results in more complex leaves, while overexpression of the cytokinin-degrading enzyme gene *AtCKX3* leads to simple leaves and only simplified primary leaflets (Shani *et al.*, 2010).

The TCP transcription factor can interact with ASYMMETRIC LEAVES 2 (AS2) to regulate downstream genes, thus regulating leaf development in Arabidopsis (Z. Li *et al.*, 2012). AS2 is a crucial regulator of leaf adaxial–abaxial petitioning and development of a delicate network of vascular bundles and venous systems (Ikezaki *et al.*, 2010; Machida *et al.*, 2015; Iwakawa *et al.*, 2020). AS2 usually forms a complex with AS1 to regulate the downstream gene transcription (Guo *et al.*, 2008; Yang *et al.*, 2008). Its regulatory mechanism is mainly manifested in two aspects: AS1/AS2 may promote the development of SAM to leaf primordia by directly inhibiting the expression of *KNOX I* (*Knotted1-like homeobox I*) gene in SAM (Ori *et al.*, 2000; Guo *et al.*, 2008; Li *et al.*, 2016; Lin *et al.*, 2021), and AS1/AS2 regulates the adaxial side of leaf primordia. But the detailed mechanisms aren't still fully uncovered in diverse species.

Previous research has shown that SlTCP24 and SlTCP29 belong to the members of the CIN clade of TCP transcription factors (Parapunova *et al.*, 2014). In this study, we evaluated the potential impact of SlTCP24/29 proteins on tomato leaf development. Simultaneous knockout of *SlTCP24* and *SlTCP29* resulted in increased leaflets and elevated cytokinin content. We found that SlTCP24 and SlTCP29 could form both homodimers and heterodimers. Leaf polarity regulator SlAS2 was shown to interact with SlTCP24 and SlTCP29, inhibiting their dimerization. Furthermore, SlTCP24 and SlTCP29 were found to activate *Cytokinin oxidase 2* (*CKX2*) expression by binding to its promoter, resulting in downregulation of the cytokinin signaling pathway and impairment of compound leaf development in tomato. These results indicate that SlTCP24/29 are a key component in the regulation of compound leaf development and cytokinin accumulation in tomato, providing a new way to improve leaf type and regulate the hormone content of tomato and other plants.

Materials and Methods

Plant materials

Solanum lycopersicum cv Alisa Craig (LA2838A) was used as the wild-type control and for generation of transgenic plants, including CRISPR/Cas9-mediated *SlTCP24* and *SlTCP29* knockout plants, and overexpression lines *p35S:SlTCP24* and *p35S:SlTCP29*. To construct the *p35S:SlTCP24* and *p35S:SlTCP29* plasmids, the full-length *SlTCP24*, and *SlTCP29* genes were amplified using gene-specific primers (Supporting Information Table S1) and cloned into *pHellsgate8* vector driven by the cauliflower mosaic virus 35S (CaMV 35S) promoter (Yang *et al.*, 2011). To generate the *SlTCP24* and *SlTCP29* knockout constructs, two target sites were designed in the first exon of *SlTCP24* or *SlTCP29* and introduced into the *pTX041* vector (Deng *et al.*, 2018). *Agrobacterium tumefaciens*-mediated transformation was performed to generate transgenic tomato plants (Sharma *et al.*, 2009). Two representative lines each from sixty, twenty-four, four, eight, and ten transgenic lines for *SlTCP24-OE*, *SlTCP29-OE*, *SlTCP24-KO*, and *SlTCP29-KO*, and double-gene knockout *SlTCP24/29-KO* were selected respectively for detailed characterization in this study. Plants were grown in plastic pots containing a nutrition matrix in a glasshouse at 24 ± 2°C in a photoperiod containing 16 h : 8 h, light : dark. The plastic pots were rotated once every 2 wk to ensure that the plants received similar quantities of light.

Measurement of leaflet numbers

Leaflets on adult leaves were counted in four dissection orders: first, second, third, and intercalary. The young leaves are small and usually lacked lateral leaflets. So we selected the mature leaf of the fifth node from the top for observation and taking photographs. For the statistics of leaflet number, we selected the leaves between the second and third inflorescence. The *t*-test was used to assess whether there was a statistically significant difference in the mean between the two groups ($P = 0.05$).

Quantitative real-time PCR assay

Total RNA was extracted from frozen leaf tissues using the TRIzol reagent. Around 5 μg sample of RNA was reverse transcribed into complementary DNA (cDNA) by HiScript® II 1st Strand

cDNA Synthesis Kit (Vazyme, Nanjing, China). Quantitative real-time (qRT)-PCR was performed to determine the transcript levels of genes and carried out in 96-well blocks with Roche LightCycler® 480 system following the reference (J. Li *et al.*, 2012). Microsoft® EXCEL was used to calculate the relative levels of gene expression. The expression of the *β-actin* gene (SGN-U580609) was used as an internal control. Primers used in this analysis are listed in Table S1.

Yeast two-hybrid assay

The full-length open reading frames (ORFs) from the *SlTCP24* and *SlTCP29* genes were cloned into the bait vector *pGBKT7* to yield *pGBKT7-SlTCP24* and *pGBKT7-SlTCP29*. The full-length ORFs of *SlAS2* and *SlAS2-like* were cloned into the prey vector *pGADT7* using gene-specific primers (Table S1). Yeast strain AH109 cells were co-transformed with the recombinant prey and bait plasmids using the lithium chloride–polyethylene glycol (PEG) method and plated on a synthetic medium (SD/−Trp−Leu). After three days of cell growth at 30°C, colonies were transferred to SD/−Trp−Leu−His−Ade medium to determine protein–protein interactions. A combination of *pGBKT7-SlTCP24* or *pGBKT7-SlTCP29* and *pGADT7* was used as a negative control.

Luciferase complementation imaging analysis

The full-length coding sequences (CDSs) of *SlAS2* and *SlTCP29* without the stop codon were cloned into the *Bam*HI-*Sal*I sites of the *pCAMBIA1300-nLUC* vector under the control of the 35S promoter for expression of the fusion protein with the N-terminus of the LUC fragment. The full-length CDSs of *SlAS2*, *SlTCP24*, and *SlTCP29* were cloned into the *Kpn*I-*Sal*I sites of the *pCAMBIA1300-cLUC* vector under the control of the 35S promoter for expression of the fusion protein with the C-terminus of the LUC fragment. Luciferase complementation imaging (LCI) assays were performed as described previously (Chen *et al.*, 2008). Briefly, the constructs were transformed into *Agrobacterium* strain GV3101, which was then incubated at 28°C overnight with shaking. The bacterial suspension was adjusted to the final OD_{600} of 0.5 with the infiltration buffer. Bacterial suspensions of different construct combinations were used to infiltrate the tobacco (*Nicotiana benthamiana*) leaves. After infiltration, the plants were kept to grow for two days in a 16 h : 8 h, light : dark cycle. Three days later, the leaf was treated with luciferin. The firefly luciferase (LUC) signals were observed with a charge-coupled device camera (Lumazone Pylon 2048 B; Roper Scientific, Tucson, AZ, USA).

Co-immunoprecipitation (Co-IP) assay *in vivo*

For *in vivo* co-immunoprecipitation assays, 5-wk-old tobacco leaves were chosen to be cut into thin strips and digested with the enzymes mix (0.15 g cellulase, 0.04 g macerozyme, 0.8 M mannitol, 2 M KCl, 1 M $CaCl_2$, and 0.2 M MES, pH 5.7) at 24 ± 2°C for 3 h. Filtered and centrifuged the filtrate in a 50 ml round bottom tube. Removed the supernatant and resuspended it in 10 ml of W5. Harvest protoplasts in a new round bottom 50 ml tube with the addition of MMG. Tobacco protoplasts were transformed with the different plasmid combinations that expressed different tagged SlTCP24, SlTCP29, or SlAS2. The empty vector *Mer* was used as a negative control. Cells were harvested 12 h after the plasmid transformation. Proteins were extracted with an extraction buffer (Xiong *et al.*, 2019). After centrifugation at 13 000 ***g*** for 10 min at 4°C, the supernatant was collected, incubated with the anti-FLAG matrix beads for 2 h at 4°C, and gently shaken in dark conditions. After four washes with a washing buffer and one wash with 50 mM Tris–HCl (Xiong *et al.*, 2019), the beads were resuspended in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer and boiled for 5 min at 95°C. The released immunoprecipitated protein complex was resolved on a 10% SDS-PAGE gel and analyzed by immunoblotting with an anti-HA or anti-myc antibody.

RNA-seq analysis

For transcriptome data analysis, the leaves of the fifth node downward from the top meristem of the 12 wk old WT and *SlTCP24/29-KO* lines were selected, and three biological replicates were performed. A unique identifier (UID)-mRNA seq libraries were constructed with three biological replicates of WT and *SlTCP24/29-KO* lines, and sequenced at SEQHEALTH (Wuhan, China) with PE-150 sequencing. FastQC software was used to assess the quality of the raw data. Trimmomatic software was used to clean raw data. A gene whose expression levels changed at least twofold with a *P*-value of < 0.05 and an absolute value of logFC > 1 between WT and *SlTCP24/29-KO* lines was considered as a differentially expressed gene (DEG) and listed in Dataset S1. Gene expression matrix heatmap production was generated using GraphPad Prism 8.

