Phytoplasma effector Zaofeng6 induces shoot proliferation by decreasing the expression of ZjTCP7 in Ziziphus jujuba

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Abstract

The jujube witches' broom (JWB) phytoplasma is associated with witches' broom, dwarfism, and smaller leaves in jujube, resulting in yield losses. In this study, eight putative JWB effector proteins were identified from potential mobile units of the JWB genome. Among them, Zaofeng6 induced witches' broom symptoms in Arabidopsis and jujube. Zaofeng6-overexpressing Arabidopsis and unrooted jujube transformants displayed witches' broom-like shoot proliferation. Transient expression of Zaofeng6 induced hypersensitive response-like cell death and expression of hypersensitive response marker genes, like harpin-induced gene 1 (H1N1), and the pathogenesis-related genes PR1, PR2, and PR3 in transformed Nicotiana benthamiana leaves, suggesting that Zaofeng6 could be a virulence effector. Yeast two-hybrid library screening and bimolecular fluorescence complementation confirmed that Zaofeng6 interacts with ZjTCP7 through its first two α-helix domains in the cell nuclei. ZjTCP7 mRNA and protein abundance decreased in Zaofeng6 transgenic jujube seedlings. The expression of some genes in the strigolactone signaling pathway (ZjCCD7, ZjCCD8, and CYP711A1) were down-regulated in jujube shoots overexpressing Zaofeng6 and in zjtcp7 CRISPR/Cas9 mutants. Zaofeng6 induces shoot proliferation through decreased expression of ZjTCP7 at the transcriptional and translational levels.

Introduction

Phytoplasmas are wall-less, bacterial pathogens that belong to the class Mollicutes. Transmitted by phloem-feeding insect vectors, usually leafhoppers (class Cicadellidae), they infect more than 1000 plant species, causing serious economic loss worldwide [1, 2]. Phytoplasma genomes are 600–1600 kb in size, representing the smallest known genomes. Since phytoplasma lack the genes for some major metabolic pathways, they inhabit the nutrient-rich phloem sieve elements in plant hosts to absorb essential compounds nutrients [3]. A large portion of the phytoplasma genome consists of repeats that resemble composite transposable elements, named potential mobile units (PMUs), which are believed to be involved in phytoplasma host adaptation [4].

Phytoplasmas encode a functional Sec-dependent pathway and secrete effectors into host cells, modulating host defense and morphogenesis [5]. Effector proteins carry cleavable signal peptides and lack transmembrane regions. After the cleavage of their signal peptide, mature effector proteins are secreted into host cells, where they interact with target proteins in the plant cell cytoplasm or nuclei [6]. The majority of phytoplasma effectors studied so far are encoded on PMUs [7]. There are also some effectors, such as TENGU, encoded outside the PMU regions [15].

Three phytoplasma effectors and their homologs have been the focus of several recent studies. The aster yellows witches' broom phytoplasma genome encodes 56 secreted proteins (SAPs) that are putative effectors [8]. SAP11, identified from the apple proliferation phytoplasma ('Candidatus Phytoplasma mali'), targets and/or destabilizes at least three CIN-TCP proteins and affects jasmonic acid, salicylic acid, and abscisic acid (ABA) biosynthesis [10]. Another SAP11 homolog, SWP1 from wheat blue dwarf phytoplasma, induces shoot proliferation by destabilizing the TCP TF BRC1 [11].
The effector SAP54 degrades MADS-box transcription factors in transgenic Arabidopsis lines, producing leaf-like flowers [12]. Homologs of SAP54 were identified in at least 20 diverse phytoplasma species [13, 14]. Another phytoplasma effector is TENGU, from onion yellows phytoplasma. Arabidopsis thaliana lines stably expressing TENGU display witches’ broom, dwarfism, and flower sterility [15, 16].

