Short Research Article

Molecular Characterization and Activity Analysis of Promoters from Two Cucumber Translationally Controlled Tumor Protein Genes (*CsTCTPs*)

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ABSTRACT

Aims: The aim of the paper was to isolate and characterize the promoters of two cucumber TCTP genes (*CsTCTP1* and *CsTCTP2*) and evaluate their active domains.

Study Design: *CsTCTP1* and *CsTCTP2* promoter activity were analyzed under treatments with different exogenous hormones.

Place and Duration of Study: In 2017, these experiments were conducted in College of Bioscience and Biotechnology of Shenyang Agricultural University (Lab 240).

Methodology: $CsTCTP_{pro}$::*GUS* constructs were generated by using double digests method. Transient expression was mediated by *Agrobacterium tumefaciens* GV3101. Histochemical and Fluorometric GUS Assays were follow by the biochemical method.

Results: Bioinformatics analysis revealed some hormone- and defense-related response elements. Histochemical and fluorometric GUS assays demonstrated that the 0.7-kb *CsTCTP1* promoter (proT1-0.7kb) and 0.7-kb and 1.3-kb *CsTCTP2* promoters (proT2-0.7kb and proT2-1.3kb) had strong transcriptional activity. In addition, we used exogenous hormones (abscisic acid [ABA], salicylic acid [SA], and ethylene [ETH]) for treatment. The results showed that proT1-0.7kb and proT2-1.3kb activity were upregulated in the ABA treatment group, suggesting that these promoter sequences may contain ABA-related response elements. However, in the SA and ETH treatment groups, the activity of all the promoter fragments of *CsTCTP1* and *CsTCTP2* declined to different degrees, suggesting that SA and ETH may have negative regulatory effects on *CsTCTP1* and *CsTCTP2* promoters.

Conclusion: Taken together, these results suggest that the proT1-0.7kb promoter of *CsTCTP1* and proT2-1.3kb promoter of *CsTCTP2* may contain ABA-related response elements, and SA and ETH may have negative regulatory effects on the *CsTCTP1* and *CsTCTP2* promoters. This study will help to further understand the expression patterns and the regulatory mechanism of gene transcriptional regulation.

Key words: Bioinformatics; CsTCTP; Fluorometric GUS assay; Histochemical assay; Promoter

1. INTRODUCTION

Correct and efficient gene expression ensures the adaptation of plants to their environment. Gene expression is regulated by noncoding elements over the lifespan of a plant. Promoters are DNA sequences that specifically bind to RNA polymerase and transcription factors to determine the initiation of gene transcription, and they contain many important *cis*-acting elements. Promoters receive signals from a variety of sources (such as cell receptors) and control the level of transcription initiation, which determines gene expression to a great extent and plays an important role in plant gene expression regulation [1, 2]. Different promoters contain different regulatory elements. The analysis of relevant regulatory elements has become particularly important to help promote the

understanding of gene expression patterns and further improve plant performance under unfavorable conditions.

Translationally controlled tumor protein (TCTP), a multifunctional protein, is highly expressed and ubiquitously distributed in all eukaryotes [3]. TCTP levels are regulated transcriptionally and posttranscriptionally [4-6]. In plants, TCTP plays a key role in stress response and signal transduction [7-10]. In rubber tree (*Hevea brasiliensis*), *HbTCTP* is regulated by drought, temperature, salt treatment, ethylene treatment, wounding, H_2O_2 treatment, and methyl jasmonate treatment [11]. In the developing seed of soybean (*Glycine max*), GmTCTP proteins interact with GmCDPKSK5 proteins and may function in high temperature and humidity stress responses [12]. Cucumber (*Cucumis sativus*) has two TCTPs. In 2014, CsTCTP1 (XP-004134215) was first found to play a vital role in the response of cucumber to *Sphaerotheca fuliginea* at the protein level through two-dimensional gel analysis [13]. In our subsequent study, the *CsTCTP1* and *CsTCTP2* genes were both found to be negative modulators in the cucumber defense response to *S. fuliginea*. *CsTCTP1* participates in the defense response to *S. fuliginea* through regulating the expression of certain defense-associated genes and/or abscisic acid (ABA) signaling pathway-associated genes, and *CsTCTP2* participates by regulating the expression of target of rapamycin (TOR) signaling pathway-associated genes [14].

Although *CsTCTP* genes are induced by *S. fuliginea*, little is known about the function of *CsTCTP* promoters or *CsTCTP* expression regulation. In this study, the promoters of *CsTCTP1* and *CsTCTP2* were isolated and analyzed. Histochemical and fluorometric GUS assays were used to test their active sites. In addition, promoter activity was analyzed under treatments with different exogenous hormones (ABA, salicylic acid [SA], and ethylene [ETH]). This study will help to further understand the expression patterns and the regulatory mechanism of gene transcriptional regulation.