Yeast one-hybrid assay

The full-length CDSs of *SlTCP24* and *SlTCP29* were cloned into the *pGADT7* vector. Two possible TCP-binding sites on the *SlCKX2* promoter were identified by JASPAR. The promotor fragment (−126 to −573) of *SlCKX2* containing the binding site was amplified with the specific primers (Table S1). It was cloned into the *Kpn*I-*Xho*I sites of the *pAbAi* vector to generate a bait construct. After digesting with *Bst*BI, the *pAbAi* bait vector was used to transform yeast strain Y1H Gold and integrated into the yeast genome to create reporter strains. The prey vector was introduced into the reporter strains and grown for three days on SD/−Leu−Ura medium at 30°C. Positive clones were picked up and diluted in double-distilled water to a final OD_{600} of 0.1. The suspension was spotted on SD/−Leu−Ura medium with or without the antibiotic aureobasidin A (AbA, 80 ng ml^{-1}). The combination of *pGADT7* and *pAbAi-SlCKX2-P* served as a negative control.

Dual-luciferase transactivation assay

For the binding activity assays, the 1881-bp genomic fragment upstream of the *SlCKX2* start codon was cloned into the

*Kpn*I-*Nco*I sites of the *pGreen II 0800-LUC* vector as the reporter. The 35S promoter drove the *Renilla reniformis* luciferase (REN) reporter gene in the same vector as an internal control. The full-length CDSs of *SlTCP24* and *SlTCP29* were cloned into the *Bam*HI-*Xho*I sites of the *pGreen II 62-SK* vector as effectors. The *pGreen II 62-SK* empty vector was used as the negative control. Transient expression assays were performed as described previously (Hellens *et al.*, 2005). Briefly, the recombinant constructs were transformed into *Agrobacterium* strain GV3101 with the *pSoup* helper plasmid and infiltrated into tobacco leaves. Two days after infiltration, LUC and REN activities were measured using the Dual-Luciferase Reporter Assay System (Promega) with a Dual-Luciferase Reporter Gene Assay Kit (Yeasen Biotechnology, Shanghai, China). The LUC-to-REN ratio was calculated as a measure of transcriptional activity. The primers used to generate the constructs are listed in Table S1.

Electrophoretic mobility shift assay

The full-length CDSs of *SlTCP24* and *SlTCP29* were cloned to *pET15d Xho*I sites to generate the *MBP-SlTCP24* and *MBP-SlTCP29* constructs. Recombinant proteins were expressed in *Escherichia coli* strain BL21 (DE3) and purified using His-Tagged Protein Purification Kit (CWBIO, Jiangsu, China). Hot probes were synthesized and labeled with 5′-carboxyfluorescencein (5-FAM; Tsingke Biotechnology, Beijing, China). Electrophoretic mobility shift assays (EMSA) were performed using a Chemiluminescent EMSA Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Fluor Chem M (Protein Simple, San Jose, CA, USA) was used to detect the gel signals, which were placed carefully onto the glass platen. Data were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Primers used for EMSA are listed in Table S1.

Measurement of endogenous phytohormones

One-month-old *SlTCP24-KO*, *SlTCP29-KO*, *SlTCP24/29-KO*, and WT lines were selected, and all leaves with a width of < 2 cm were removed for blending, and *c.* 0.5 g of leaf powder from each was used for the measurement of endogenous phytohormones. Three biological replicates were performed for each sample. Extraction and quantification of endogenous phytohormones by liquid chromatography–tandem mass spectrometry (LC–MS) were performed as described previously (Eggert & von Wiren, 2017).

Statistical analysis

At least three independent replicates were performed in each experiment. The data were presented as means ± standard deviation of independent biological replicates. Statistical analysis of the bioassays was carried out using the Spss v.21.0 statistical package, and the significance of treatment differences was analyzed with Turkey's test at $P < 0.05$ or $P < 0.01$.

Results

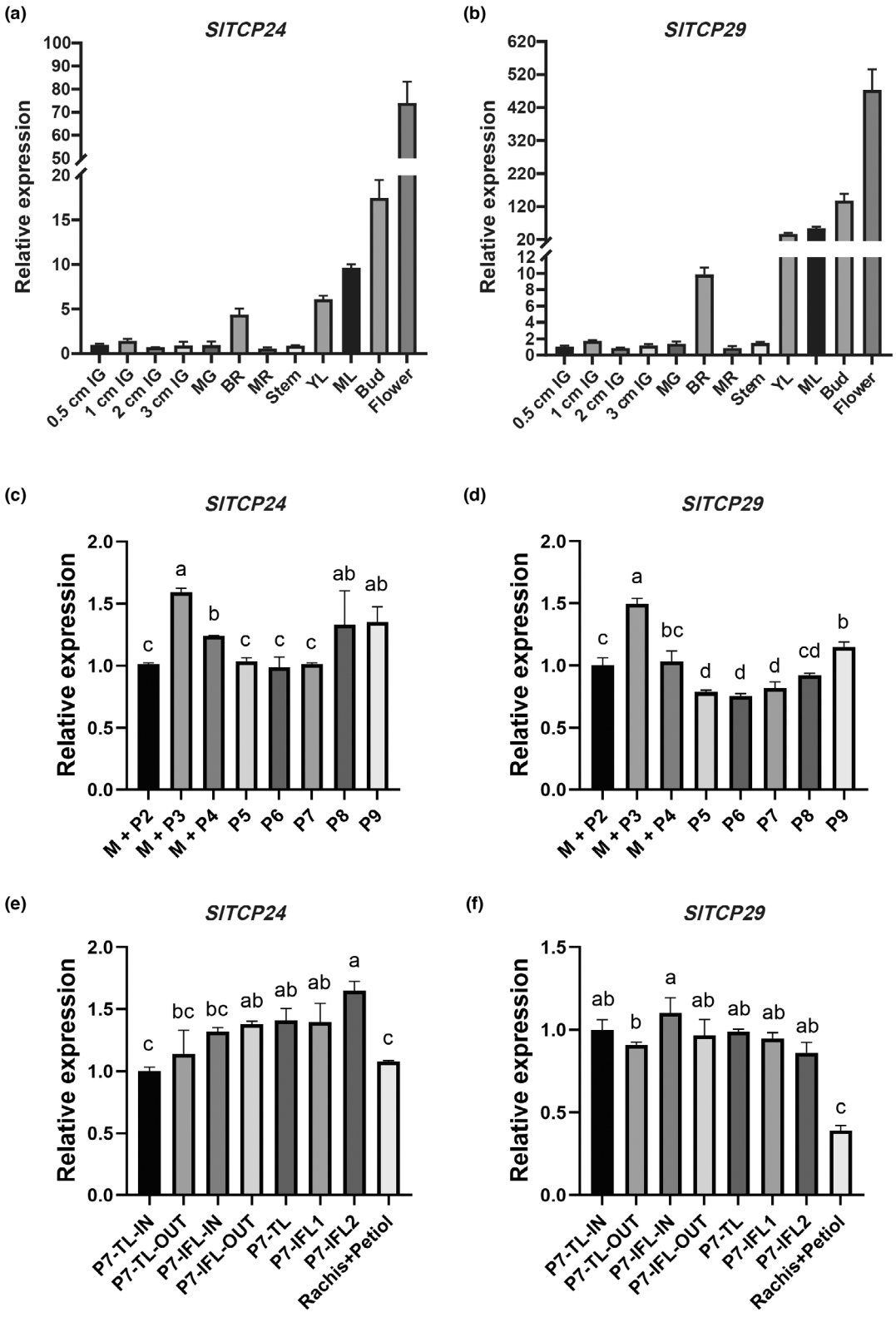
The expression of *SlTCP24* and *SlTCP29* in different tissues

qRT-PCR was used to examine the expression levels of *SlTCP24* and *SlTCP29* in different tomato tissues and observed that the expression patterns of the two *TCP* genes were similar in all tissues examined (Fig. 1a,b). Their expression levels were found to be higher in leaves and flowers (Fig. 1a,b), suggesting that *SlTCP24* and *SlTCP29* may play an important role in the development of leaves and flowers. Furthermore, we examined the expression levels of *SlTCP24* and *SlTCP29* during leaf development. We discovered that *SlTCP24* and *SlTCP29* were expressed at all stages of leaf development, with *SlTCP24* and *SlTCP29* being expressed at a relatively higher level in the SAM with youngest leaf primordia 1–3 (M + P3) stage (Fig. 1c,d). The expression in the marginal region of leaflets was not significantly different compared with internal regions for *SlTCP24* and *SlTCP29*, whereas the expression levels of *SlTCP24* and *SlTCP29* were higher in leaves than in the rachis and petiole (Fig. 1e,f).

Synergetic function of *SlTCP24* and *SlTCP29* in compound leaf development

35 TCP transcription factors were found in the latest tomato genome database ITAG4.1. The phylogenetic tree was constructed based on the TCP genes from tomato and Arabidopsis, SlTCP24 and SlTCP29 belong to the same CIN LIKE clade and are close with AtTCP24 (Fig. S1a). We used CRISPR/Cas9 to generate loss-of-function mutations in SlTCP24 and SlTCP29 to validate their specific functions. Because *SlTCP24* and *SlTCP29* show high similarity, two unique targets were designed on *SlTCP24* and *SlTCP29* to get single-gene knockout plants, and two targets shared by *SlTCP24* and *SlTCP29* were designed to get double-genes knockout plants (Fig. 2a). Two lines with different gene-knockout (KO) mutations were selected to represent

Fig. 1 Expression patterns of *SlTCP24* and *SlTCP29* in tomato plants. (a, b) Expression of *SlTCP24* and *SlTCP29* in different tissues of tomato. Total RNA was isolated from 0.5, 1, and 3 cm IG (immature green fruit), MG (mature green fruit), BR (breaker fruit), MR (mature red fruit), Stem, YL (young leaf), ML (mature leaf), Bud, and Flower. The values are means ± SD ($n = 3$). The relative expression of *SlTCP24/29* in different tissues was determined using qRT-PCR. The data were normalized with comparison to the value of 0.5 cm IG, which was set at 1.0. (c, d) Expression of *SlTCP24* and *SlTCP29* at different stages of early leaf development in wild-type tomato. M + P2, SAM with youngest leaf primordia 1 and 2; M + P3, SAM with youngest leaf primordia 1–3; M + P4, SAM with youngest leaf primordia 1–4; P5, fifth youngest leaf primordium; P6-P9, sixth to ninth youngest leaf primordium, respectively. The values are means ± SD ($n = 3$). The data were normalized with comparison to the value of M + P2, which was set at 1.0. (e, f) Expression of *SlTCP24* and *SlTCP29* in different parts of the leaf in wild-type tomato. P7, developing leaf at the seventh youngest leaf primordial stage (P7); TL, terminal leaflet; IFL1, first leaflet; IFL2, second leaflet; IN, inner parts of a leaflet; OUT, outer parts of a leaflet. Statistically significant differences were determined using a one-way ANOVA. Different letters indicate statistically significant differences.



each of the *SlTCP29-KO*, *SlTCP24-KO*, and *SlTCP24/29-KO* lines (Fig. 2b). The information of their mutation sites is listed in Table S2.