Chinese jujube (Ziziphus jujuba Mill.), or red date, is one of the oldest cultivated fruit trees in the world and is the most economically valuable species in the family Rhamnaceae [17, 18] with 2 million hm² cultivated in China [19]. The most destructive disease in its production is jujube witches’ broom (JWB), also called zaofeng disease, (“zao” is jujube and “feng” is uncontrolled in Chinese) [20, 21]. JWB phytoplasma infection modifies the developmental processes of jujube trees, which then show a wide range of symptoms, such as flower alterations (virescence, phyllody), witches’ broom, stunting, general decline and proliferation (Fig. 1), resulting in total losses in yield and fruit quality [22]. JWB is associated with the presence of ‘Candidatus Phytoplasma ziziphi’ (16SrV-B subgroup) [23].

The pathogenic mechanism of JWB has been studied at the phenotypic, physiological, biochemical, and molecular levels. The nutritional and mineral contents (such as N, P, K), plant hormone contents (such as zeatin, IAA, GA3, ABA) [24], defense-related enzyme activities (such as peroxidase, superoxide dismutase, polyphenol oxidase) [25], and photosynthetic activity [26] of diseased jujube trees have been compared with healthy trees. Gene expression patterns were compared between healthy and diseased trees by transcriptomic and proteomic analyses [27–29]. The expression patterns of TCP [30], lipoxygenase (LOX) [31], SQUAMOSA promoter-binding protein-like [32], basic leucine zipper [33], and the mitogen-activated protein kinase kinase kinase [34] gene family members were analyzed. Despite this, the pathogenic effectors of jujube witches’ broom remain unknown, as for most phytoplasmas. In this study, putative JWB phytoplasma effectors were mined from PMUs of the JWB phytoplasma genome. All of them were transformed into Arabidopsis to screen for induced disease symptoms. Roles of the effector Zaofeng6 in inducing shoot proliferation were analyzed.

### Results

#### Identification of putative jujube witches’ broom phytoplasma virulence effectors

Twenty-seven hypothetical proteins containing a signal peptide and lacking a transmembrane domain were predicted as secreted JWB proteins. Five JWB phytoplasma PMUs were identified in this study, JWB PMU1 (154393–171 168), JWB PMU2 (267636–288 697), JWB PMU3 (395202–418 693), JWB PMU4 (633467–651 376), and JWB PMU5 (132010–154 386) (Fig. S1). Eight of these secreted JWB proteins were encoded in separate PMUs and identified as putative effectors (Zaofeng1 to Zaofeng8), numbered based on their location in the JWB phytoplasma genome (Table 1, Fig. S1). Eight of these secreted JWB proteins were encoded in separate PMUs and identified as putative effectors (Zaofeng1 to Zaofeng8), numbered based on their location in the JWB phytoplasma genome (Table 1, Fig. S1). The physicochemical properties signal peptide scores. The size of the putative effector proteins ranged from 111 (Zaofeng6) to 136 (Zaofeng5) amino acids. Their isoelectric points ranged from 5.64 (Zaofeng5) to 9.70 (Zaofeng1), and their molecular weights ranged from 13009.14 Da (Zaofeng4) to 16110.60 Da (Zaofeng5). Phylogenetic analysis showed that Zaofeng6 belonged to the SAP11-like protein cluster.
Table 1. Predicted biochemical properties of the putative effectors in JWB phytoplasma.

