Molecular characterization, expression analysis and heterologous expression of two translationally controlled tumor protein genes from *Cucumis sativus*

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Abstract

The translationally controlled tumor protein (TCTP) is a family of abundant and ubiquitous proteins involved in several important primary functions. Cucumbers harbor two TCTP genes, *CsTCTP1* and *CsTCTP2*, however, their functional roles remain unclear. In this study, we isolated *CsTCTP1* and *CsTCTP2* (XP-004134215 and XP-004135602, respectively) promoters, full-length cDNA and genomic sequences from *Cucumis sativus*. Bioinformatics analysis, containing cis-acting elements, structural domains, phylogenetic tree and conserved motifs, suggested the conservation and divergence of *CsTCTP1* and *CsTCTP2*, thus providing knowledge regarding their functions. Expression analysis indicated that *CsTCTP1* and *CsTCTP2* exhibited tissue-specific expression and were regulated by biotic or abiotic stresses in *C. sativus*. Furthermore, *CsTCTP1* and *CsTCTP2* were regulated by ABA and may be associated with the TOR (target of rapamycin) signaling pathway. In a prokaryotic expression analysis, *CsTCTP1* and *CsTCTP2* showed positive responses to salt and heat stresses and a negative response to drought and HgCl₂ stresses. TCTP may exert multiple functions in various cellular processes.

Introduction

The translationally controlled tumor protein (TCTP) is a highly conserved protein in eukaryotic phyla. It was initially discovered in Ehrlich ascites tumor cells and has subsequently been studied in animals [1, 2]. Extant data suggest that TCTP exerts multiple functions in various cellular processes such as cell growth, differentiation, organ size, apoptosis, signaling pathway, stimulus and immune responses [3–6]. TCTP functions as a GEF (guanine nucleotide exchange factor) of Ras GTPase Rheb in *Drosophila melanogaster* and is related to the TOR
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(target of rapamycin) signaling pathway [7]. The high conservation and ubiquitous presence of this protein underscores its similar mechanisms among plant and nonplant homologs. The notion that its role is similar was first supported by the fact that the *Arabidopsis TCTP* gene can rescue the corresponding *Drosophila TCTP* mutant [8, 9].

The first report of plant *TCTP* was obtained from *Medicago sativa* [10]. Several *TCTP* genes have been identified from different plant species by comparative transcriptomic and proteomic studies [11, 12]. Furthermore, *AtTCTP* overexpression enhanced drought tolerance in *Arabidopsis* and played a role in ABA-mediated stomatal movement [13]. RNAi of *Rpf41* (a homolog of *TCTP*) in *Robinia pseudoacacia* decreased the root and stem length, fresh weight and nodule number per plant [14]. To date, work on *TCTP* in plants has focused mostly on *Arabidopsis*; however, few experiments have been conducted. The Arabidopsis genome consists of two *TCTP* sequences, *AtTCTP*1 (NP-188286) and *AtTCTP*2 (NP-187205). The molecular function of the first sequence is better studied. The latter sequence was once thought to be a pseudo-gene; however, *AtTCTP*2 appears to play crucial roles in plant regeneration according to Toscano-Morales [15]. These published data suggest a role of *AtTCTP*1 in the regulation of growth, the control of programmed cell death and the response to stress signals [9, 16]. Silencing of the *AtTCTP*1 gene in *Arabidopsis* caused slow vegetative growth, leaf expansion and lateral root formation [17]. *AtTCTP*2 is essential for viability and enhances plant regeneration. Indeed, silencing of the *AtTCTP*2 gene in *Arabidopsis* displays a lethal phenotype [18]. The molecular function and physiological mechanism of *TCTP* in plants are still not fully understood; elucidating them is of immense importance.