2. MATERIALS AND METHODS

2.1 Plant Material and Treatments

Cucumber seeds (B21-a-2-1-2) with high resistance to *S. fuliginea* were obtained from the Liaoning Academy of Agricultural Sciences. The seeds were sterilized with 50% NaClO for 15 min and 75% ethyl alcohol for 45 s, then washed with ddH_2O 4 or 5 times. The treated seeds were germinated on solid Murashige and Skoog medium in a greenhouse under a light intensity of 40 lx at 24 °C. When grown to two true leaves, the seeds were carefully transferred into soil matrix and maintained in a growth chamber with a photoperiod of 16 h light, 8 h dark.

2.2 Isolation of CsTCTP1 and CsTCTP2 Promoters, pT1 and pT2

Total genomic DNA was isolated from the cucumber leaves using a genomic DNA kit. Using the genomic DNA as a template, the primer pairs *pT1-1-SalI-F/pT1-NcoI-R* (-1,308/+88, positions relative to the transcriptional start site [TSS]) and *pT2-1-SalI-F/pT2-NcoI-R* (-1,305/+69) were used to clone the promoter (Supplementary Table 1; underlining represents restriction sites).

Amplification reactions were carried out as follows: 94 °C for 5 min, followed by 38 cycles of amplification (94 °C for 30 s, 60–65 °C for 30 s, 72 °C for 2 min 15 s), and then 72 °C for 10 min. Polymerase chain reaction (PCR) products were purified using a DNA gel extraction kit.

2.3 Bioinformatics Analyses

TSSs and possible core promoter regions were predicted by using the BDGP (http://www.fruitfly.org/seq_tools/promoter.html) and TSSP (http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter) tools. The *cis*-acting elements, distributions, and biologic

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al functions of *CsTCTP1* and *CsTCTP2* promoter sequences were analyzed by using the Plan tCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.4 Plasmid Construction

The upstream regions of *CsTCTP1* (-1,308 to +88) and *CsTCTP2* (-1,305 to +69) were divided into three fragments by size (1.3, 0.7, and 0.3 kb). Different *CsTCTP1* and *CsTCTP2* promoter fragments were amplified by PCR with specific forward primers (*pT1-2-SacI-F* [-714/+88], *pT1-3-SalI-F* [-306/+88], *pT2-2-SacI-F* [-732/+69], *pT2-3-SalI-F* [-324/+69]) and common reverse primers (*pT1-NcoI-R, pT2-NcoI-R*) (Supplementary Table 1). PCR products were digested with restriction enzymes and subcloned into pCAMBIA1301 vectors, replacing the *CaMV35S* promoters (previously eliminated by corresponding restriction enzyme digestion) to generate six $CsTCTP_{pro}$::*GUS* constructs (Fig. 1).

2.5 Agrobacterium-Mediated Transient Expression

Agrobacterium tumefaciens GV3101 cells harboring the $35S_{pro}$*::GUS* or $CsTCTP_{pro}$::*GUS* vector were cultured to OD600=0.5 in infiltration medium (100 mM $MgCl_2$, 200 μM acetosyringone, 20 mM MES, pH 5.6), then diluted to OD600=0.5. The diluted culture was injected into 5-week-old tobacco leaves (*Nicotiana benthamiana*) using a syringe without a needle [15]. *A. tumefaciens* GV3101 cells were also transformed into tobacco plants as negative controls. Three replicates within each independent experiment and three independent biological replicates were performed.

2.6 Histochemical and Fluorometric GUS Assays

The tobacco plants after injection were routinely grown in a culture chamber (25 °C, 16 h light, 8 h dark) and sprayed with exogenous hormones (100 μM ABA, 1 mM SA, or 10 μL/L ETH) after 24 hours of treatment. The control group was treated with H_2O and collected after 24 h of continuous cultivation, and infiltrated leaf discs were used for histochemical GUS staining according to the method of Jefferson [16]. The plant samples were immersed in GUS staining solution (1 mg/mL X-Gluc, 100 mM NaH_2PO_4, 100 mM Na_2HPO_4, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1% Triton X-100, 1 mM EDTA, pH 7) for 16 h at 37 °C, then washed with 70% absolute ethanol to remove chlorophyll until the negative control was colorless.