| Putative JWB protein | Protein ID* | Length (amino acids) | Molecular weight (Da) | Isoelectric point | Signal peptide score | Location Information on PMUs | Protein Annotation |
|----------------------|-------------|----------------------|-----------------------|-------------------|----------------------|-----------------------------|--------------------|
| Zaofeng1             | AYJ01076.1  | 121                  | 14611.23              | 9.70              | 0.554                | JWB PMU5 (147712–148077)   | hypothetical protein |
| Zaofeng2             | AYJ01077.1  | 128                  | 15232.83              | 9.19              | 0.458                | JWB PMU5 (148263–148649)   | hypothetical protein |
| Zaofeng3             | AYJ01078.1  | 122                  | 14249.43              | 7.89              | 0.573                | JWB PMU5 (148824–149192)   | hypothetical protein |
| Zaofeng4             | AYJ01094.1  | 112                  | 13009.14              | 9.66              | 0.533                | JWB PMU1 (161619–161957)   | hypothetical protein |
| Zaofeng5             | AYJ01296.1  | 136                  | 16110.60              | 5.64              | 0.500                | JWB PMU3 (405218–405628)   | hypothetical protein |
| Zaofeng6             | AYJ01297.1  | 111                  | 13153.31              | 9.35              | 0.462                | JWB PMU3 (406706–407041)   | hypothetical protein |
| Zaofeng7             | AYJ01300.1  | 114                  | 14011.14              | 9.10              | 0.387                | JWB PMU3 (408484–408828)   | hypothetical protein |
| Zaofeng8             | AYJ01459.1  | 114                  | 13519.72              | 9.66              | 0.554                | JWB PMU4 (640778–641122)   | hypothetical protein |

* Protein ID in JWB phytoplasma genome (Gene bank accession code CP025121) [35]

and shared 48% identity with SAP11_AYWB. Zaofeng3 was highly homologous to the phytoplasma effector SAP54 (87% identity with SAP54_ANWB). The other putative effectors had low identity (less than 25%) with reported phytoplasma effectors, and therefore might be novel effectors (Fig. S2).

**Overexpression of Zaofeng6 in Arabidopsis and jujube plants resulted in shoot proliferation phenotype**

The gene fragments of the eight putative effectors without signal peptide were cloned and expressed in Arabidopsis to screen their ability to induce phytoplasma-like symptoms. Most of the Zaofeng6-overexpressing (OE) Arabidopsis (T<sub>0</sub>) had significantly greater numbers of leaves and bolts, while the leaves and flowers were smaller than those of the wild-type (WT) and empty vector (EV) plants 50 days after root emergence (Fig. 2a and b). This phenotype is similar to the proliferation observed in Arabidopsis infected with JWB phytoplasma by a leafhopper (Hishimonus selitatus) insect vector [22, 36–38] (Fig. S3). Zaofeng3-OE and Zaofeng8-OE Arabidopsis also showed phytoplasma-like symptoms. Overexpression of the other four putative effectors did not produce symptoms in the transgenic Arabidopsis (data not shown). In the study, Zaofeng6 was selected for further analysis of its effect on jujube growth. Zaofeng6-OE jujube shoots produced more shoots and smaller leaves than WT and EV (Fig. 2c), which is consistent with the phenotype of JWB-infected jujube (Fig. 1f).

**Zaofeng6 triggered hypersensitive response and induced the expression of defense-related genes in tobacco leaves**

Transient overexpression assays showed that Zaofeng6 induced hypersensitive response-like cell death in N. benthamiana (Fig. 3a). Zaofeng6 could not suppress cell death caused by mouse Bcl-2 associated X (BAX) protein (Fig. 3c, right). Cell death was further confirmed by trypan blue staining (Fig. 3b and d).

To further analyze the effect of Zaofeng6, defense response marker genes were examined by qRT-PCR (Fig. 3e–i). The expression of HIN1, PR1, PR2, and PR3 were significantly up-regulated in tobacco leaves by transiently expressing Zaofeng6 compared with WT and transient expression of the EV. In particular, the expression level of HIN1, PR1, and PR2 increased 20- to 50-fold.