Through a 2-D gel analysis, *TCTP* (XP-004134215) was found to be involved in the response of cucumber to *Sphaerotheca fuliginea* at the protein level [12]. However, the functional characterization of *CsTCTPs* remains unclear. Cucumber harbors 2 *TCTP* genes. Here, we report a detailed expression analysis and molecular characterization of *CsTCTP*1 and *CsTCTP*2 (XP-004134215 and XP-004135602, respectively). The expression patterns of *CsTCTP*1 and *GcTCTP*2 were determined in different tissues and under various treatments. In addition, *E. coli* BL21 strains overexpressing *GsTCTP*1 and *CsTCTP*2 exhibited varying degrees of tolerance to heat, salt, drought and mercury stress. The study provides new insight into *TCTP* in cucumber and will be helpful in further improving cucumber performance under stresses.

**Materials and methods**

**Plant materials and treatments**

Two cucumber sister lines, one that is highly resistant (B21-a-2-1-2) and one that is highly susceptible (B21-a-2-2-2) to *S. fuliginea*, were obtained from Liaoning Academy of Agricultural Sciences. These lines were selected from a segregated population derived from fourth generation selfing of a cultivar from South Korea. The two lines are similar in terms of plant type, commodity characteristics, resistance to Fusarium wilt and downy mildew but differ in their traits of resistance to powdery mildew.

Cucumber seeds of both lines were grown in soil with a 16 h photoperiod at 25˚C/18˚C (day/night). For biotic stress, at 3rd-4th leaf stage, the second leaf blades of B21-a-2-1-2 and B21-a-2-2-2 were inoculated with *S. fuliginea* by gently rubbing mildewed leaves. For abiotic stress and stimuli treatments, 3rd-4th leaf stage seedlings of B21-a-2-1-2 were treated with 45˚C (for heat stress) or 4˚C (for cold stress), irrigated with water containing 20% PEG (m/v, for drought stress), or sprayed with water containing 100 mM NaCl (for salinity stress), 10 mM CaCl$_2$, 10 μM H$_2$O$_2$, 100 μM ABA, 100 μM MeJA, 1 mM SA or 1% (v/v) Ethrel. During the treatments, six-time points (0, 12, 24, 48, 72, and 144 h) under biotic stress, three-time points...
(12, 24, and 48 h) under stimuli treatments, and five-time points (0, 3, 6, 12, and 24 h) under abiotic stresses. Then, the second leaves collected at each time point were placed in liquid nitrogen and stored at -80˚C until further experiments.

**Extraction of genomic DNA, total RNA and cDNA synthesis**

Genomic DNA was isolated from the two cucumber sister lines B21-a-2-1-2 and B21-a-2-2-2 seedling leaves with a Plant Genomic DNA kit (TianGen Biotech, China) according to the manufacturer’s instructions. Total RNA from various samples was isolated using RNeasy Pure Plant Kit, and cDNA was generated using a FastQuant cDNA first strand synthesis kit (TianGen Biotech, China) according to the manufacturer’s instructions. The DNA and RNA were detected on 1.2% agarose gels, and the purity of the DNA and RNA was determined by spectrophotometry (Biodrop).

**Cloning of CsTCTP1 and CsTCTP2 promoter, DNA and cDNA sequences**

The nucleotide sequence of *Cucumis sativus* L. TCTPs (CsTCTP1, accession: XP-004134215 and CsTCTP2, accession: XP-004135602) was retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Amplifications of CsTCTP1 and CsTCTP2 cDNAs were performed with primers 1- cDNA F/1- cDNA R and 2- cDNA F/2- cDNA R on first-strand cDNA templates of B21-a-2-1-2 and B21-a-2-2-2 in a Mastercycler (BIO-RAD) under the following conditions: 94˚C for 5 min, 94˚C for 30 s, 58˚C for 30 s, 72˚C for 1 min, 32 cycles, and 72˚C for 10 min. The PCR products were gel-purified, cloned into pMD18-T vector (TaKaRa, China) and sequenced (Sangon Biotech, China).