We also checked their expression in *SlTCP24/29-KO* and *SlTCP24/29-OE* lines, the level of overexpressing for *SlTCP29* was much higher than *SlTCP24*. The expression decreased in

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several KO plants for the corresponding genes (Fig. S2a,b). The leaf phenotype was investigated in these KO and OE lines. In *SlTCP24-KO-24* single-gene knockout lines, there was an increase in the number of leaflets, and the leaf margin serration liked to be deepened. No obvious differences were found in *SlTCP24-KO-26* (Fig. 2c,d). The number of leaflets increased

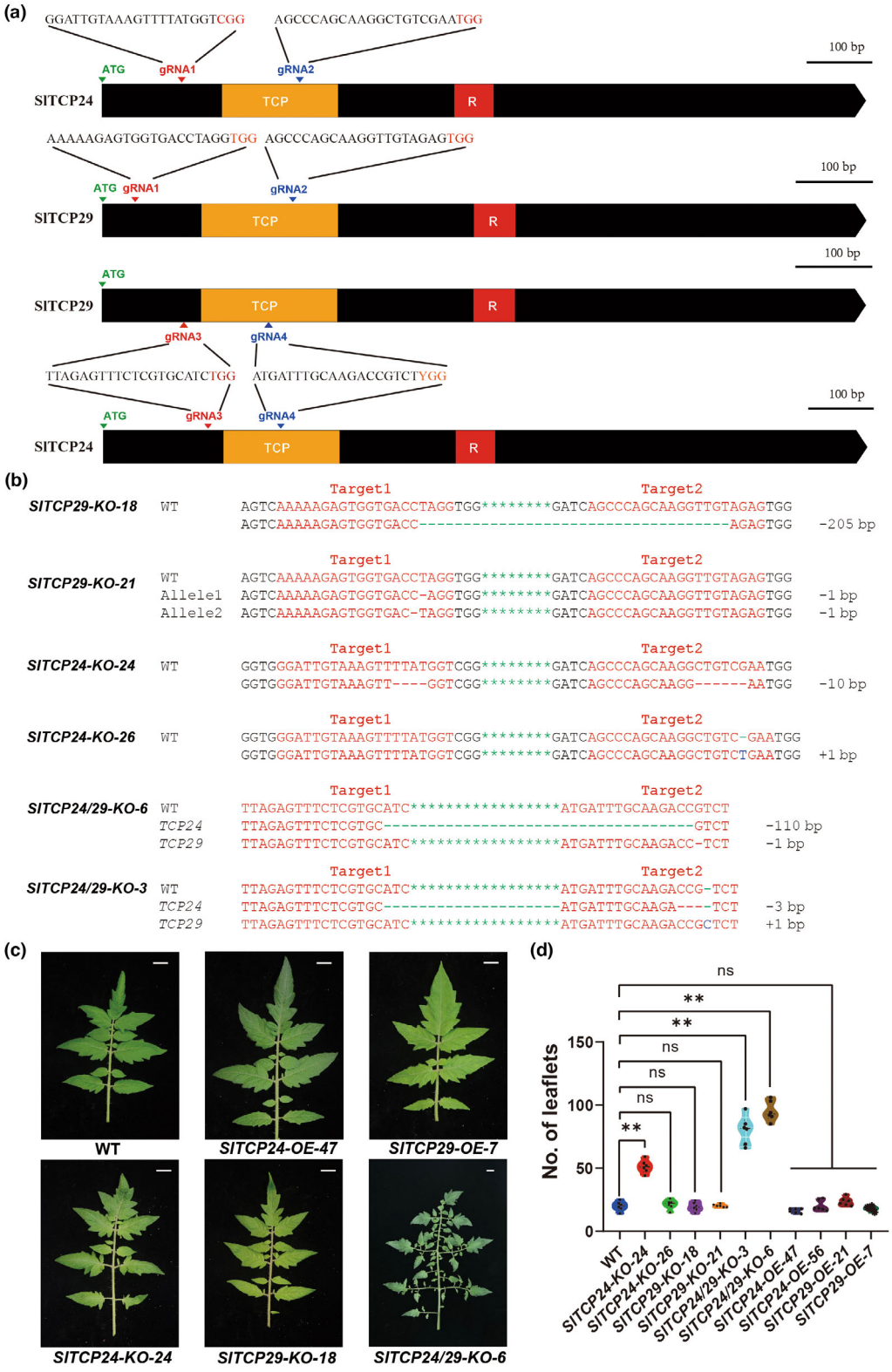

Fig. 2 Leaf morphological phenotypes of *SlTCP24/29-KO* single-gene and double-gene knockout mutants created via the CRISPR/Cas9 approach. (a) Schematic diagrams of target sites in the *SlTCP24* and *SlTCP29* cDNA sequences. TCP, the conserved TCP domain of *c*. 60 amino acid residues. R, the conserved arginine (R)-rich motif of 18–20 residues. gRNA, guide RNA for targeted gene editing by CRISPR/Cas9. (b) Genomic DNA sequences within the target regions in selected gene-knockout lines of *SlTCP24-KO*, *SlTCP29-KO*, and *SlTCP24/29-KO*. Letters in red font indicate the target sequences. Nucleotide insertions are shown in blue, nucleotide deletions are represented by dash lines, and omitted nucleotide sequences are indicated by green asterisks. The numbers on the right side indicate deletions (−) or insertions (+) in mutant lines as compared to the WT. (c) Leaf phenotypes from 8-wk-old *SlTCP24/29-KO* and *SlTCP24/29-OE* plants. Bar, 2 cm. (d) Numbers of leaflets in different *SlTCP24/29-KO* and *SlTCP24/29-OE* lines. Values are means ± SD ($n = 6$), and a one-way ANOVA and Dunnett's test was conducted. ns, no significant difference at $P > 0.05$; **, statistically significant difference at $P < 0.01$.

drastically in the *SlTCP24/29-KO* double-gene knockout lines, and the leaf became more compound than WT and the blade size was reduced (Fig. 2c,d). However, there was no apparent leaf morphology phenotype in overexpressing lines of *SlTCP24* or *SlTCP29* (Fig. 2c,d). Based on these findings, we conclude that *SlTCP24* and *SlTCP29* are required for the development of normal compound leaves and the functions of the two genes may be redundant in compound leaf formation in tomato.

Interaction of SlTCP29 and SlTCP24 with SlAS2

AS2 has been found to form a protein complex with TCPs in Arabidopsis to regulate leaf development (Z. Li *et al.*, 2012). To test whether SlTCP24 and SlTCP29 interacted with SlAS2 in tomato, we first made a phylogenic tree for lateral organ boundaries domain (LBD) protein family (Fig. S1b). Phylogenetic analysis showed that LBD proteins could be divided into four clades, AS2 has a homology gene LBD26 in Arabidopsis and two homology genes *SlAS2* and *SlAS2-like* in tomato (Fig. S1b); then, we performed yeast two-hybrid assays using SlTCP24 or SlTCP29 as bait and SlAS2 or SlAS2-like protein as prey. Our findings revealed that both SlAS2 and SlAS2-like proteins could interact with SlTCP24 or SlTCP29 in yeast (Figs 3a, S3). We also investigated the transcript level of *SlAS2* and *SlAS2-like* in *SlTCP24/29-KO* lines, but no a regular relationship was found among *TCPs-KO* lines, implying that the regulation of *SlTCP24* and *SlTCP29* to *SlAS2* and *SlAS2-like* was not in the transcript level, maybe in the protein level (Fig. S4a,b).

We next used the luciferase complementation imaging assay to verify whether SlAS2 could interact with SlTCP29 or SlTCP24 in plant cells. Luciferase (LUC) activity signals were detected in tobacco leaves co-infiltrated with *Agrobacterium* strains expressing *SlAS2-nLUC* and *cLUC-SlTCP29* or *cLUC-SlTCP24* (Fig. 3b,c). By contrast, no LUC signal was observed in three negative controls (Fig. 3b,c). The results further confirmed that SlAS2 interacts with SlTCP24 or SlTCP29 in plant cells.

Furthermore, we performed a Co-IP assay to confirm their interaction. In this experiment, tobacco protoplasts were transformed with two constructs (*SlAS2-HA* and *SlTCP29-FLAG*) or (*SlAS2-myc* and *SlTCP24-FLAG*) using PEG-mediated transformation. *Mer*, an empty vector was used as a negative control. The results showed that both recombinant proteins (SlAS2-HA and SlTCP29-FLAG) or (SlAS2-myc and SlTCP24-FLAG) were expressed at the expected molecular mass in the protoplasts (Fig. 3d,e, input panel). Expected interaction proteins were co-precipitated and could be detected by corresponding antibodies (Fig. 3d,e, Co-IP panel), suggesting that SlTCP29 or SlTCP24 can interact with SlAS2 in plant cells.

