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The Identification of Cucumber TDC Genes and Analyses of Their Expression and Functions under Abiotic Stress Conditions

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Abstract: Melatonin is a crucial regulator of plant growth and development as well as stress tolerance. However, we only have a limited understanding of the functions of endogenous melatonin. Tryptophan decarboxylase (TDC) serves as the initial rate-limiting enzyme in the melatonin synthesis pathway. To date, no cucumber *TDC* gene has been cloned and characterized. In this study, we identified two *TDC* genes (*CsTDC1* and *CsTDC2*) in the cucumber genome. The subcellular localization analysis indicated that CsTDC1 and CsTDC2 are predominantly localized in the cytoplasm and plasma membrane. Tissue-specific expression analyses revealed that *CsTDC1* and *CsTDC2* are expressed in both vegetative and reproductive organs. Many cis-elements related to stress, hormone, and light responses as well as development were identified in the *CsTDC* promoter regions. Furthermore, the expression of *CsTDC1* and *CsTDC2* was strongly induced by treatments with various abiotic stresses and exogenous hormones. The transient overexpression of *CsTDC1* and *CsTDC2* in tobacco leaves resulted in increases in the TDC activity and melatonin content, along with improved tolerance of tobacco leaves to salt, drought, and low-temperature stresses. Notably, the overexpression of *CsTDC2* had a more pronounced effect than the overexpression of *CsTDC1*. Accordingly, both *CsTDC* genes, but especially *CsTDC2*, may be important for regulating cucumber growth, development, and stress tolerance. The study findings provide a theoretical and experimental basis for future functional analyses of endogenous melatonin in cucumber.

Keywords: cucumber; melatonin; TDC; abiotic stress

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1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), which is an indole hormone commonly found in animals, plants, and microorganisms, was first detected in plants in 1995 [1,2]. Numerous studies have demonstrated that melatonin plays a significant role in regulating various plant growth and developmental processes, including seed germination, root development, leaf senescence, flower development, fruit maturation, fruit storage, and seedling growth [3,4]. Moreover, melatonin can function as an antioxidant, thereby enhancing plant tolerance to abiotic stresses (e.g., drought, salinity, alkalinity, and temperature extremes) [5,6]. Thus, it may be useful for improving future agricultural production. However, most of the physiological effects of melatonin were determined via the administration of exogenous melatonin. Although plants can increase their endogenous melatonin levels by absorbing exogenous melatonin, the effects of exogenous and endogenous melatonin may differ [5,7]. Accordingly, plant melatonin synthase genes will need to be identified and functionally characterized to more comprehensively elucidate endogenous melatonin effects.

Phytomelatonin biosynthesis involves four main synthetic routes, all of which require tryptophan as the initial substrate and at least six enzymes, namely tryptophan decarboxylase (TDC), tryptophan hydroxylase (TPH), tryptamine 5-hydroxylase (T5H),

serotonin N-acetyltransferase (SNAT), N-acetylserotonin methyltransferase (ASMT), and caffeic-O-methyltransferase (COMT) [8,9]. Among these enzymes, TDC belongs to a group of aromatic-L-amino acid decarboxylases (AADCs), which catalyze the conversion of aromatic L-amino acids to aromatic monoamines and play a crucial role in the synthesis of secondary metabolites in plants [10]. Two important AADCs in plants are TDC and tyrosine decarboxylase (TyrDC) [11], of which TDC uses tryptophan as a substrate, whereas TyrDC uses tyrosine, but both enzymes are involved in the synthesis of various alkaloid metabolites [10,12]. Additionally, TDC is responsible for catalyzing the rate-limiting reaction during the synthesis of melatonin. More specifically, in the first enzymatic reaction leading to the synthesis of melatonin, tryptophan is decarboxylated by TDC, resulting in the formation of tryptamine [5,13].