**Zaofeng6 interacted with the Z. jujuba TCP transcription factor ZjTCP7**

A yeast two-hybrid (Y2H) library was constructed with jujube cDNA to screen proteins that interact with Zaofeng6 in jujube. Eleven jujube proteins were found to interact with Zaofeng6 (Table S5). An interaction network between Zaofeng6 and these jujube proteins was constructed using Cytoscape. The interaction network predicted that Zaofeng6 might interfere with multiple processes, including plant defense, growth, development, and morphogenesis (Fig. S5). Among the 11 proteins that interact with Zaofeng6, LOC107418855 was annotated as the TCP transcription factor ZjTCP7, a homolog of Arabidopsis BRC1 [30], a negative regulator of branching in the strigolactone signaling pathway (Fig. S5).

The eight members of class II in the TCP family in Z. jujuba were screened for interaction with Zaofeng6 by Y2H assays. A clear interaction between Zaofeng6 and ZjTCP7 was found, while there was a weaker interaction between Zaofeng6 and ZjTCP16 (Fig. 4b). The interaction between Zaofeng6 and ZjTCP7 was further confirmed by bimolecular fluorescence complementation (BiFC) assays in N. benthamiana. Strong fluorescence signals were observed in the cell nucleus when ZjTCP7 was co-expressed with Zaofeng6 (Fig. 4d).

In addition to the N-terminal signal peptide, Zaofeng6 is predicted to contain three α-helix domains (Fig. 4a). A series of derivatives of Zaofeng6 (Zaofeng6Δα1, Zaofeng6Δα1Δα2, Zaofeng6Δα3, Zaofeng6Δα2Δα3, and Zaofeng6Δα1Δα3) were constructed, fused to the BD, and co-expressed with ZjTCP7 in yeast cells for Y2H assays. Full-length Zaofeng6 and Zaofeng6Δα3 interacted with ZjTCP7 (Fig. 4c). This interaction was further confirmed by BiFC assays (Fig. 4d). No fluorescence signals were detected with other Zaofeng6 derivatives, indicating that the first two α-helix domains of Zaofeng6 are required for its interaction with ZjTCP7.
The expression of ZjTCP7 was significantly decreased in unrooted jujube shoots overexpressing Zaofeng6

The effect of Zaofeng6 on the mRNA level of ZjTCP7 was analyzed. The expression of ZjTCP7 was significantly decreased in the Z. jujuba shoots overexpressing Zaofeng6 compared with those carrying EV (Fig. 5a). Likewise, the protein abundance of ZjTCP7 was remarkably lower in Zaofeng6 transformants compared to WT and EV transformants (Fig. 5b). Taken together, Zaofeng6 down-regulated the expression of ZjTCP7 at both the transcriptional and translational levels.
Figure 4. Zaofeng6 interacted with ZjTCP7 through its first two α-helix domains.

CRISPR-induced zjtcp7 jujube mutants exhibit shoot proliferation phenotype

CRISPR-Cas9 was used to generate zjtcp7 mutant jujube shoots. Sequencing and alignment showed that the target site in ZjTCP7 was successfully deleted by the CRISPR/Cas9 system in the primary transformants (Fig. S6). The zjtcp7 mutant jujube shoot had shorter internodes and a larger number of shoots. The proliferation phenotype from knockout of ZjTCP7 was similar to that caused by the overexpression of Zaofeng6 in jujube shoots (Fig. 5c, Fig. 2c).

Genes involved in strigolactone signal pathway were down-regulated in the Zaofeng6 transformants and zjtcp7 mutants

The interaction between ZjTCP7 and promoter regions of the strigolactone (SL) signal pathway genes ZjCCD7, ZjCCD8, ZjCYP711A1, and ZjMAX2 was screened by yeast one-hybrid (Y1H). The promoter regions of ZjCCD7, ZjCCD8, and ZjCYP711A1 interacted with ZjTCP7, while ZjMAX2 did not (Fig. 5d). The expressions of these three genes (ZjCCD7, ZjCCD8, and ZjCYP711A1) were significantly decreased in both the Zaofeng6-OE transformants and the zjtcp7 mutants (Fig. 5e). In Zaofeng6-OE and zjtcp7 jujube shoots, the expression level of ZjCCD8 was 10-fold lower than that in WT. These data suggested that Zaofeng6 might regulate the expression of these genes through ZjTCP7.