Genomic PCRs were performed with primers 1- DNA F/1- DNA R and 2- DNA F/2- DNA R on DNA templates of B21-a-2-1-2 and B21-a-2-2-2 as follows: 94˚C for 5 min, 94˚C for 30 s, 62˚C for 30 s, 72˚C for 2 min, 36 cycles, and 72˚C for 10 min. For promoter analysis, the promoter regions of CsTCTP1 and CsTCTP2 (approximately -2.0 kb upstream of translation initiation site) were also isolated from B21-a-2-1-2 and B21-a-2-2-2 genomic DNA based on 1- P F/1- P R primers and 2- P F/2- P R primers. PCR conditions used were as follows: 94˚C for 5 min, 94˚C for 30 s, 60˚C for 30 s, 72˚C for 2 min, 38 cycles, and 72˚C for 10 min. The PCR products were gel purified, cloned and sequenced.

**qRT-PCR analysis**

Quantitative real-time PCR was conducted using the SYBR Green I 96-1 system (Roche fluorescence quantitative PCR instrument, Basle). Reaction mixtures consisted of 4.5 μL of 2xSuperReal PreMix Plus (TianGen Biotech, China), a mixture of primers (0.2 μL of forward and reverse primer for proper gene), 4.3 μL of RNase-Free ddH2O and 1 μL of cDNA. The PCR program was set up in seven stages: (1) 95˚C for 15 min (pre-incubation), (2) 95˚C for 10 s, (3) 58˚C for 20 s, (4) 72˚C for 30 s, (3) repeated 40 times (amplification), (5) 95˚C for 0.5 s, (6) 60˚C for 1 min and (melt) (7) 50˚C for 30 s (cooling). The primers were synthesized by BGI Tech (China), and the PCR reaction quality was estimated based on melting curves. 18s rRNA was used as an internal control for determining transcript levels in cucumber. The gene-specific primers employed are shown in S1 Table. Two independent biological replicates and three technical replicates for each biological replicate were run, and the significance was determined by t-test using SPSS statistical software (P < 0.05).
**CsTCTP1 and CsTCTP2 overexpression in bacteria**

The insert fragment was digested with Kpn I + Sac I from pMD18-T vector and then ligated into the expression vector pET30a (+) digested with the same enzymes, resulting in constructed vectors (pET30a-CsTCTP1 or pET30a-CsTCTP2). The constructed vectors and empty vector (pET30a) were subsequently transformed into E. coli BL21 (DE3). The transformed cells were inoculated into LB containing 50 μg/mL kanamycin at 28˚C, 170 rpm until OD$_{600}$ reached 0.6.

IPTG (final concentration to 1 mM) induced prokaryotic expression products for 0 h, 3 h, 6 h and 12 h at 28˚C. Meanwhile, BL21 + pET30a (control) was induced in 1 mM IPTG for 0 h and 12 h. The cells from 1 mL of culture were harvested by centrifuging at 10 000 rpm for 10 min at 4˚C, dissolved in 20 μL 2 × SDS loading buffer and boiled for 5 min. All samples were analyzed by SDS-PAGE.

For assays of tolerance to various stresses, bacterial cells containing constructed vectors and the empty vector were induced in 1 mM IPTG for 12 h at 28˚C. The cells were diluted to 0.6 (OD$_{600}$) and further diluted to 10$^{-3}$ and 10$^{-4}$. Then, 10 μL of 10$^{-3}$ and 10$^{-4}$ diluted cells was spotted on LB plates containing 50 μg/mL kanamycin at 37˚C, 45˚C and 55˚C (for heat stress) or on LB plates with NaCl (250 mM, 500 mM and 750 mM, for salt stress), mannitol (0.4 M, 0.8 M and 1.2 M, for drought stress), or HgCl$_2$ (15 μM, 25 μM and 35 μM, for mercury stress) at 37˚C overnight. The cells were also added to LB containing 50 μg/mL kanamycin at 45˚C or to LB liquid medium adding 500 mM NaCl, 0.8 M mannitol or 25 μM HgCl$_2$ at 37˚C, 200 rpm. Cell growth was measured every 2 h by OD$_{600}$ [19–21]. Assays of response to stresses were repeated at least three times. Each treatment was performed at least twice.