β-Glucuronidase activity was quantified by using fluorometric GUS assays. About 100 mg of tobacco leaves were frozen and milled in liquid nitrogen, and the total protein was extracted by resuspending the samples in 1 mL of extraction buffer (50 mM $NaPO_4$, pH 7, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100). The homogenate was centrifuged at 12,000 rpm for 10 min, then the supernatant was recovered. The concentration of total protein was determined according to the method of Bradford [17] using bovine serum albumin as a standard. GUS activity was measured as described by Jefferson [16], and fluorescence of 4-methyl umbelliferone was recorded using a spectrofluorimeter with excitation at 365 nm and emission at 455 nm and expressed as nanomoles of 4-methyl umbelliferone generated per μg of protein per min.

3. RESULTS AND ANALYSIS

3.1 Isolation and Structural Analysis of *CsTCTP1* and *CsTCTP2* Promoters

The upstream 1400-bp fragment of the ATG of the *CsTCTP1* and *CsTCTP2* genes and its short segments were successfully cloned by reverse transcription PCR. The PCR amplification products of the *CsTCTP1* and *CsTCTP2* promoters were detected by 1% agarose gel electrophoresis, and the results showed that these specific fragments were the same size as expected (Fig. 2).

Using the BDGP, PlantCARE, and PLACE tools, the structures of the *CsTCTP* promoters were

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predicted and analyzed. The results showed that the core promoter region of *CsTCTP1* was located at -129 to -79 bp, the TSS was C, which was located at -88 bp, and the TATA box was located at -28 bp upstream of the TSS (Fig. 3). The core promoter region of *CsTCTP2* was located at -109 to -59 bp, the TSS was A, which was located at -68 bp, and the TATA-box was located at -28 bp upstream of the TSS (Fig. 4).

The *CsTCTP1* promoter contained three ABRE elements and one TCA element, which are involved in ABA and SA responsiveness, respectively. The *CsTCTP1* promoter also had some defense response elements, such as TC-rich repeats (involved in defense and stress responsiveness), HSE (involved in heat stress responsiveness), and MBS (MYB binding site involved in drought-inducibility) (Supplementary Table 2). The *CsTCTP2* promoter had ABRE and ERE elements, which are involved in ABA and ethylene responsiveness, respectively. Four TC-rich repeats (involved in defense and stress responsiveness) were found in the *CsTCTP2* promoter (Supplementary Table 3).

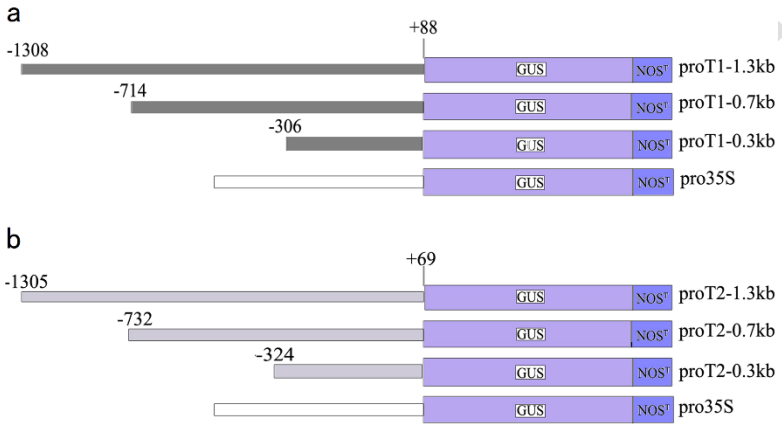

Fig. 1 Construction of *CsTCTP*$_{pro}$::*GUS*. Three differently sized *CsTCTP* promoter regions (0.3 kb$_{pro}$, 0.7 kb$_{pro}$, 1.3 kb$_{pro}$) were fused to the *GUS* reporter gene. NOS^T, *Agrobacterium tumefaciens NOS* gene transcription terminator.

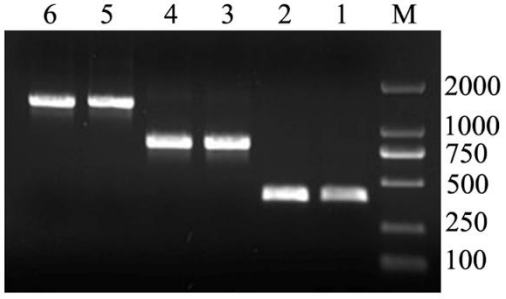

Fig. 2 Electrophoresis profile of the PCR products of the *CsTCTP1* and *CsTCTP2* promoters and their short segments. M: DL2000 DNA marker; 1,3,5: *CsTCTP1pro*; 2,4,6: *CsTCTP2pro*.