Hypothetical model of Zaofeng6 induction of proliferation in Z. jujuba

A hypothetical model of how Zaofeng6 induces shoots proliferation symptoms in jujube is suggested (Fig. 6). In diseased jujube plants, JWB phytoplasma secretes a variety of effector proteins, including Zaofeng6, into host cells. Zaofeng6 interacts with ZjTCP7, significantly decreasing its expression at both the transcriptional and translational levels. Down-regulation of ZjTCP7 causes jujube shoot proliferation. Another possibility is that the lower levels of ZjTCP7 protein down-regulates some
Figure 5. Overexpression of Zaofeng6 decreased the expression of ZjTCP7 and several genes in strigolactone (SL) signaling pathway.

genes like ZjCCD7, ZjCCD8, and ZjCYP711A1 in the SL signaling pathway. Alteration of the SL pathway may induce shoot proliferation.

Discussion

The presence of PMUs is a unique characteristic of the phytoplasma genomes that have been thus far characterized. The PMUs in phytoplasma typically carry effector and other genes that may have contributed to the adaptation of phytoplasma to their pathogenic lifestyle [39]. Four of the PMUS in JWB phytoplasma (JWB PMU1, JWB PMU2, JWB PMU3, and JWB PMU4) were identified in 2018 by Wang [35]. Here we found one more PMU (JWB PMU5), which carried characteristic genes such as tra5, hflB, smc, trnA, and dna8, but lacked genes such as sigF, ssb, and himA compared with other PMUs. More importantly, JWB PMU5 carries three candidate effector genes (Zaofeng1, Zaofeng2, and Zaofeng3).

Plants possess effector-triggered immunity (ETI), which often leads to localized programmed cell death (PCD) or to necrosis, contributing to the hypersensitive response (HR). The HR occurs at the site of pathogen invasion to restrict further spread of pathogens [40]. In response, effectors can attenuate the necrosis caused by PCD [41]. Zaofeng6 could induce HR-like programmed cell death, which suggested that Zaofeng6 might trigger ETI. Transformation with Zaofeng6 of jujube and
Arabidopsis induced a witches’ broom phenotype, which implied that Zaofeng6 could be considered a virulence effector that alters plant shoot branching.

ZjTCP7 is homologous to Arabidopsis BRC1, a class II TCP, which regulates the SL signaling pathway through interaction with ZjCCD7, ZjCCD8, ZjMAX2, and ZjCYP711A1. In Arabidopsis, BRC1 and its paralog BRC2 are considered to be key integrators that negatively control shoot branching within axillary buds [42, 43]. CRISPR/Cas9-mediated knockout of Populus BRC1 and BRC2 revealed that both genes function in controls of bud outgrowth and leaf development [44]. A previous study identified 21 jujube TCP family members, 10 of which, including the BRC1 homolog ZjTCP7, were differentially expressed during JWB phytoplasma infection [30]. In Zaofeng6-OE jujube shoots, levels of both the ZjTCP7 mRNA and ZjTCP7 protein were decreased. CRISPR/Cas9-mediated mutation of zjtcp7 showed the SL signaling pathway gene expression patterns and a proliferation phenotype similar to the Zaofeng6-OE transformants.

Materials and methods
Plant material and phytoplasma strain
In vitro-grown Z. jujuba ‘Huizao’ seedlings were maintained and used for transformation as previously reported [45]. A. thaliana and N. benthamiana were cultured in a growth chamber under a 16 h light/8 h dark photoperiod at 23°C. Phytoplasma-infected Z. jujuba ‘Huizao’ shoots were collected in jujube orchards in Xinzheng, Henan province and maintained through in vitro micropropagation as a phytoplasma source [29]. The presence of phytoplasma in both A. thaliana and Z. jujuba were verified by PCR with primers R16F2n/R16R2. Amplified products were recovered and sequenced at Songon (Shanghai, China). The sequence showed 100% similarity with JWB phytoplasma 16S rRNA gene sequence.