The *TDC* gene family in plants is relatively small; the first plant TDC-encoding gene was isolated from *Catharanthus roseus* [14]. The subsequent advancements in genome sequencing technology have facilitated the identification of additional *TDC* genes in various plants, including *Oryza sativa* [15], *Ophiorrhiza pumila* [16], *Citrus* species [17], *Aegilops variabilis* [18], and *Camptotheca acuminata* [19]. The regulatory functions of the proteins encoded by these genes have been gradually elucidated. However, the *Nicotiana tabacum* and Arabidopsis genomes lack *TDC* genes [13]. Earlier research indicated that upregulated *TDC* expression in plants leads to increases in tryptamine, serotonin, and melatonin levels [20–22], whereas the downregulated expression of *TDC* genes has the opposite effect [15]. These results reflect the importance of TDC for the synthesis of melatonin in plants. A recent study showed that silencing *TDC* expression in rice via RNA interference (RNAi) results in semi-dwarfism [15]. In contrast, another study demonstrated that the overexpression of *TDC* in rice leads to stunted growth and low fertility [22]. Additionally, *TDC* expression can enhance the ability of plants to tolerate drought and saline conditions [7], suggestive of a key regulatory role influencing plant growth and stress responses. However, TDC functions have not been thoroughly characterized and additional research is required to clarify TDC regulatory effects and the underlying mechanisms.

Cucumber (*Cucumis sativus* L.) is a vegetable crop that is cultivated worldwide, but it is susceptible to environmental stresses [23]. Numerous studies have revealed the positive effects of exogenous melatonin on plant growth and stress tolerance, but there have been relatively few studies on the functions of endogenous melatonin. Therefore, the genes responsible for melatonin synthesis in different species should be identified and their regulatory functions will need to be determined. Although *TDC* gene family members have been identified in some species, there is only limited available information regarding cucumber *TDC* genes and their functions. Thus, in this study, we identified two *TDC* genes in the cucumber genome. These genes were characterized on the basis of analyses of their promoter cis-elements, tissue-specific expression patterns, and responses to different stresses and plant hormones. To further verify the biological functions of *CsTDC* genes, we conducted a subcellular localization analysis and transiently overexpressed the genes in tobacco to preliminarily explore their roles in plant responses to salt, drought, and low-temperature stresses. The study findings may form a theoretical foundation for future in-depth investigations on the potential functions of *CsTDC* genes and the regulatory effects of endogenous melatonin.

2. Materials and Methods

2.1. Identification and Analysis of TDC Genes in Cucumber

The previously reported TDC protein sequences in rice were obtained and used as queries to search for homologous TDC sequences in the Cucumber (Chinese Long) v3 Genome Database (http://cucurbitgenomics.org/organism/20, accessed on 20 April 2023) using the BLASTP program. The candidate TDC proteins were verified using the online tools SMART (http://smart.embl-heidelberg.de/, accessed on 20 April 2023) [24] and Pfam (http://pfam.xfam.org, accessed on 20 April 2023) [25]. The molecular weight and pI value were calculated using the online program ExPASy (http://web.expasy.org/compute_pi/,

accessed on 20 April 2023). The online software CELLO (version 2.5) (http://cello.life.nctu.edu.tw, accessed on 20 April 2023) [26] was used to predict the subcellular localization of the identified TDC proteins.

2.2. Analysis of Cis-Elements in the Promoter Regions

To investigate the cis-elements in the promoter regions of the two identified *TDC* genes, we downloaded the genomic sequence 2 kb upstream of the initiation codon (ATG) of each gene from the cucumber genome database (Figure S1. The putative cis-regulatory elements in the promoter sequences were analyzed using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html, accessed on 25 April 2023) [27].

2.3. Plant Materials and Treatments

Cucumber seedlings (*Cucumis sativus* L. cv. 'Changchunmici') were cultivated in a climate-controlled chamber (12-h light, 25 °C:12-h dark, 18 °C) at the College of Life Science, Gannan Normal University, Ganzhou, China. After an approximately 10-day cucumber fruit growth period, the following tissues were collected from each plant: basal old leaves (OL), upper young leaves (YL), middle mature leaves (ML), young stems (YS), roots (R), blooming female flower petals (FF), ovaries (O), blooming male flowers (MF), tendrils (T), fruits (F), and seeds (S). The collected samples were promptly frozen in liquid nitrogen and stored at −80 °C for the gene expression analysis.

For each stress and exogenous hormone treatment, 12 cucumber seedlings (half-expanded first true leaf) were transplanted to a plastic basin (33 cm × 25 cm × 11 cm) containing 5 L half-strength Hoagland nutrient solution [23]. When the seedlings reached the two-leaf stage, the nutrient solution was supplemented with 100 mM NaCl, 10% PEG 6000, 50 mM $NaHCO_3$, 100 µmol ABA, 100 µmol JA, 100 µmol SA, 50 µmol 2,4-D, 50 µmol ETH, or 50 µmol GA. For the temperature treatments, the seedlings were transferred to a light incubator maintained at 40 °C (high-temperature stress) or 5 °C (low-temperature stress). The nutrient solution was refreshed every 2 days. Cucumber seedling roots and leaves were collected at 0, 0.5, 1, 3, 6, 12, and 24 h post-treatment. The collected samples were promptly frozen in liquid nitrogen and stored at −80 °C for the gene expression analysis.