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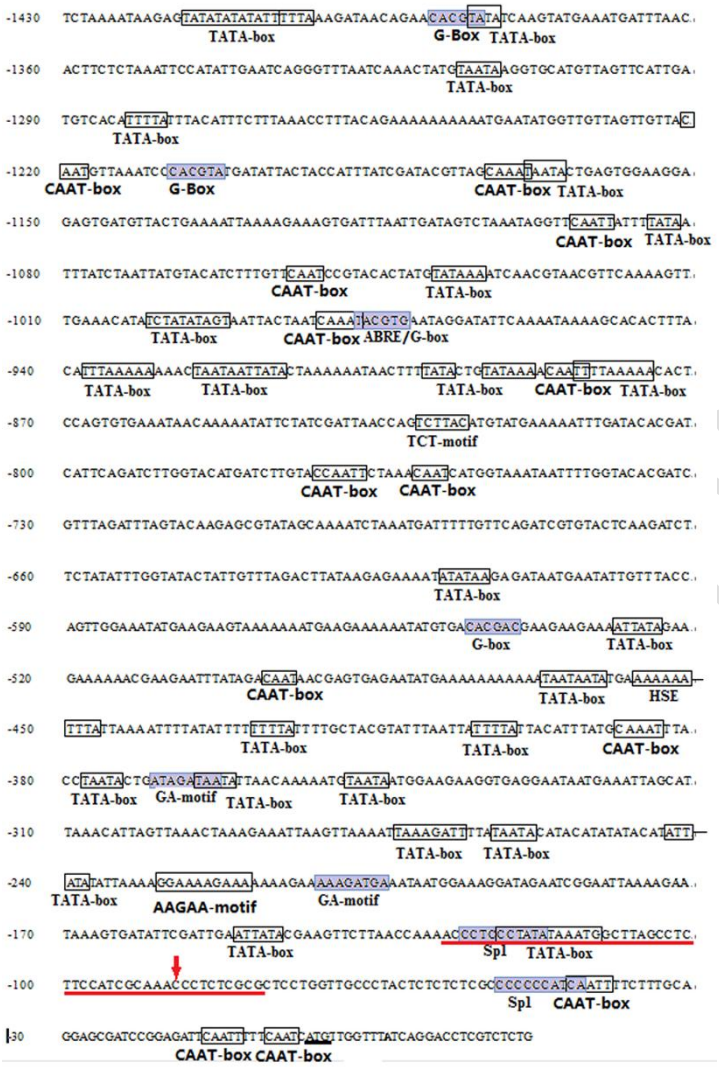

Fig. 3 Sequence and structural analysis of the *CsTCTP1* promoter. Red underline: the core region of the promoter; arrow: transcription start site; black underline: start codon; box: *cis*-regulatory element.

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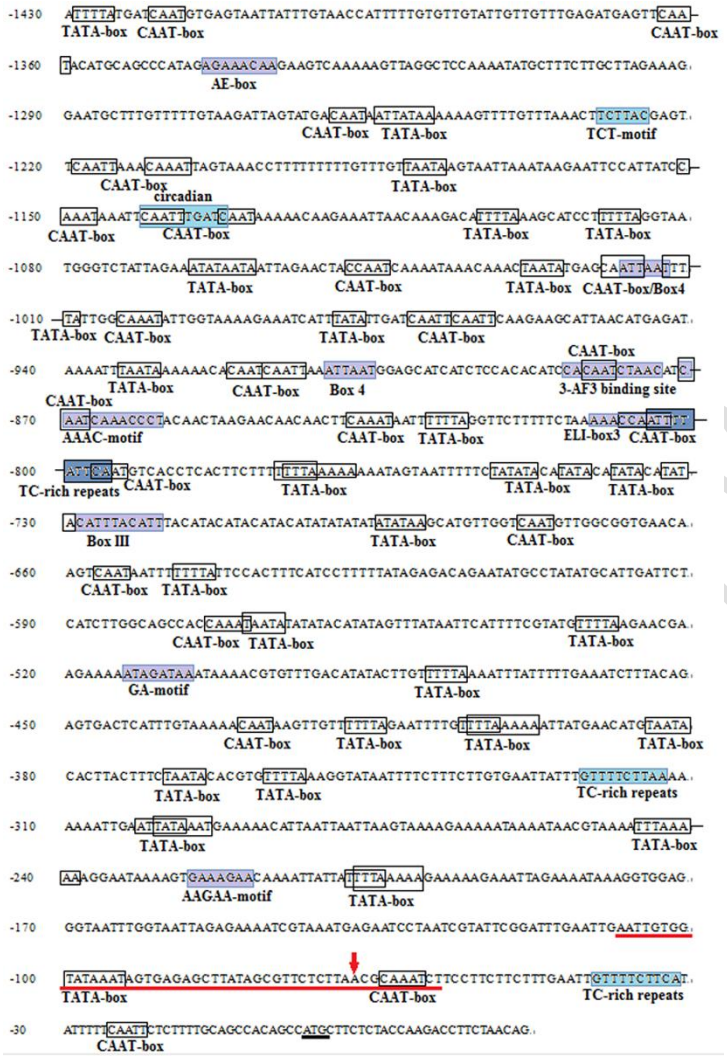

Fig. 4 Sequence and structural analysis of the *CsTCTP2* promoter. Red underline: the core region of the promoter; arrow: transcription start site; black underline: start codon; box: *cis*-regulatory element.