**Statistical and bioinformatics analysis**

Primer design and sequence alignment were conducted in DNAman. Promoter analysis was performed using BDGP, PLACE (www.dna.affrc.go.jp) and Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html). The phylogenetic tree was conducted in MEGA 6.0. The sequence was analyzed with MEME software (http://meme-suite.org/tools/meme), ProtParam Tool (http://web.expasy.org/protparam/), Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/), SMART (http://smart.embl-heidelberg.de/) and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

**Results**

**Isolation and characterization of CsTCTP1 and CsTCTP2**

The cucumber genome harbors two TCTP genes. Here, we termed the gene that shows high similarity to AtTCTP1 as CsTCTP1 (accession: XP-004134215) and the other gene as CsTCTP2 (accession: XP-004135602). Using the RT-PCR method, the promoters, cDNA and DNA sequences of CsTCTP1 and CsTCTP2 were amplified, cloned and sequenced. Subsequently, we compared the sequences of the two sister cucumber lines (B21-a-2-2-2 and B21-a-2-1-2) obtained here with other cucumber TCTP sequences available in the NCBI databases and found no differences between them.

Using 1/2-P F and 1/2-P R primers on cucumber genomic DNA, we obtained the CsTCTP1 promoter sequence that contains 2096 bp upstream of the translation start site and has an AT content of 74% and the CsTCTP2 the promoter sequence that contains 155 bp upstream and has an AT content of 74%. The two promoter sequences shared 45% nucleotide identity, suggesting that CsTCTP1 and CsTCTP2 may exhibit different expression profiles. The transcriptional start sites (TSS) of CsTCTP1 and CsTCTP2 were found 87 bp and 67 bp upstream from
the translation start site, respectively. The putative TATA-box was found at position -28 with respect to the TSS. Putative cis-regulatory elements were also deciphered in the CsTCTP1 and CsTCTP2 promoter sequences using Plant CARE and PLACE databases; the results are summarized in S1 and S2 Tables. We identified ABRE, TC-rich repeats and circadian in both cucumber promoters, which are important cis-acting elements involved in abscisic acid responsiveness, defense and stress responsiveness and circadian control. Numerous cis-elements associated with light responsiveness, such as ACE, TCT-motif and G-box, were also found in both promoters. TCA-element, HSE, and MBS were identified only in the CsTCTP1 promoter sequence. TCA-element, HSE, and MBS are involved in responsiveness to salicylic acid, heat and drought responsiveness, respectively. Two structure-related cis-acting elements known as 3-AF3 binding site and Box III were found at position 618 (+) and 773 (+) only in the CsTCTP2 promoter. Searching the CsTCTP2 promoter also resulted in the discovery of several cis-elements, including ethylene, zein metabolism regulation, anaerobic induction, endosperm expression, fungal elicitor and shoot-specific responsive elements.

A genomic DNA sequence containing a 2211 bp coding region of the CsTCTP1 gene was obtained by amplification with 1-DNA F and 1-DNA R primers, and a 1679 bp coding region of the CsTCTP2 gene was obtained by amplification with 2-DNA F and 2-DNA R primers as described in the “Materials and methods” section. Our results showed that the CsTCTP1 coding region is composed of four introns (i1, i2, i3 and i4 with 578 bp, 181 bp, 84 bp and 496 bp, respectively) and five exons (e1, e2, e3, e4 and e5 with 28 bp, 74 bp, 129 bp, 158 bp and 118 bp, respectively); CsTCTP2 coding region is constituted by four introns (i1, i2, i3 and i4 with 509 bp, 113 bp, 89 bp and 112 bp, respectively) and five exons (e1, e2, e3, e4 and e5 with 28 bp, 74 bp, 129 bp, 158 bp and 118 bp, respectively), which matches the cDNA sequence. The full-length cDNA of CsTCTP1 was 872 bp in size with a predicted 507 bp open reading frame (ORF), 147 bp 5'-UTR and 218 bp 3'-UTR; the full-length cDNA of CsTCTP2 was 856 bp in size with a predicted 507 bp ORF, 63 bp 5'-UTR and 286 bp 3'-UTR.