SlAS2 inhibits dimerization of SlTCP24 and SlTCP29

Previous studies have shown that TCP transcription factors can form both homo- and heterodimers (Danisman *et al.*, 2013). SlTCP24 has been reported to interact with itself in yeast (Parapunova *et al.*, 2014). We asked whether SlTCP24 and SlTCP29 could form heterodimers and whether SlTCP29 could interact with itself to form homodimers. Results of yeast two-hybrid assays revealed that SlTCP29 could interact with itself and with SlTCP24 in yeast (Fig. 4a). Analysis of luciferase complementation imaging (LCI) assays further confirmed that SlTCP24 and SlTCP29 could form a heterodimer and SlTCP29 could form a homo-dimer with itself in plant cells (Fig. 4b,c). The Co-IP analysis also confirmed the interaction between SlTCP24 and SlTCP29 (Fig. 4d). Because SlAS2 could also interact with SlTCP29 and SlTCP24 in yeast and plant cells (Fig. 3), we asked whether and how SlAS2 affected the dimerization of TCP proteins. Luciferase complementation imaging assays were performed to investigate this influence (Fig. 4e). When the combinations of TCP proteins (*SlTCP29-nLUC* and *cLUC-SlTCP24* or *SlTCP29-nLUC* and *cLUC-SlTCP29*) were expressed without the presence of *SlAS2*, the luciferase signals were strong (Fig. 4e, right panels). However, in the presence of overexpressed *SlAS2*, the luciferase signals were much weaker (Fig. 4e, left panels). These observations indicated that both the homo-dimerization and heterodimerization of tomato TCP proteins are inhibited by the presence of *SlAS2*. Taken together, our results suggest that SlAS2 interacts with SlTCP29 and SlTCP24, and these interactions lead to inhibition of dimerization of SlTCP29 and SlTCP24 proteins.

Regulation of expression of cell division-related genes by *SlTCP24* and *SlTCP29*

To investigate what genes were regulated by SlTCP24 and SlTCP29 transcription factors, we performed transcriptome sequencing of leaves from both WT and *SlTCP24/29* double-gene knockout lines. A total of 1961 genes were found to be differentially expressed between WT and *SlTCP24/29-KO* lines (Dataset S1). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the differentially expressed genes (DEGs) indicated that some metabolism pathways significantly enriched were downregulated (Fig. 5a) and some of them were upregulated

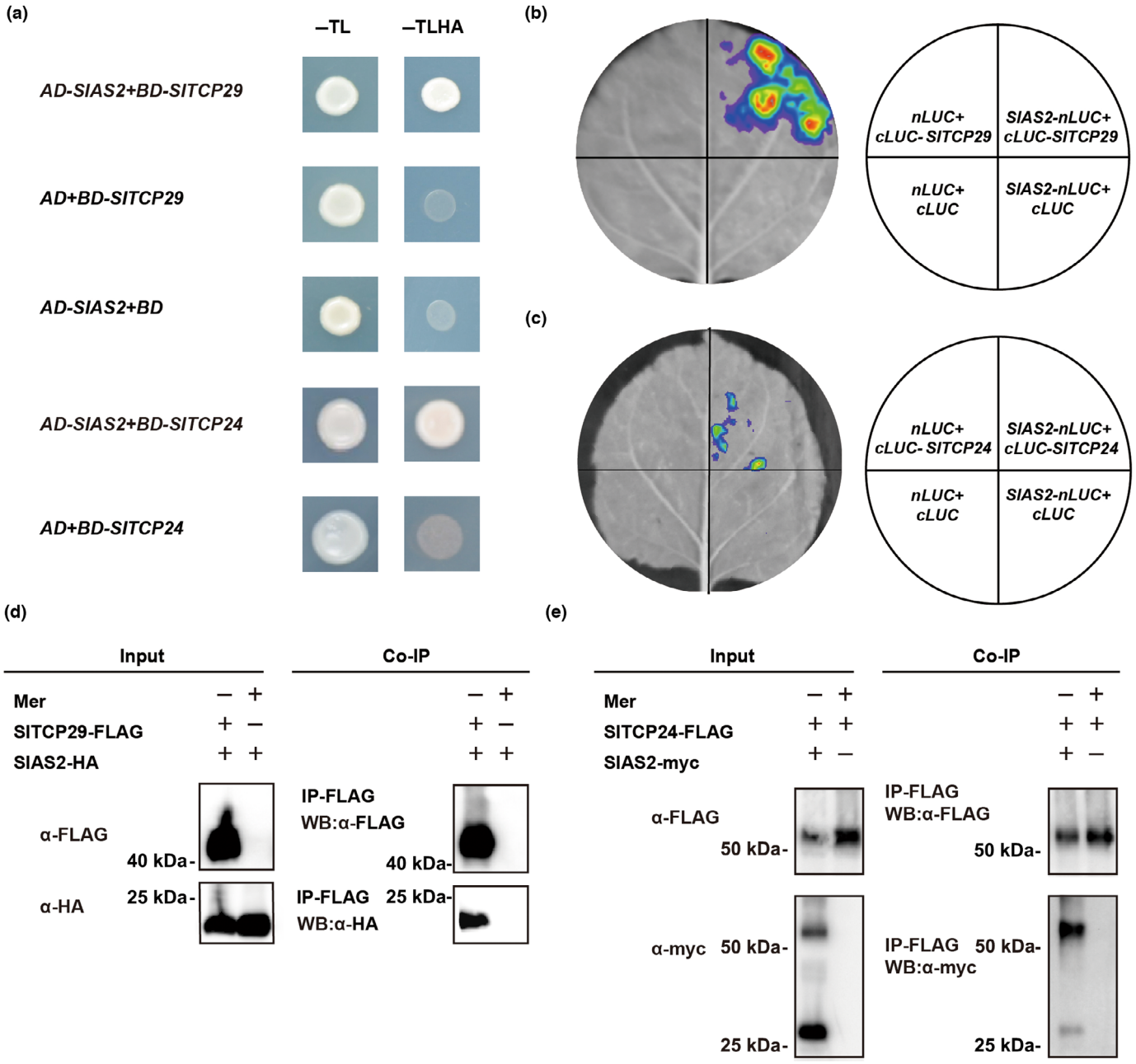

Fig. 3 Interaction between SlTCP24/29 and SlAS2. (a) Yeast two-hybrid assays showing the interactions between SlTCP24/29 and SlAS2 proteins. The *BD-SlTCP24/29* and *AD-SlAS2* plasmids were co-transformed into yeast strain AH109. Yeast cells were grown on a synthetic medium (SD/−Trp−Leu) (−TL) and selected for protein–protein interaction on a selective medium (SD/−Trp−Leu−His−Ade) (−TLHA). (b, c) Luciferase complementation imaging (LCI) assays showing protein interactions of SlAS2 with SlTCP29 (b) or SlTCP24 (c). Tobacco (*N. benthamiana*) leaves were divided into four parts and infiltrated with *Agrobacterium* strains harboring *SlAS2-nLUC* and *cLUC-SlTCP24* or *cLUC-SlTCP29*. Three pairs of constructs, including *SlAS2-nLUC* + *cLUC*, *nLUC* + *cLUC-SlTCP24/29*, and *nLUC* + *cLUC*, were used as negative controls. Images were captured with a charge-coupled device camera 3 d postinfiltration (dpi). (d, e) Co-immunoprecipitation assays showing the interaction between SlTCP29 and SlAS2 (d) and SlTCP24 and SlAS2 (e). *SlAS2-HA* and *SlTCP29-FLAG* or *SlAS2-myc* and *SlTCP24-FLAG* were used for co-transformation of tobacco protoplasts. Proteins were extracted from protoplasts, immunoprecipitated using anti-FLAG matrix beads, and analyzed by western blotting using anti-HA, anti-FLAG, or anti-myc antibodies. *Mer* is an empty vector as a control.

(Fig. 5b). Among the DEGs, thirteen genes involved in the zeatin metabolism pathway were downregulated (Fig. 5c), including *cytokinin oxidase 2* (*CKX2*) and *cytokinin oxidase 5* (*CKX5*), both implicated in cytokinin (CK) degradation, four zeatin *O*-glucosyltransferase genes and five zeatin *O*-xylosyltransferase genes, which are involved in zeatin inactivation by glycosyl conjugation. qRT-PCR was performed to verify the expression of several genes (Fig. 5d). These results showed that genes involved in cytokinin degradation and conjugation are downregulated when both *SlTCP24* and *SlTCP29* genes are knocked out.