3.2 Analysis of *CsTCTP1* and *CsTCTP2* Promoter Activity in Transgenic Tobacco

In our previous study, we found that there were no differences in the sequences of the promoters between two sister cucumber lines, B21-a-2-2-2 and B21-a-2-1-2 [18]. In this study, we chose cucumber line B21-a-2-1-2 as the material for sequence isolation. Based on the results of the analysis of the *Arabidopsis* TCTP promoter [19] and the structural analysis of the *CsTCTP* promoters, we generated transgenic plants with three short segments of *CsTCTP* promoters that were fused to GUS, and we then determined the activity of the promoters.

With the transgenic tobacco plants of *CsTCTP*$_{pro}$::*GUS* vectors, histochemical and fluorometric GUS assays were conducted to analyze promoter activity in the plants (Fig. 5). The results showed that the 0.7-kb *CsTCTP1* promoter (proT1-0.7kb) and 0.7-kb and 1.3-kb *CsTCTP2* promoters (proT2-0.7kb and proT2-1.3kb) had strong GUS expression, which was comparable to that of the control plant with the 35S promoter (*35S*$_{pro}$*::GUS*). The proT1-0.7kb promoter showed the strongest GUS activity compared to proT1-0.3kb and proT1-1.3kb, corresponding to approximately five-fold GUS activity of *35S*$_{pro}$*::GUS*. The proT2-0.7kb and proT2-1.3kb promoters showed the strongest GUS activity

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compared to proT2-0.3kb, corresponding to approximately three-fold GUS activity of *35S$_{pro}$::GUS*.

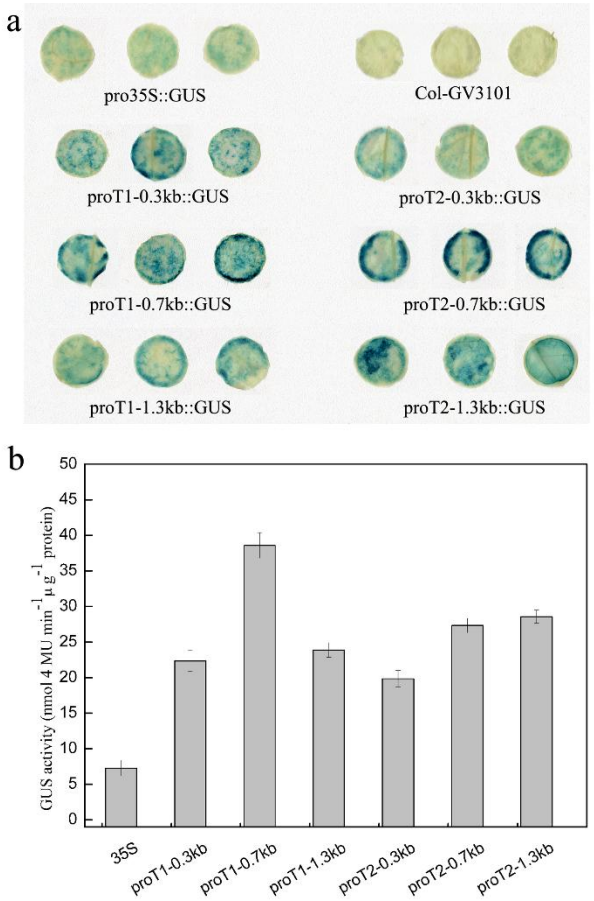

Fig. 5 Promoter activity analyses with upstream regions of *CsTCTP1* and *CsTCTP2*. a. Histochemical GUS analysis. b. Fluorometric GUS analysis. Each measurement was repeated three times. GV3101 and *pro35S::GUS* leaf discs were used as negative and positive controls, respectively.

3.3 Effects of Exogenous Hormones on *CsTCTP1* and *CsTCTP2* Promoter Activity

CsTCTP1 and *CsTCTP2* are regulated by exogenous hormone treatments [18]. Furthermore, their promoters contain several *cis*-elements associated with ABA, SA, and ETH. To explore the effects of exogenous hormones on *CsTCTP1* and *CsTCTP2* promoter activity, the transgenic tobacco leaves were divided into three groups and sprayed with 100 μM ABA, 1 mM SA, or 10 μL/L ETH. After 24 h of treatment, each group was tested by histochemical and fluorometric GUS assays.