cDNA sequence analysis showed that CsTCTP1 encodes a polypeptide of 168 amino acids with a predicted molecular weight of 18 kDa and a pI of 4.56; CsTCTP2 encodes a polypeptide of 168 amino acids with a predicted molecular weight of 19 kDa and a pI of 4.35. Sequence comparison of CsTCTP1 and CsTCTP2 showed 77% identity at the amino acid level and 73% at the nucleotide level. Phylogenetic tree analysis showed that CsTCTP1 was closely related to Cucumis melo TCTP; CsTCTP2 was closely related to Arabidopsis thaliana TCTP2. Conserved motif analysis showed that five types of motifs were common among all eukaryotic phyla analyzed here and that all TCTPs had a highly conservative motif 1 and motif 4 at N-terminal (Fig 1). Thus, the function of different TCTP genes may be similar but do not overlap completely.

Based on data analysis in SMART and InterProScan, CsTCTP1 and CsTCTP2 have several typical TCTP domains, such as a Ca\(^{2+}\) binding domain (80–110), cell cycle-controlling polo kinase (111–168), Na\(^+\)/K\(^+\) ATPase (107–168), tubulin binding domain, putative binding domain to Rab GTPase, TCTP1 (45–55) and TCTP2 (125–147) (Fig 2). Our results suggested that CsTCTP1 and CsTCTP2 are both typical TCTP proteins and that TCTP might have a specialized function in cucumber plants.

Expression analysis of CsTCTP1 and CsTCTP2 in cucumber

Expression patterns of CsTCTP1 and CsTCTP2. CsTCTP1 was found to be differentially expressed in both highly resistant and highly susceptible cucumber leaves under S. fuliginea stress in our previous study. To gain insight into CsTCTP1 and CsTCTP2 function, we detected their expression patterns in various tissues by qRT-PCR. Consistent with previous studies
Fig 1. Phylogenetic and conserved protein motifs of TCTPs. The phylogenetic tree of TCTP protein sequences included Homo sapiens TCTP (P13693), Mus musculus TCTP (P63028), Drosophila melanogaster TCTP (Q9VGS2), Pseudocapsa prolifica TCTP (JA088771), Cucumis sativus TCTP1 (XP-004134215), Cucumis sativus TCTP2 (XP-004135602), Cucurbita maxima TCTP (ABC02401), Cucumis melo TCTP1 (AAF40198), Cucumis melo TCTP2 (XP-0084560611), Arabidopsis thaliana TCTP 1 (NP-188286), Arabidopsis thaliana TCTP 2 (NP-187205), Zea mays TCTP (Q8H6A5), Triticum aestivum TCTP (Q8LM8), Oryza sativa TCTP (XP_015610660), Hevea brasiliensis TCTP (Q9ZSW9), Brassica oleracea TCTP (Q94W6), Larix kaempferi TCTP (AGW01241), Pseudotsuga meziotiai TCTP (Q92RX0), Schizosaccharomyces pombe TCTP (Q10344), and Physcomitrella patens TCTP (Q10344). The construction of the tree was conducted with Mega 6.0. Numbers above the branches indicate bootstrap values.

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Fig 2. Putative structural domains of CsTCTP1 and CsTCTP2.

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CsTCTP1 and CsTCTP2 were expressed in all examined tissues (roots, stems, leaves and cotyledons). Among these tissues, CsTCTP1 was highly expressed in the stems of both sister cucumber lines B21-a-2-1-2 and B21-a-2-2-2, while CsTCTP2 was highly expressed in the stems of B21-a-2-1-2 and in the roots of B21-a-2-2-2 (Fig 3). These results demonstrated that CsTCTP1 and CsTCTP2 are both ubiquitously expressed in all analyzed plant tissues and exhibit tissue-specific expression.