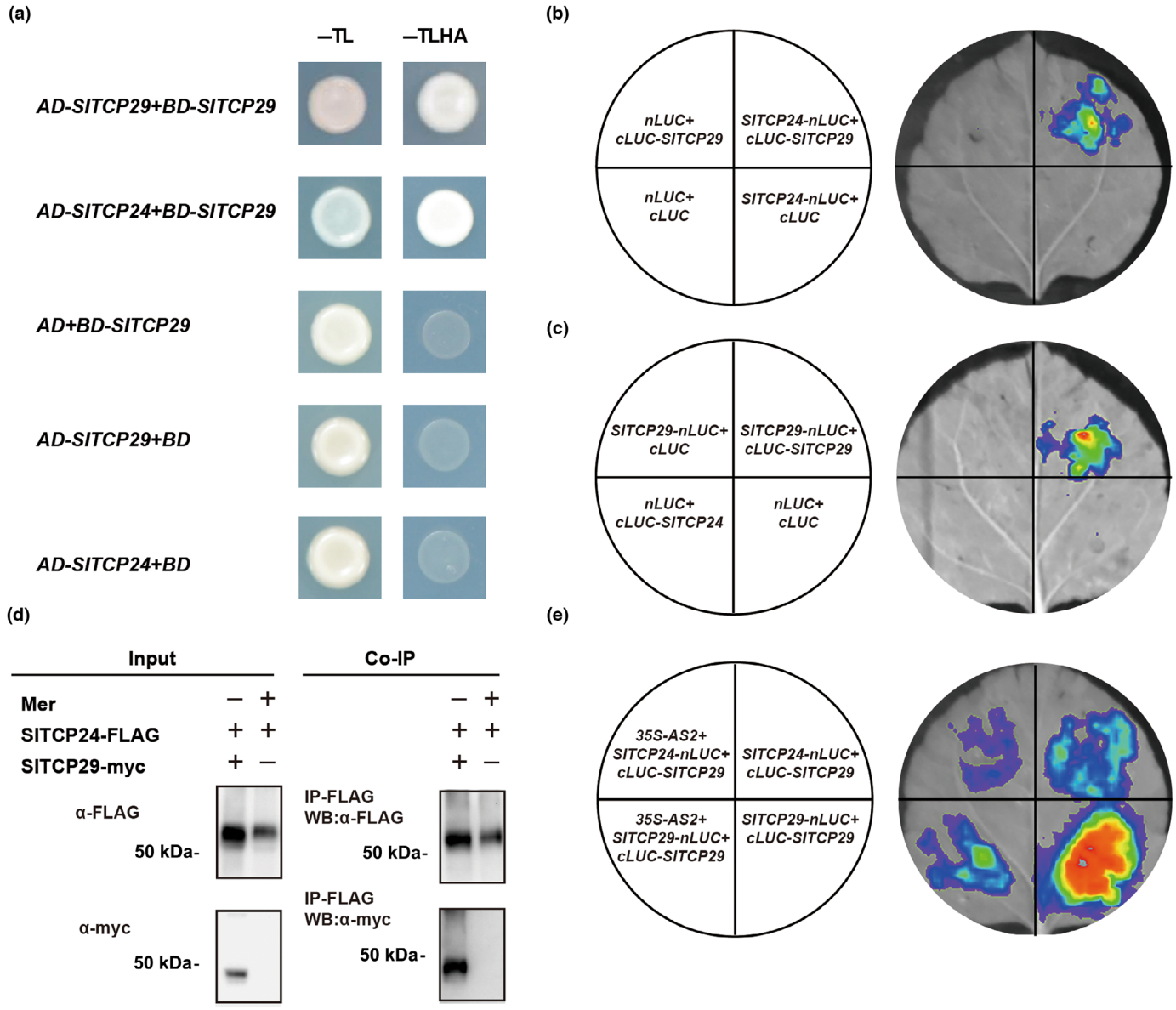

Fig. 4 Effect of SlAS2 on dimerization of SlTCP24/29. (a) Yeast two-hybrid assays showing the interactions between SlTCP24 and SlTCP29, and between SlTCP29 and SlTCP29. The combinations of *BD-SlTCP29* and *AD-SlTCP24*, *BD-SlTCP29*, and *AD-SlTCP29* were used to co-transform yeast strain AH109. Yeast cells were grown on a synthetic medium (SD/−Trp−Leu) (−TL) and selected for protein–protein interaction on a selective medium (SD/−Trp−Leu−His−Ade; −TLHA). (b, c) Luciferase complementation imaging (LCI) assays showing protein interactions between SlTCP24 and SlTCP29 (b) and between SlTCP29 and SlTCP29 (c). Tobacco leaves were divided into four parts and infiltrated with *Agrobacterium* strains harboring *SlTCP24-nLUC* and *cLUC-SlTCP29* or *SlTCP29-nLUC* and *cLUC-SlTCP29*. Three pairs of constructs, including *SlTCP24/29-nLUC* + *cLUC*, *nLUC* + *cLUC-SlTCP24/29*, and *nLUC* + *cLUC*, were used as negative controls. Images were captured with a charge-coupled device camera 3 d postinfiltration (dpi). (d) Co-immunoprecipitation assays showing the interaction between SlTCP29 and SlTCP24. *SlTCP29-myc* and *SlTCP24-FLAG* were used for co-transformation of tobacco protoplasts. Proteins were extracted from protoplasts, immunoprecipitated using anti-FLAG matrix beads, and analyzed by western blotting using anti-myc or anti-FLAG antibodies. *Mer* is an empty vector as a control. (e) LCI assays showing the effect of SlAS2 on the stringency of the interactions between SlTCP24 and SlTCP29 and between SlTCP29 and SlTCP29. Tobacco leaves were divided into four parts. The interactions between SlTCP24 and SlTCP29 and between SlTCP29 and SlTCP29 were assayed in the absence of SlAS2 (right panels) or in the presence of SlAS2 (left panels).

Activation of *SlCKX2* gene expression by SlTCP24 and SlTCP29

It has been reported that class II TCP proteins preferred to bind to the sequence GTGGNCCC in gene promoters (Manassero *et al.*, 2013). Two such TCP-binding sites were found in the *SlCKX2* promoter (Fig. 6a). To examine whether SlTCP24 or SlTCP29 could bind to these TCP-binding sequences, we performed yeast one-hybrid assays in which the *SlCKX2* promoter was used to drive the expression of the aureobasidin A resistance gene (*AUR1-C*). The empty vector *pGADT7* was used as a negative control. Yeast cells co-transformed with *pGADT7-SlTCP24* or *pGADT7-SlTCP29* and *pAbAi-SlCKX2-P* were found to be able to grow in SD/−Ura−Leu medium supplemented with AbA (80 ng ml^{-1};

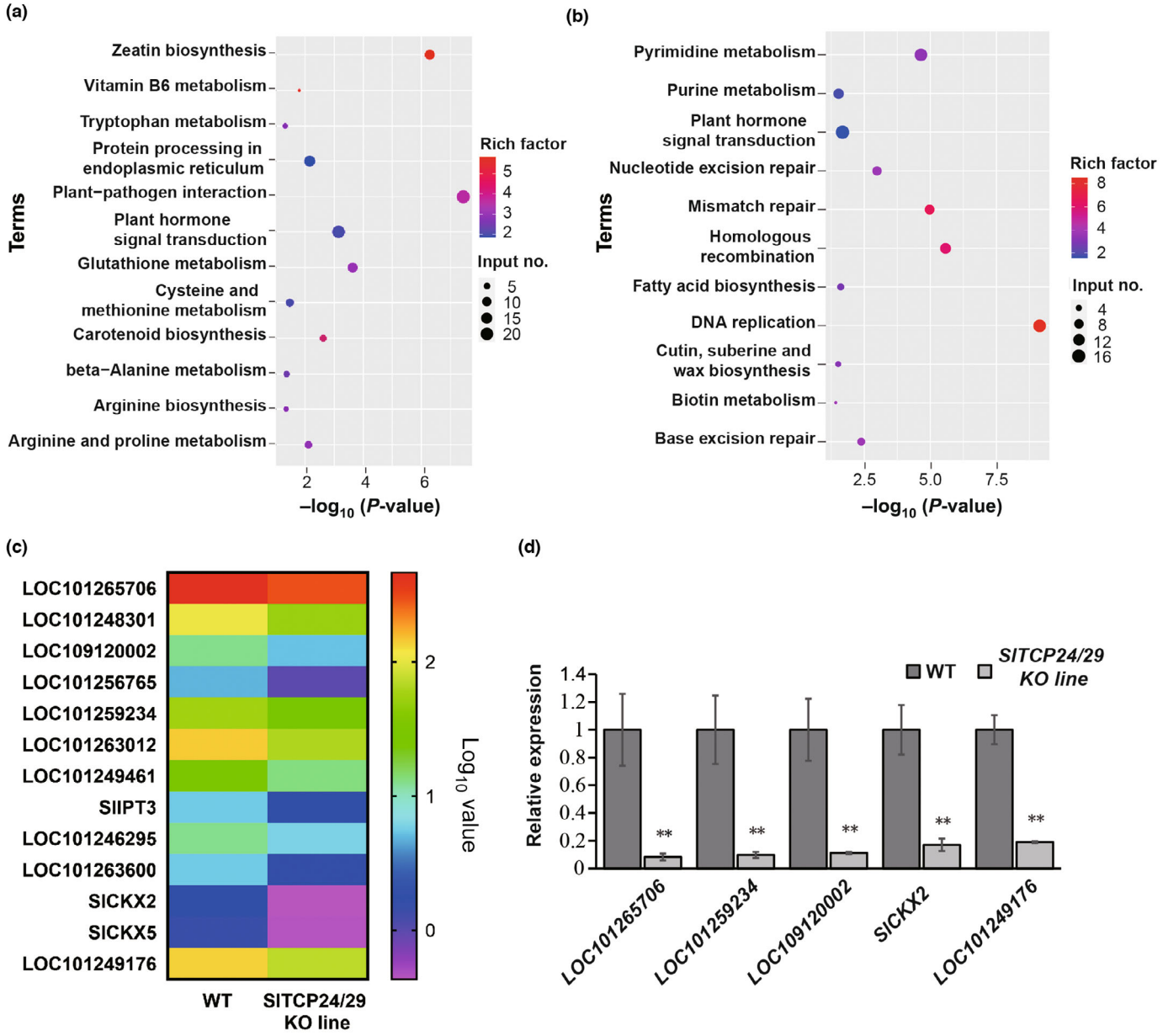

Fig. 5 Transcriptome analysis of gene expression in *SlTCP24/29* double-gene knockout line. (a) KEGG-enriched pathways with significantly downregulated genes in the *SlTCP24/29-KO* line as compared with the WT. The rich factor = $m \times N/(n \times M)$, and m is the number of differentially expressed genes in a specific KEGG pathway, N is the number of genes with KEGG annotation in the reference genome, *n* is the number of differentially expressed genes in *N*, and *M* is the number of genes in a specific KEGG pathway in the reference genome. (b) KEGG-enriched pathways with significantly upregulated genes in the *SlTCP24/29-KO* line as compared with the WT. (c) Heatmap of zeatin biosynthesis genes with decreased expression in the *SlTCP24/29-KO* line. (d) Validation of the transcriptome data using qRT-PCR. The expression values of each gene in WT were set as 1.0. The values are means ± SD ($n = 3$). **, statistically significant differences at $P < 0.01$.