The GUS activity of the proT1-0.7kb and proT2-1.3kb promoters was remarkably enhanced under ABA treatment compared to under H_2O treatment (Fig. 6). Further, the GUS activity of proT2-0.7kb was reduced, but it did not change significantly for the other promoters (proT1-0.3kb, proT1-1.3kb, proT2-0.3kb). When under SA or ETH treatment, the GUS activity of all the promoters was reduced to varying extents compared to under H_2O treatment (Fig. 7 and 8). These results are supported by the previous bioinformatics analysis, which revealed SA and ETH response elements in the promoters of *CsTCTP1* and *CsTCTP2*, and it can be speculated that SA and ETH may negatively regulate the promoters of *CsTCTP1* and *CsTCTP2*.

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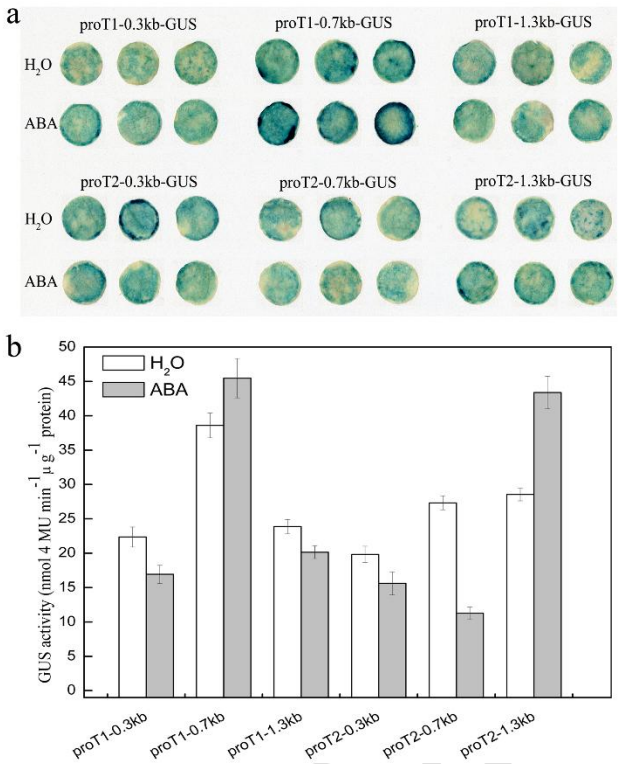

Fig. 6 GUS staining and fluorescence quantitative assay after ABA treatment. The H_2O-treated group was used as the control.

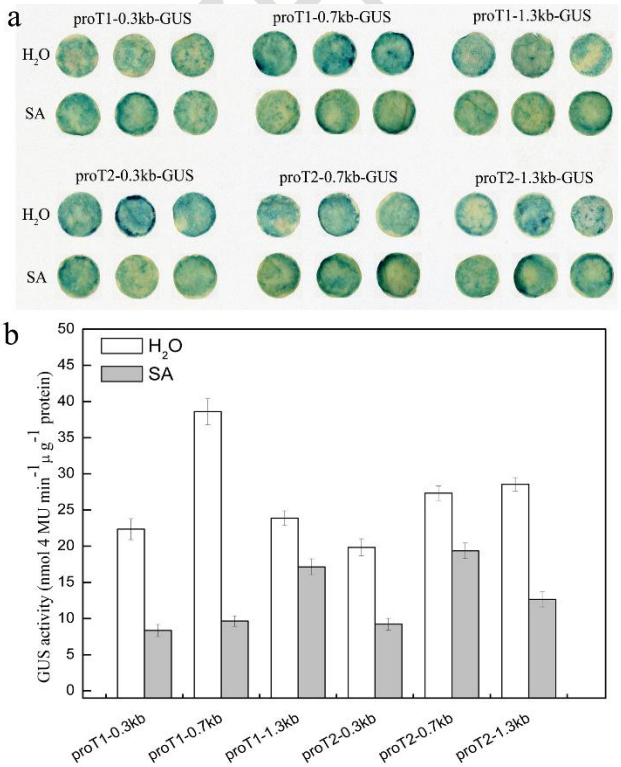

Fig. 7 GUS staining and fluorescence quantitative assay after SA treatment.