Expression of CsTCTP1 and CsTCTP2 in response to S. fuliginea. TCTP may be regulated by Erysiphe graminis (the causal agent of wheat powdery mildew) [24]. Interestingly, we also found that CsTCTP1 seems to be involved in response to S. fuliginea, the causal agent of powdery mildew in cucumber in previous work. To examine whether CsTCTP1 and CsTCTP2 are affected by S. fuliginea, the expression patterns of CsTCTP1 and CsTCTP2 in both highly resistant and highly susceptible cucumber leaves at each corresponding time point were analyzed (Fig 4). During the cucumber-S. fuliginea interactions, the CsTCTP1 transcript level increased continuously for 144 h in the susceptible variety B21-a-2-2-2. In the resistant
variety B21-a-2-1-2, CsTCTP1 transcripts reached the highest level at 24 h of S. fuliginea treatment. CsTCTP2 appeared to be highly expressed in the susceptible line than that in the resistant line after infestation. Also, CsTCTP2 transcripts showed similar expression profiles in the two lines. CsTCTP2 initially increased and then decreased, with the maximum accumulation in the resistant and susceptible lines observed at 48 h and 72 h post inoculation.

**Fig 4.** Relative expression levels of *CsTCTP1* and *CsTCTP2* in resistant (B21-a-2-1-2) and susceptible (B21-a-2-2-2) varieties inoculated with *S. fuliginea*. The expression level in B21-a-2-2-2 was normalized as 1. Data represent means ± SEs of three biological replicates. Asterisk or asterisks indicate significant differences at P < 0.05 or P < 0.01, respectively, compared with B21-a-2-2-2 by Student's t-test.

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Fig 5. Relative expression levels of CsTCTP1 and CsTCTP2 in a resistant (B21-a-2-1-2) variety under various abiotic stresses. The expression level at 0 h was normalized as 1. Data represent means ± SEs of three biological replicates. Letters indicate significant differences at P < 0.05 compared with 0 h treatment by Student’s t-test.

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Fig 6. Relative expression levels of CsTCTP1 and CsTCTP2 in a resistant (B21-a-2-1-2) variety under various treatments. The expression level of the water treatment (12 h) was normalized as 1. Data represent means ± SEs of three biological replicates. Asterisk or asterisks indicate significant differences at $P < 0.05$ compared with water treatment (12 h) by Student’s t-test.

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respectively. It is worth mentioning that the maximum expression of CsTCTP1 and CsTCTP2 appeared to be earlier in the resistant variety than in the susceptible variety when inoculated with *S. fuliginea*.

**Expression of CsTCTP1 and CsTCTP2 in response to various extracellular stimuli.**

Expression of CsTCTP1 and CsTCTP2 was also observed under abiotic stresses in the leaves of the resistant variety B21-a-2-1-2 (Fig 5). Under low-temperature and high-temperature stresses, CsTCTP1 and CsTCTP2 demonstrated a wave expression pattern and reached the highest level at 6 h and 3 h, respectively. During high salt treatment, CsTCTP1 and CsTCTP2 slightly decreased and then increased, with the highest level at 24 h and 12 h, respectively. Under drought treatment, CsTCTP1 and CsTCTP2 were constantly up-regulated; the maximum accumulation occurred at 3 h and 24 h, respectively. These results reveal that CsTCTP1 and CsTCTP2 might play important roles in a variety of stress responses in cucumber plants.

TCTP is also regulated by various extracellular signals [13, 17, 25]. To address whether CsTCTP1 and CsTCTP2 are affected by extracellular signals, we investigated their time-dependent accumulation patterns under different treatments using qRT-PCR. As shown in Fig 6, there was no visible change in CsTCTP1 and CsTCTP2 in leaves treated with only H2O. Under CaCl2, H2O2, ABA, MeJA, SA and Ethrel treatments, CsTCTP1 and CsTCTP2 showed different expression patterns. In the case of ABA treatment, CsTCTP1 and CsTCTP2 genes were
Fig 8. Abiotic stress assay. Spot assay of BL21+pET30a, BL21+pET30a-CsTCTP1 and BL21+pET30a-CsTCTP2 on LB plates containing 50 μg/mL kanamycin at 37˚C, 45˚C and 55˚C (for heat stress) or on LB plates with NaCl (250 mM, 500 mM and 750 mM, for salt stress), mannitol (0.4 M, 0.8M and 1.2M, for drought stress), or HgCl₂ (15 μM, 25 μM and 35 μM, for mercury stress) at 37˚C overnight. Liquid culture assay on LB liquid medium containing 50 μg/mL kanamycin at 45˚C or on LB liquid medium with 500 mM NaCl, 0.8M mannitol, or 25 μM HgCl₂ at 37˚C. All data points are mean ± SE (n≥3).