Fig. 6b), suggesting that SlTCP24/29 transcription factors recognized the *SlCKX2* promoter and could drive the expression of the AbA resistance gene (*AUR1-C*). We also used the same system to test the *SlCKX5* promoter but could not detect the binding of SlTCP24/29 to the *SlCKX5* promoter (Fig. S5a). Thus, we conclude that SlTCP24/29 could bind to and activate the *SlCKX2* promoter, but not the *SlCKX5* promoter.

To test whether the activation of the *SlCKX2* promoter by SlTCP24/29 could take place in plant cells, we constructed a promoter::reporter plasmid (*pSlCKX2:LUC*) in which the luciferase (LUC) reporter gene was driven by the *SlCKX2* promoter (Fig. 6c). The promoter::reporter construct was expressed transiently in tobacco (*N. benthamiana*) leaves in the presence of effectors SlTCP24 or 29, the LUC reporter signal and the relative ratio of LUC/REN increased significantly compared with the control (Fig. 6d,e), suggesting that the effector either SlTCP24 or SlTCP29 could activate the *SlCKX2* promoter and enhance the LUC reporter gene expression. When both *35S:SlTCP24* and

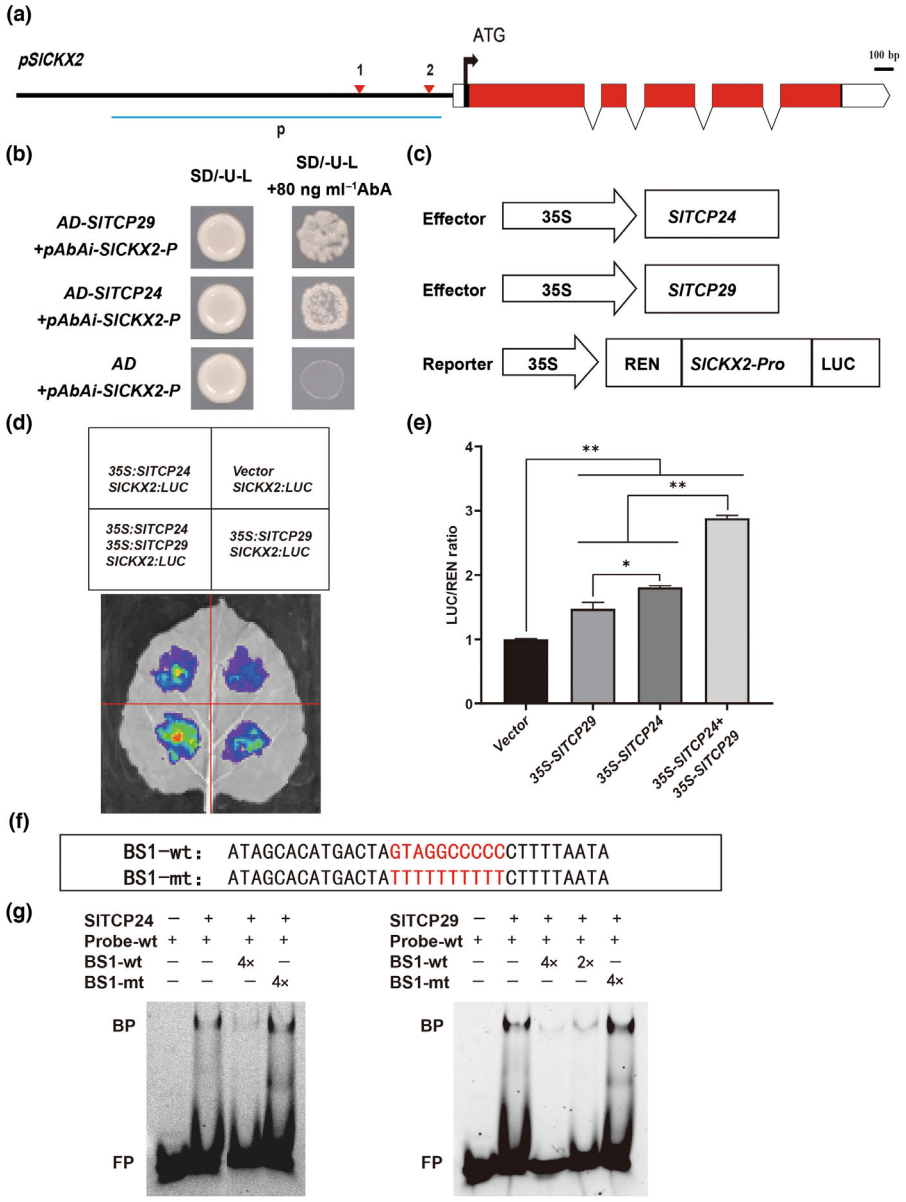

Fig. 6 Activation of *SlCKX2* expression by SlTCP24 and SlTCP29. (a) Schematic diagram of the promoter and *SlCKX2* gene. Two putative TCP-binding sites on the *SlCKX2* promoter are indicated by red triangles. Red rectangles and black twisting lines represent the exons and introns of *SlCKX2*. P indicates the DNA fragment used as the promoter of *SlCKX2* in yeast one-hybrid assay. (b) Yeast one-hybrid assay showing the binding of SlTCP24 and SlTCP29 to the *SlCKX2* promoter. Yeast strain Y1H Gold was transformed with the bait vector *pAbAi-SlCKX2* and the prey vector *AD-SlTCP24* or *AD-SlTCP29*. Yeast cells were grown on SD/−Leu−Ura medium (SD/−U−L) and selected for protein-promoter binding in the presence of antibiotic aureobasidin A (AbA, 80 ng ml^{-1}). (c) Schematic diagrams of plasmids used as effectors and reporter for the dual-luciferase experiment. The full-length open reading frame (ORF) of *SlTCP24/29* was cloned into *pGreen II 62-SK* to generate the effector constructs, *pGreen II 62-Sk-SlTCP24/29*. The promoter fragment of *SlCKX2* (−1 to −1881) was used to drive the expression of luciferase in *pGreen II 0800-LUC* to create *pGreen II 0800-SlCKX2-Pro*. (d) Luciferase imaging assays. LUC activities were expressed under the control of the *SlCKX2* promoter in the presence of SlTCP24, SlTCP29, or SlTCP24/29. Empty effector plasmid was used as a control. The intensity of luminescent signal indicates the strength of activation of the promoter by effectors. (e) Luciferase activity was detected by dual-luciferase reporter assay. The LUC/REN ratio of tobacco leaves transformed with the empty effector construct and the reporter construct was set to 1. LUC, firefly luciferase activity; REN, *Renilla reniformis* luciferase activity. The values are the means ± SD ($n = 6$). A one-way ANOVA and Dunnett's test were conducted. * and ** indicate statistically significant differences with $P < 0.05$ and $P < 0.01$, respectively. (f) Diagram of the wild-type and mutated probes used for electrophoretic mobility shift assay (EMSA). BS1-wt is the wild-type probe synthesized based on the TCP-binding site 1 (BS1) of the *SlCKX2* promoter. BS1-mt is a mutant probe in which the *cis*-element sequences were replaced with TTTTTTTTTT. Nucleotides of the core TCP-binding sequence are shown in red. (g) EMSA assays showing the binding of SlTCP24 or SlTCP29 to the binding-site 1 (BS1) probe of the *SlCKX2* promoter. Probe-wt is the double-strand DNA fragment labeled with 5′-carboxyfluorescencein (5-FAM). BS1-wt and BS1-mt are unlabeled double-strand DNA fragments used for competition for effector binding. BP, bound probe; FP, free probe.