Identification of jujube witches’ broom phytoplasma putative effectors
Putative effectors were mined from the fully sequenced JWB phytoplasma genome [35] (Genbank accession code CP025121) according to Bai et al. [8]. Genes annotated in the JWB phytoplasma genome as hypothetical protein were selected for prediction of signal peptide and transmembrane domains [35]. Signal peptides were predicted in all proteins by SignalP 4.1 [46]. Trans-membrane domains were predicted by the TMHMM2.0 program [47]. The secreted JWB proteins that were encoded in PMUs were identified as JWB phytoplasma putative effectors. PMUs were identified by the presence of flanking tra5 insertion sequences and DNA replication genes (dnaG, dnaB, ssb, tmk) [48]. Finally, these eight putative effectors were named based on their order on the JWB phytoplasma genome [48].

Generation of transgenic Arabidopsis and Z. jujuba lines
To generate transgenic plants overexpressing the identified effectors, eight putative effector genes without signal peptides were cloned into the pSAK277 overexpression vector using gene-specific primers (Table S1). Agrobacterium tumefaciens carrying each of the eight effectors and the empty pSAK277 vector were transformed into Arabidopsis by floral dip [49]. To generate Zaofeng6-overexpression jujube, in vitro-grown Z. jujuba ‘Huizao’ were used as explants for the leaf disc transformation method [45]. Insertion of the effector sequences were detected in both transgenic Arabidopsis seedlings
and unrooted jujube shoots using specific primers of pSAK277 vector (Table S1). The specific primers of pSAK277 were designed according to the sequences of pSAK277 vector with Primer Premier 5.0.

**Generation of zjtcp7 mutant jujube shoots**

The coding sequence of ZjTCP7 (LOC107418855) was cloned into the Cas9 vector PXEE401BG-mche+PNGGRT3X. The primers (Table S1) containing the target sites were designed as reported [50]. Upstream and downstream primers of the target sites were annealed into pairs in an 85°C oven. Then the annealed product was connected with the enzyme-digested Cas9 vector by SE Seamless Cloning and Assembly Kit (ZOMANBIO, Beijing, China). To generate zjtcp7 jujube mutants, in vitro jujube shoots were used as explants for leaf disc transformation [45].

**Phenotypic analysis**

The phenotypes of T0 transgenic Arabidopsis carrying Zaofeng6 were compared with WT and Arabidopsis infected with JWB phytoplasma by leafhopper (Hishi-monus sellatus) [22, 36–38]. Fifteen Arabidopsis seedlings were selected to record the growth indexes of rosette leaf size and number at 50 days after root emergence and the number of leaves on each bolt. The presence of phytoplasma in leafhopper and Arabidopsis thaliana were verified by PCR with phytoplasma 16SrRNA amplification primers (R16F2n/R16R2) [56, 57]. The phenotypes of transgenic jujube shoots carrying either the EV or Zaofeng6 (Fig. S4) were photographed 50 days after Agrobacterium infection.

**Hypersensitive response assays**

Zaofeng6 was transiently overexpressed in N. benthamiana leaves by A. tumefaciens infiltration with a syringe [41]. The pSAK277 EV was transiently overexpressed as negative control. Transient co-expression assays and trypan blue staining were carried out according to Wang [41]. As a positive control, the gene encoding BAX [51] protein was cloned using primers BAXF/BAXR (Table S4). Leaves were harvested 7 days after infiltration. Three leaves from each treatment were inoculated as one biological replicate. Cleared leaves were photographed under a single-lens reflex camera.