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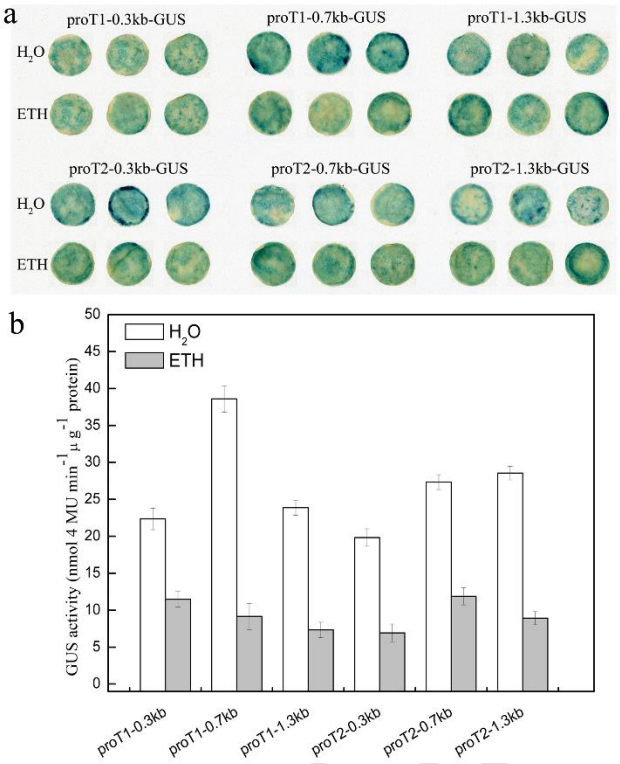

Fig. 8 GUS staining and fluorescence quantitative assay after ETH treatment.

4. DISCUSSION

TCTP is a highly conserved protein that exists in eukaryotes [3]. TCTP is considered to play an essential role in the regulation of growth and development in eukaryotes [20-23]. However, gene expression regulation by upstream promoters is not understood. In this study, the reporter gene used was an intron containing the *GUS* gene (Fig. 5), which ensured that the expression was plant cell-specific. The results showed that all of the six promoter deletion fragments of *CsTCTP1* and *CsTCTP2* had the function of initiating gene expression. The transcription activity of proT1-0.7kb in tobacco corresponded to about five-fold activity compared with $35S_{pro}$*::GUS*, which showed that proT1-0.7kb (-720 bp to -1313 bp) contained a *cis*-acting element similar to the enhancer. It was reported that the 0.3-kb *AtTCTP* promoter in *Arabidopsis* exhibited the strongest transcription activity, while the 0.7-kb *CsTCTP1* promoter and 0.7-kb and 1.3-kb *CsTCTP2* promoters in this study exhibited the strongest transcription activity (Fig. 5). One explanation for these results is that: (a) the *CsTCTP1* promoter shares only 41.37% nucleotide identity with the *Arabidopsis AtTCTP* promoter, while the *CsTCTP2* promoter shares only 38.25% nucleotide identity with the *AtTCTP* promoter, and (b) transient transformation is different from stable transformation. Indeed, transient expression has some deficiencies that limit its application. The results of this experiment still need to be further validated by the combination of stable expression analysis. However, transient expression can be a quick and preliminary way to understand the functional properties of *CsTCTP* promoters. Furthermore, the tobacco system is more mature and accepted.

In plants, SA is an important defense signaling molecule. In this study, SA may have had a negative regulatory effect on the *CsTCTP1* and *CsTCTP2* promoters. This work proved once again that *CsTCTP1* and *CsTCTP2* are both negative modulators in the cucumber defense response to *S.*

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fuliginea. Despite showing the same negative effects on *S. fuliginea*, *CsTCTP1* and *CsTCTP2* displayed slightly different expression patterns. The 1,396-bp *CsTCTP1* promoter and 1,374-bp *CsTCTP2* promoter sequences (relative to ATG) showed only 44.09% nucleotide identity. This low sequence homology can be explained by the different regulatory mechanisms of *CsTCTPs*. Compared with H_2O-treated tobacco, the ABA-treated group showed that the activity of proT1-0.7kb and proT2-1.3kb increased. Combined with bioinformatics predictions, this may be explained by the presence of defensive stress response elements in their promoter sequences. The GUS activity of proT2-0.7kb was reduced, probably due to the presence of negative regulators of ABA within the range of -324 bp to -732 bp. However, the activity of proT1-0.3kb, proT1-1.3kb, and proT2-0.3kb did not change significantly, so it is speculated that no relevant hormone response elements existed in these fragments.

Promoter prediction is still a challenging task, although different methods have been proposed for prediction, but for new resistance-related promoter clones, *cis*-acting elements have been identified by specific sequences between the elements. The transcription factors that interact with these elements will be the focus of future promoter studies.