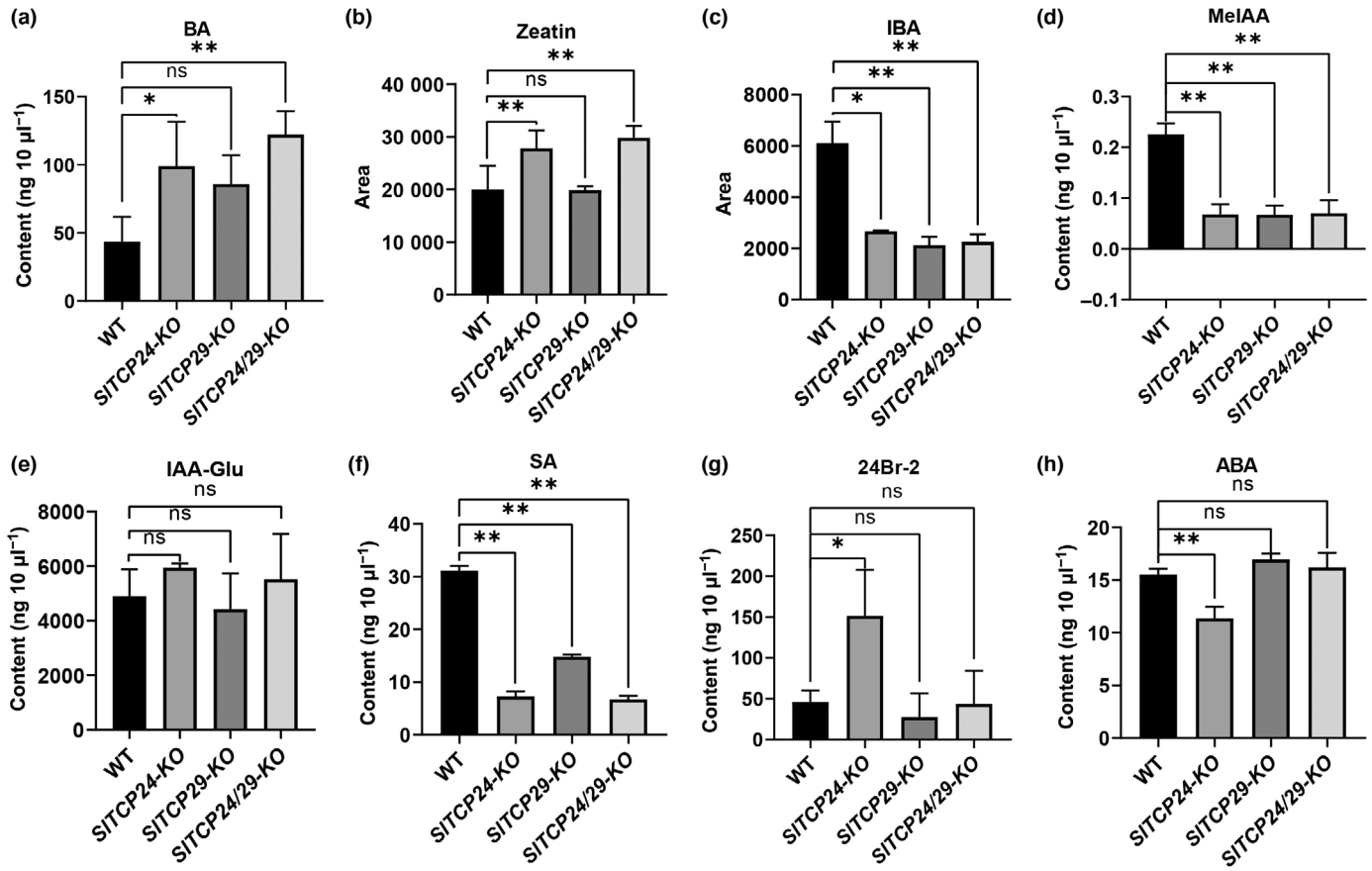

Fig. 7 Hormone contents in *SlTCP24/29* single-gene knockout and double-gene knockout lines. The content of several hormones including BA, 6-benzylaminopurine (a); Zeatin (b); IBA, indole-3-butyric acid (c); MeIAA, methyl indole-3-acetic acid (d); IAA-Glu, indole-3-acetic acid-glutamate (e); SA, salicylic acid (f); 24Br-2, brassinosteroid (g); ABA, abscisic acid (h). The values are the means ± SD ($n = 3$), a one-way ANOVA and Dunnett's test was conducted. * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively. ns, no significant difference.

35S:SlTCP29 were co-transformed with *pSlCKX2:LUC*, the LUC reporter signal and the ratio of LUC/REN reached the highest level (Fig. 6d,e). These findings indicated that SlTCP24 and SlTCP29 had additive effects to promote the expression of *SlCKX2* in plant cells.

Furthermore, we used electrophoretic mobility shift assays (EMSA) to test whether SlTCP24 and SlTCP29 could bind directly to the conserved TCP-binding sequence, GTGGNCCC. A double-strand DNA fragment of 33 bp corresponding to the putative TCP-binding site 1 of the *SlCKX2* promoter containing GTAGGCCCCC (Fig. 6f) was synthesized and labeled 5′-carboxyfluorescencein (5-FAM). The mobility of the promoter fragment was shifted to a band of much higher molecular mass in the presence of purified recombinant SlTCP24 or SlTCP29 proteins (Fig. 6g). This mobility shift could be reduced by addition of an excessive amount (4-fold) of unlabeled promoter DNA fragment as a competitor for DNA binding, suggesting that the binding was specific to the DNA sequence. When the conserved TCP-binding sequence, GTGGNCCCCC, of the promoter DNA fragment was replaced by TTTTTTTTTT (Fig. 6f), incubation with the excessive amount (fourfold) of this mutant version of the promoter DNA fragment failed to compete for binding to the SlTCP24/29 and could not reduce the intensity of the shifted band (Fig. 6g). Thus, SlTCP24 and SlTCP29 seem to bind to the conserved TCP-binding sequence, GTGGNCCCCC of the binding site 1 in the *SlCKX2* promoter. We also confirmed that SlTCP24/29 could not bind to the putative binding site 2 of the *SlCKX2* promoter on EMSA (Fig. S5b). Taken together, these findings suggest that SlTCP24 and SlTCP29 directly bind to the TCP-binding site 1 of *SlCKX2* promoter and activate its expression.

Elevated cytokinin content in leaves of *SlTCP24/29-KO* double-gene knockout plants

Based on the analysis of *trans*criptome sequencing and the regulation of *SlCKX2* expression, we wondered whether the cytokinin content was changed in leaves of the *SlTCP24/29-KO* double-gene knockout plants. We determined the levels of several active hormones and their conjugates in young leaves of *SlTCP24/29-KO* lines using liquid chromatography assays. The contents of cytokinin (6-benzylaminopurine (BA) and zeatin) were found to be significantly higher in the *SlTCP24-KO* and *SlTCP24/29-KO* lines than the WT control plants (Fig. 7a,b), which was consistent with the result of the downregulated *SlCKX2* expression in the *SlTCP24/29-KO* lines (Figs 5, 6). These findings suggested

that SlTCP24 and SlTCP29 could regulate the cytokinin content by regulating the *SlCKX2* expression. In addition to the change in cytokinin content, we also found that the contents of indole-3 butyric acid (IBA), methyl indole-3 acetic acid (MeIAA), and salicylic acid (SA) were reduced significantly in the *SlTCP24-KO*, *SlTCP29-KO*, and *SlTCP24/29-KO* lines (Fig. 7c,d,f). The contents of other phytohormones and their conjugates remained essentially unchanged in *SlTCP24/29* knockout lines (Fig. 7e,g,h). The mechanisms by which *SlTCP24* and *SlTCP29* regulate the levels of phytohormones could be complex and remains to be investigated further. In summary, the findings of the present work demonstrate that two TCP-family transcription factors, SlTCP24 and SlTCP29, synergistically regulate tomato leaf development by changing the expression of genes involved in the cytokinin inactivation pathway. Understanding the roles of the two key regulators of leaf morphogenesis has provided a new target of regulatory components for genetic engineering and breeding programs aimed at improving the morphology and light harvesting efficiency of compound leaves in crops such as tomato.

Discussion

SlTCP24 and *SlTCP29* are active regulators in the cytokinin degradation pathway

Recent studies have demonstrated that cytokinin (CK) is a key phytohormone that promotes morphogenesis of plant organs (Shani *et al.*, 2006; Shwartz *et al.*, 2016). The CINCINATA (CIN) clade TCP transcription factors are known to affect leaf shape by promoting differentiation, and the morphogenetic window is dependent on the low CIN-TCP activities early in leaf development (Ori *et al.*, 2007; Ori, 2012; Schommer *et al.*, 2014; Koyama *et al.*, 2017; Challa *et al.*, 2019). *LANCEOLATE* (*LA*), encoding a TCP transcription factor, plays an essential role in compound leaf development and plant responses to CK in tomato (Israeli *et al.*, 2021). *LA* promotes differentiation by decreasing the plant sensitivity to CK and increasing the GA levels and/or enhancing plant responses (Israeli *et al.*, 2021). In Arabidopsis, the *LA* homolog *TCP4* reduces CK responses during leaf development (Efroni *et al.*, 2013). However, to our knowledge, no direct evidence is reported for TCP transcription factors in the regulation of the CK biosynthesis/accumulation pathway.

Our findings show that *SlTCP24/29* downregulated the expression of zeatin biosynthesis genes and that SlTCP24 and SlTCP29 also directly bound to the promoter of *SlCKX2* and activated its expression (Fig. 6). SlTCP24 and SlTCP29 also interacted with SlAS2, attenuated the activation effect of SlTCP24/29 on the zeatin degradation gene *SlCKX2*. Cytokinin oxidase, encoded by *CKX* genes, is an enzyme that plays a key role in regulating the cytokinin level by irreversible cleavage of active *trans*-zeatin (Werner *et al.*, 2006). Notably, overexpression of *CKX*s reduces the level of endogenous cytokinin and causes various developmental defects (Werner *et al.*, 2001, 2003). For example, the expression of *AtCKX3* in tomato leaves has been found to lead to the formation of simplified leaves (Shani *et al.*, 2010), while overexpression of *AtCKX3* under either leaf or root-specific promoters does not affect the concentrations of cytokinin in tomato leaves (Glanz-Idan *et al.*, 2022). In our study, the expression of *SlCKX2* was significantly lower in *SlTCP24/29-KO* lines than in the wild-type control (Fig. 5), which was consistent with the function of SlTCP24 and SlTCP29 directly activating *SlCKX2* gene expression. The cytokinin contents were found to be much higher in *SlTCP24/29-KO* lines than in WT (Fig. 7a,b). These findings suggest that *SlTCP24/29* could regulate the cytokinin content by promoting its degradation rather than decreasing plant responses to this hormone. Unlike *LA*, the TCP transcription factors represent a new mechanism by which the cytokinin synthesis and leaf morphogenesis are regulated. This study provides reliable evidence for the first time to establish a link between the TCP transcription factors and the regulation of cytokinin synthesis in plants.