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upregulated at all time points, with the maximum upregulation observed at 12 h and 48 h post treatment, respectively. These results suggest that CsTCTP1 and CsTCTP2 are likely to be involved in ABA responses in cucumber plants.

Ectopic expression of CsTCTP1 and CsTCTP2 in E. coli BL21 confers salt and heat tolerance

TCTP is involved in responses to a wide range of stimuli, such as mercury, heat, cold, drought, salt, ABA, ET and pathogens [13, 23, 26, 27]. To test whether overproduction of CsTCTP1 and CsTCTP2 can improve in vivo stress tolerance, CsTCTP1 and CsTCTP2 were cloned in pET30a and overexpressed in E. coli BL21. SDS-PAGE showed the molecular mass of CsTCTP1 to be very close to the predicted mass of 25 kDa harboring the His-tag, while CsTCTP2, although less close to the predicted mass, appeared to have the 34 kDa additional protein visualized according to results from our group (Fig 7).

Control (BL21 + pET30a) and overexpressed cells (pET30a-CsTCTP1 or pET30a-CsTCTP2) were exposed to salt, high temperature, drought, and HgCl\(_2\), and their growth was monitored (Fig 8). The results revealed that overexpressing CsTCTP1 and CsTCTP2 resulted in a higher tolerance to salt treatment than that of the control cells, and overexpressing CsTCTP2 also resulted in a higher tolerance to high temperature. Furthermore, overexpressed cells and control cells showed similar growth on LB liquid medium with different supplements (Fig 8). Recombinant CsTCTP1 and CsTCTP2 cells had a slower growth and a lower tolerance than did the control in LB containing mannitol and HgCl\(_2\).

Discussion

The high degree of homology and the common existence of TCTP in animals and plants underscore its vital roles in growth, development and responses to biotic or abiotic stresses in different organisms. However, plant TCTPs have not been studied extensively, especially in cucumber plants. In this study, the 2096 bp CsTCTP1 promoter and 2015 bp CsTCTP2 promoter sequences (relative to the translation start site) showed only 45% nucleotide identity. By contrast, the CsTCTP1 and CsTCTP2 coding sequences share 73% identity. Indeed, CsTCTP1 and CsTCTP2 displayed slightly different expression patterns. A putative cis-element associated with defense and stress responses (TC-rich repeats) was identified in the CsTCTP1 and CsTCTP2 promoter sequences. Similarly, the CsTCTP2 promoter sequence contains Box-W1, which is related to fungal elicitor responses. These data are in line with the expression data showing that CsTCTP1 and CsTCTP2 genes are affected by abiotic and biotic stresses. In addition, CsTCTP1 and CsTCTP2 slightly differed in their evolution and structures. It is reasonable to assume that such differences are reflected in their functions.

In Drosophila, dTCTP as a GEF directly associates with dRheb GTPases and positively regulates the TOR signaling pathway [4, 28]. TOR signaling pathway is known as a central coordinator of nutrient, energy and stress signaling networks [29]. In Arabidopsis, AtTCTP can bind to four AtRab GTPases (AtRABA4a, AtRABA4b, AtRABF1 and AtRABF2b) and interact with Drosophila dRheb GTPases. Similarly, dTCTP can bind to the four Arabidopsis AtRab GTPases [9]. Furthermore, eukaryotic GTPases act as molecular switches for diverse cellular processes. This GTPase-binding property might explain how TCTP is a multi-functional protein [17, 30]. In silico analysis of CsTCTP1 and CsTCTP2 functional domains indicated that both share the key GTPase binding surface. Although the GEF property of CsTCTP has not been shown in vitro, it is clear that CsTCTP is an important component of the TOR pathway.