5. CONCLUSION

In summary, the 0.7-kb *CsTCTP1* promoter (proT1-0.7kb) and 0.7-kb and 1.3-kb *CsTCTP2* promoters (proT2-0.7kb and proT2-1.3kb) had strong transcriptional activity. The proT1-0.7kb promoter of *CsTCTP1* and proT2-1.3kb promoter of *CsTCTP2* may contain ABA-related response elements, and SA and ETH may have negative regulatory effects on the *CsTCTP1* and *CsTCTP2* promoters.

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Supplementary Table 1. *CsTCTP1* and *CsTCTP2* gene promoter primer sequences

Primer name	Primer sequence （5’ to 3 ’）
pT1-1-SalI-F	GTCGACAACAGAACACGTATATCAAGTATG
pT1-2-SacI-F	GAGCTCATCATTCAGATCTTGGTACAT

pT1-3-SalI-F	GCGTCGACTTTATGCAAATTTACCTAATAC
pT1-NcoI-R	CATGCCATGGGATTGAAAAATTGAATCTC
pT2-1-SalI-F	GTCGACGAGATGAGTTCAATACATGCAGC
pT2-2-SacI-F	CGAGCTCGATTCAATGTCACCTCACTTCTT
pT2-3-SalI-F	GCGTCGACGGAACATGTAATACACTTACTTTC
pT2-NcoI-R	CCATGGGGGCTGTGGCTGCAAAAGAGAATT

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Supplementary Table 2. Partial *cis*-acting element of *CsTCTP1* gene promoter

Site Name	Sequence	Strand		Function
		+	—	
5'UTR Py-rich stretch	TTTCTTCTCT	0	1	element conferring high transcription levels
ABRE	TACGTG	1	2	involved in the abscisic acid responsiveness
ARE	TGGTTT	1	0	regulatory element essential for the anaerobic induction
Box I	TTTCAAA	0	1	light responsive element
Box 4	ATTAAT	2	0	involved in light responsiveness
CAAT-box	CAATT、CAAT、CAAAT	14	9	common cis-acting element in promoter and enhancer regions
G-Box	CACGTA	2	1	involved in light responsiveness
G-box	TACGTG、CACGAC	2	2	involved in light responsiveness
GA-motif	ATAGATAA、AAAGATGA	2	0	part of a light responsive element
GAG-motif	AGAGAGT	0	1	part of a light responsive element
GT1-motif	GGTTAA	0	3	light responsive element
HSE	AAAAAATTTC	1	0	involved in heat stress responsiveness
MBS	CAACTG	0	1	MYB binding site involved in drought-inducibility
Sp1	CC(G/A)CCC	3	0	light responsive element
TC-rich repeats	ATTTTCTTCA	0	3	defense and stress responsiveness
TCA-element	CCATCTTTTT	0	1	salicylic acid responsiveness
TCT-motif	TCTTAC	1	0	part of a light responsive element
Circadian	CAANNNNATC	0	2	cis-acting regulatory element involved in circadian control

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Supplementary Table 3. Partial *cis*-acting element of *CsTCTP2* gene promoter

Site Name	Sequence	Strand		Function
		+	—	
AAAC-motif	CAATCAAAACCT	1	0	light responsive element
ABRE	CACGTG、TACGTG	0	1	abscisic acid responsiveness
ACE	AAAACGTTTA	0	1	light responsiveness
ARE	TGGTTT	0	1	essential for the anaerobic induction
Box 4	ATTAAT	2	2	involved in light responsiveness
Box I	TTTCAAA	0	1	light responsive element
Box-W1	TTGACC	0	1	fungal elicitor responsive element
CAAT-box	CAAT、CAAAT、CAATT	27	17	common cis-acting element in promoter and enhancer regions
ELI-box3	AAACCAATT	1	0	elicitor-responsive element
ERE	ATTTCAAA	0	1	ethylene-responsive element
G-Box	CACGTT、CACGTG	0	2	involved in light responsiveness
G-box	CACGTT、CACGTG	0	3	light responsiveness
GA-motif	ATAGATAA、AAGGAAGA	1	1	part of a light responsive element
GCN4-motif	TGAGTCA	0	1	cis-regulatory element involved in endosperm expression
MRE	AACCTAA	0	1	MYB binding site involved in light responsiveness
Skn-1-motif	GTCAT	0	1	required for endosperm expression
TA-rich region	TATATATATATATATATATATA	1	0	Enhancer
TC-rich repeats	ATTTTCTTCA、GTTTTCTTAC	3	1	defense and stress responsiveness
TCT-motif	TCTTAC	1	1	part of a light responsive element
Circadian	CAANNNNATC	1	0	cis-acting regulatory element involved in circadian control

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