In addition, we noted that the amount of MeIAA and IBA in the knockout lines decreased significantly compared with the control lines (Fig. 7c,d). A decrease in auxin synthesis would also cause the leaves more complex (Ben-Gera *et al.*, 2012; Xiong & Jiao, 2019). In the present study, we checked the transcriptome data and found that several genes of the tryptophan metabolism pathway were significantly downregulated in the double knockout lines. It indicates that SlTCP24/29 may also be involved in regulating the biosynthesis of IAA. Auxin often works together with other hormones to regulate leaf development (Bai & DeMason, 2006). Whether SlTCP24/29 may regulate compound leaf development by coordinating auxin with cytokinin content or whether crosstalk with two hormones remains to be studied further.

SlTCP24 is a stronger factor than *SlTCP29* in regulation of compound leaf development

The *TCP* genes exist as a gene family in plant genomes and the members exhibit partial genetic redundancy rather than complete redundancy. They perform a similar function but have distinct roles and expression patterns (Briggs *et al.*, 2006; Danisman *et al.*, 2013). It has been demonstrated that TCP transcription factors play important roles in diverse pathways in plants (Koyama *et al.*, 2007; Braun *et al.*, 2012; Danisman *et al.*, 2012; Lucero *et al.*, 2017; Zhang *et al.*, 2019; Karaaslan *et al.*, 2020). Class I TCP induces cell division and class II TCP inhibits cell division in the axillary meristem (Martin-Trillo & Cubas, 2010). Recent studies in Arabidopsis showed that this split is not so simple because the *brc1/brc2* double mutant has a high degree of branching. Furthermore, double mutants of the *Jaw-TCP* and *TCP5-like* genes exhibited a phenotype with reduced branching (van Es *et al.*, 2019). Our results indicated that the number of leaflets increased significantly only when *SlTCP24* and *SlTCP29* were both mutated. While *SlTCP24* looks to be a stronger factor than *SlTCP29* in regulating compound leaf development. We discovered a slight increase in the number of leaflets in *SlTCP24-KO* lines as compared to the wild-type control (Fig. 2c,d). SlTCP24 was more efficient than SlTCP29 in activating the *SlCKX2* promoter on the LUC reporter assays

(Fig. 6d,e), and the contents of cytokinin (6-benzylaminopurine (BA) and zeatin) were found to be significantly higher in the *SlTCP24-KO* line than *SlTCP29-KO* (Fig. 7a,b). These findings support our hypothesis that *SlTCP24* is a stronger effector than *SlTCP29* in regulating the cytokinin pathway and compound leaf development.

The regulation of *SlTCP24* and *SlTCP29* by *SlAS2* may partially explain the *KNOX*s gene function in compound leaf development

Some compound leaf mutants reported previously such as *la*, *knox5*, and *clausa* have a very similar leaves phenotype with *SlTCP24/29-KO* lines (Ori *et al.*, 2007; Kimura *et al.*, 2008; Bar *et al.*, 2016). Interestingly, the expression of some compound leaf relative genes including several *KNOX* genes varied significantly in the *SlTCP24/29-KO* lines on our transcriptome data (Fig. S6a), and qRT-PCR was performed to verify the expression of some genes (Fig. S6b), implying that SlTCP24/29 may regulate their expression. Previous research has shown that *Petroselinum* (*PTS*), a single-nucleotide deletion in the promoter region of the *KNOX5*, increased the abundance of *KNOX5* transcripts and resulted in multiple compound leaves (Kimura *et al.*, 2008). The AS1/AS2 complex inhibits the expression of *KNAT1* (*KNOX1*), and the expression of *KNAT1* promotes the transition from single-leaf fissure to compound leaves in cotton (Chang *et al.*, 2019). AS2 also interacts with LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) and JAGGED LATERAL ORGAN (JLO) to repress *KNOX* genes (Borghi *et al.*, 2007; Z. Li *et al.*, 2012, 2016). AS2 and TCPs bind to similar regions of the *KNAT1* and *KNAT2* promoters and the DNA-binding activity of TCP proteins depends on the presence of AS2 (Z. Li *et al.*, 2012). But the regulation mechanism AS2 affects the expression of *KNOX* genes is still not fully understood. In the present study, it was found that SlTCP24/29 dimerization could enhance downstream gene activation (Fig. 6d,e), whereas SlAS2 can inhibit the dimerization of SlTCP24 and SlTCP29 proteins (Fig. 4e). The regulation of SlTCP24 and SlTCP29 by SlAS2 may partially explain the *KNOX*s gene function in compound leaf development.

SlTCP29 may play an important role downstream of *CLAUSA*

CLAUSA is a critical developmental regulator in tomato. *clausa* is a classic tomato mutant with increased morphogenetic potential that results in abnormal, hyper-divided leaves with stem-like structures on the rachis (Khush & Rick, 1967). *CLAUSA* encodes a MYB transcription factor with a unique role in compound leaf species to promote exit from the morphogenetic stage of tomato leaf development (Bar *et al.*, 2016). *CLAUSA* functions as a potential negative regulator of the *KNOX I* gene and the border gene *GOBLET* (*GOB*) (Jasinski *et al.*, 2007, 2008; Naz *et al.*, 2013; Bar *et al.*, 2015). *CLAUSA* modulates the morphogenetic window by reducing cytokinin signaling and sensitivity (Bar *et al.*, 2016), GA treatment reduces leaf complexity in *clausa* mutants (Jasinski *et al.*, 2007), and *CLAUSA* functions by modulating GA levels and responses (Israeli *et al.*, 2021).

Recent studies have shown that *LA* is expressed earlier and more widely than *CLAUSA* and determines the developmental context of *CLAUSA* activity. *LA* and *CLAUSA* may promote differentiation through parallel genetic pathways (Israeli *et al.*, 2021). *SlTCP29*, *SlTCP24*, and *LA* belong to the same CIN-like TCP transcription factors and share high homology. We suspect that *SlTCP24/29* and *CLAUSA* may also regulate leaf development through parallel pathways. A previous study has shown that overexpression of *CLAUSA* causes simpler leaves with fewer leaflets (Bar *et al.*, 2016). In our study, the expression level of *CLAUSA* was significantly increased in *SlTCP24/29-KO* lines (Fig. S6b), contrary to the leaflet multiplicity phenotype. As a result, we hypothesized that there might be a feedback regulation in the *CLAUSA* pathway regulating *SlTCP24/29* and that *SlTCP24/29* may be a direct target of CLAUSA. Notably, there is a MYB binding site in the promoter of *SlTCP29*. Yeast one-hybrid assays confirmed that CLAUSA could directly bind to the promoter of *SlTCP29* (Fig. S7a). Dual-luciferase transactivation assay also showed that CLAUSA could significantly activate the expression of *SlTCP29*, but not *SlTCP24* (Fig. S7b,c). This suggests that *SlTCP29* may be an essential downstream target of CLAUSA. They can act in the same pathway, rather than in different parallel pathways like *LA* and *CLAUSA*. Since *CLAUSA* expression was increased in double knockout lines, there may be feedback regulation in the *CLAUSA* pathway regulating *SlTCP29*. The relationship between them still needs to be further verified by genetic pathways.

In conclusion, our findings indicate that CLAUSA-targeted-SlTCP29 and SlTCP24 may regulate tomato leaf development by interacting with SlAS2 and activating the expression of *SlCKX2* (Fig. S8). These results provide insights into the regulatory mechanisms of leaf development in compound leaves. Because leaves are an essential organ of plant photosynthesis, an in-depth understanding of the mechanism of tomato leaf development will contribute to improving tomato varieties.

Accession numbers

Sequence data from this article can be found in the Sol Genomics Network (SGN) database under the following accession nos: SlTCP24, Solyc08g048390; SlTCP29, Solyc08g048370; SlAS2, Solyc03g063140; SlAS2-like, Solyc11g008830; SlAS1, Solyc09g010840; SlCKX2, Solyc01g088160; SlCKX5, Solyc04g016430; SlIPT3, Solyc01g080150; TKN2, Solyc02g081120; SlCLAUSA, Solyc04g008480; SlPTS, Solyc06g072480; SlTCP2, Solyc07g062680; SlTF, Solyc05g007870; SlBIP, Solyc02g089940; SlGOB, Solyc07g062840; SlLYR, Solyc05g009380; LOC101265706, Solyc12g009930; LOC101259234, Solyc04g008310; LOC101120002, Solyc04g008330; LOC101249176, and Solyc05g053120.

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Competing interests

None declared.

Author contributions

TW and GH conceived and designed the research. GH, DZ, DL, WS and RZ performed experiments and fieldwork. GH analyzed data and wrote the manuscript. ZY, JZ, CY, ZH, SM and TW advised the research and revised the manuscript. All the authors have confirmed the final version of the manuscript.

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Data availability

The data supporting the results of this study are available in a public repository NCBI SRA (Sequence Read Archive), and can be accessed via the following link: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA997479.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Differentially expressed genes between wild-type (WT) and *SlTCP24/29-KO* line as identified by transcriptome analysis.

Fig. S1 Phylogenetic trees for TCPs and LBDs in tomato and Arabidopsis.

Fig. S2 Expression levels of *SlTCP24* and *SlTCP29* in the transgenic lines.

Fig. S3 Interactions of SlAS1, SlAS2, SlAS2-like, SlTCP29, and SlTCP24 in yeast two-hybrid system.

Fig. S4 Expression levels of *SlAS2* and *SlAS2L* in the transgenic lines.

Fig. S5 Analysis of the binding of SlTCP24/29 to the TCP-binding site.

Fig. S6 Expression levels of genes related to compound leaf development in *SlTCP24/29-KO* line.

Fig. S7 Activation of *SlTCP29* expression by SlCLAUSA.

Fig. S8 Model of proposed SlTCP24 and SlTCP29 regulation of compound leaf development in tomato.

Table S1 Primers used in experiments of this work.

Table S2 Mutation sites of gene-knockout lines.

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