Plant developed a range of defense mechanisms to protect themselves from external environmental stimuli. The expression of stress-responsive genes is an important part of the plant
response to a variety of biotic and abiotic stresses. In plants, the accumulation of the TCTP gene by fungal stress has been reported in Arabidopsis (Pseudomonas syringae) and wheat (Erysiphe graminis) [24, 31]. CsTCTP1 was identified in S. fuliginea-resistant cucumber cultivar interactions [12]. In contrast with the study conducted by Zheng [32], in the current study, the pathogen activated the early accumulation of CsTCTP1 and CsTCTP2. In this study, the maximum accumulation of CsTCTP1 and CsTCTP2 in the resistant variety B21-a-2-1-2 appeared earlier than that in the susceptible variety upon inoculation with S. fuliginea. From these results, it is clear that CsTCTP1 and CsTCTP2 both function at early stages (24 h and 48 h post infection) in plant resistance to pathogen attack. CsTCTP1 and CsTCTP2 levels are also highly regulated in response to abiotic stresses, such as heat, cold, drought and salinity stress. These results are in accordance with the in silico analysis data showing that CsTCTP1 and CsTCTP2 genes are affected by various abiotic stresses. As CsTCTP1 and CsTCTP2 genes were found to be upregulated at all treatment time points, this ABA-regulating property might explain the involvement of CsTCTP in the seemingly related ABA signaling transduction process. The results of promoter analysis and qRT-PCR showed that CsTCTP1 and CsTCTP2 might have diverse functions in a variety of stress responses and ABA signaling transduction processes in cucumber plants. Therefore, CsTCTP1 and CsTCTP2 might be good candidates for alternative stress-related genes.

Several stress-related cis-elements were identified in the promoters of CsTCTP1 and CsTCTP2, as supported by their rapid induction under various stresses. However, because of these results, it is not clear whether CsTCTP1 and CsTCTP2 are positively regulated or negatively regulated in response to various abiotic stresses. Studies on the specific function of CsTCTP1 or CsTCTP2 in plant-stress interactions are necessary. Current studies indicate that overproduction of plant stress-related genes enhances growth of E. coli cells [19, 33]. Furthermore, a cassava TCTP conferred salt stress tolerance to E. coli [21]. The fact that the pET30a-CsTCTP2 sample has a predicted protein band of 26 kDa was inconsistent with the additional protein band of about 34 kDa that was not found in the control sample. One explanation of the results is as follows: (a) adding SDS changed the protein conformation (b) the terminator of CsTCTP2 may not terminate translation effectively until it meets the terminator of the vector. Mercury (Hg) is also a growth risk factor to plants that damages many cellular-level functions and inhibits plant growth and development. It has been shown that OsTCTP plays an important role in mercury tolerance in rice [22]. Intriguingly, overexpression of CsTCTP1 and CsTCTP2 reduced the tolerance to HgCl2 stress. Overproduction of CsTCTP1 and CsTCTP2 resulted in greater sensitivity to drought and HgCl2 stresses. Collectively, CsTCTP plays an important role in cucumber abiotic stress responses. Since the response mechanisms of CsTCTP to different stresses may differ, further study of CsTCTP is necessary.

In conclusion, CsTCTP1 and CsTCTP2 promoter, full-length DNA and genomic sequences were cloned from Cucumis sativus. CsTCTP might be involved in stress responses by regulating ABA or TOR pathways in cucumber. CsTCTP is related to responses to a wide range of stimuli. CsTCTP1 and CsTCTP2 showed positive responses to salt and heat stresses and negative responses to drought and HgCl2 stresses.

**Supporting information**

S1 Table. Predicted cis-acting elements with putative functions identified in the CsTCTP1 promoter using the PLACE and PlantCARE databases.

(RTF)
S2 Table. Predicted cis-acting elements with putative functions identified in the CsTCTP2 promoter using the PLACE and PlantCARE databases.

S3 Table. List of primers used in the study.

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