**Gene expression pattern analysis**

In N. benthamiana, qRT-PCR was used to determine the transcript levels of HR-related genes (Table S4) in leaves infiltrated with water (WT) or transiently transformed with EV or Zaofeng6. In Zaofeng6-OE jujube shoots and zjtcp7 mutants, qRT-PCR was used to examine the transcript levels of ZjTCP7 and SL signal pathway genes such as ZjCCD7, ZjCCD8, ZjMAX2, and ZjCYP711A1 (Table S4). Wild-type and overexpression Zaofeng6 jujube shoots were set as negative controls. Then qRT-PCR was performed according to Ye [27].

**Yeast two-hybrid library screening**

To screen for jujube proteins directly interacting with Zaofeng6, three types of jujube leaves, symptomatic (infected and showing obvious JWB pathogenic symptoms), asymptomatic (infected but showing normal phenotype), and healthy (PCR negative and showing no symptoms) jujube trees were sampled for Y2H library construction. Library screening was performed as reported [52]. Fragments from positive yeast were sent to Sangon Biotech (Shanghai, China, https://www.sangon.com/) for sequencing. The sequences were searched by BLAST against the genome of Z. jujuba (Gene bank accession code JREP00000000) [53]. An in silico-predicted interaction network was constructed with Cytoscape software [30] according to the interaction information from the STRING database [54].

**Yeast two-hybrid (Y2H) assays**

Y2H assays were carried out as reported [11] to determine the domain required for Zaofeng6 to interact with ZjTCP7. Zaofeng6 and a series of Zaofeng6 derivatives, namely Zaofeng6Δα1, Zaofeng6Δα3, Zaofeng6Δα1Δα2, Zaofeng6Δα2Δα3, and Zaofeng6Δα1Δα3, were amplified and cloned into pGBK7T (ZOMANBIO, Beijing, China). The coding sequence of the ZjTCP7 gene [30] was ligated into the pGAD7T (ZOMANBIO) vector. The primers used for Y2H assays are shown in Table S2.

**Bimolecular fluorescence complementation analysis**

To confirm the results of Y2H, BiFC assays were carried out according to Wang [11]. The series of Zaofeng6 derivatives mentioned earlier and ZjTCP7 were cloned into pBin-NYFP and pBin-CYFP, respectively. Primers used for BiFC assays are shown in Table S3. All were transformed into A. tumefaciens, which was then pressure-infiltrated by syringe into three N. benthamiana leaves. Leaves were harvested 48 h after agro-infiltration for confocal laser observation.

**Yeast one-hybrid assay**

Y1H assay was carried out as described in a previous report [55]. Sequences 300–1500 bps upstream of the initiation codon of each selected gene were amplified from jujube genomic DNA with designed primers (Table S2) and fused to the vector pLacZi. The coding regions of ZjTCP7 were amplified and fused to the vector p842AD. Three days after transformation, the transformants were screened on SD−Trp-Ura (SD-TU) with 20 mg/L X-Gal for 3–4 days.

**Western blotting**

Western blotting assays were carried out to explore the effect of Zaofeng6 on ZjTCP7 protein abundance with ZjTCP7-specific antibody. Whole jujube transformants overexpressing Zaofeng6 or EV and zjtcp7 mutant jujube lines were sampled 50 days after Agrobacterium transfection. Three plants from each treatment were sampled.
Total protein extraction, separation, membrane transfer, and protein bands detected were carried out according to Wang [41].

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Author contributions
P.C. and L.C.C. performed the experiments and co-wrote the manuscript, contributing equally to this work. J.D.L. and J.C.F. conceived this project and supervised the experiments. X.Y., B.T., X.B.Z., J.C., and W.W. analyzed the data. Q.Q.Y. and Y.Z. contributed jujube materials and reviewed the manuscript. All authors read and approved the final manuscript.

Data availability
All data needed to evaluate the conclusions in this paper are present in the paper and/or the Supplementary Materials.

Conflict of interest statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary data
Supplementary data is available at Horticulture Research Journal online.

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