2.4. RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using the RNA prep pure Plant Kit (TANGEN). First-strand cDNA was synthesized following the instructions of the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). qPCR amplification was performed following the recommended protocol in the instruction manual of the Hieff® qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) in a LightCycler® 96 Instrument (Roche, Mannheim, Germany). Relative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method [28]. The primers used for gene expression analysis can be found in Table S1.

2.5. Subcellular Localization Analysis

The *CsTDC1* and *CsTDC2* coding sequences (without the stop codon) were amplified via PCR and then inserted into the pCAMBIA2300-GFP vector (digested with *Xba*I and *Sma*I) to generate the recombinant plasmids pCAMBIA2300-*CsTDC1-GFP* and pCAMBIA2300-*CsTDC2-GFP* (the primers are shown in Table S1). Next, *Agrobacterium tumefaciens* strain GV3101 cells were transformed with the recombinant plasmids or the empty pCAMBIA2300-GFP vector (negative control). The *A. tumefaciens* cells carrying pCAMBIA2300-GFP, pCAMBIA2300-*CsTDC1/2-GFP*, P19, or AtPIP2-mCherry (plasma membrane marker) were cultured with shaking until the optical density at 600 nm (OD_{600}) reached 0.6–0.8. The cells were collected and resuspended in infiltration buffer (10 mM MES-KOH, pH 5.6, 10 mM $MgCl_2$, and 100 µM acetosyringone) for an OD_{600} of 0.6. The cell solutions were then mixed in a 1:1:1 volume ratio (empty vector or recombinant plasmid:

P19: AtPIP2-mCherry) and then injected into the lower epidermis of tobacco (*Nicotiana benthamiana*) leaves. After 3 days, GFP fluorescence was observed using an SP8 MP confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.6. Transient Overexpression of CsTDC Genes in Tobacco Leaves

The *CsTDC1* and *CsTDC2* coding sequences were amplified via PCR and then inserted into the overexpression vector pCAMBIA 1305.4 to generate the recombinant plasmids pCAMBIA 1305.4-*CsTDC1* and pCAMBIA 1305.4-*CsTDC2* (the primers are shown in Table S1). *Agrobacterium tumefaciens* strain GV3101 cells were transformed with the recombinant plasmids or the empty pCAMBIA 1305.4 vector (negative control) and then cultured until the OD_{600} reached 0.6–0.8. The cells were collected, resuspended in infiltration buffer, and then mixed in a 1:1 volume ratio (empty vector or recombinant plasmid: P19) before being injected into 4-week-old tobacco leaves.

2.7. Determination of TDC Activity and Melatonin Content

The injected tobacco leaf samples were collected after 2 and 4 days of transient expression of *CsTDCs*. The TDC activity was analyzed according to the instructions of the plant tryptophan decarboxylase (TDC) ELISA kit (LOT: 231103122O), which was purchased from Jiangsu Meimian Industrial Co., Ltd. (Yancheng, China). The melatonin content was determined by Jiangsu Meimian Industrial Co., Ltd. using high-performance liquid chromatography (HPLC; LC-20AT, Shimadzu, Kyoto, Japan).

2.8. Transient Overexpression of CsTDC Genes and Abiotic Stress Treatments

After the *CsTDC* genes were transiently expressed in tobacco leaves for 2 days, the leaves were detached from the plants. The petioles were then immersed in 100 mL Erlenmeyer flasks filled with distilled water, 200 mM NaCl solution, or 0.5% PEG solution. The leaves were secured in place using a sponge. The treatments were conducted in a light incubator set at 23 °C with a 12 h light (70 μmol/m^2·s): 12 h dark cycle and 65% relative humidity. For the low-temperature treatment, the leaves were placed in a light incubator set at 0 °C. The post-treatment fresh weight was compared with the initial fresh weight to calculate the leaf water loss rate. Negative values indicated that the leaves absorbed water. The relative water content was measured as described by Hong et al. (2008) [29]. The MDA content and electrolyte leakage rate were determined as described by Li et al. (2023) [30].

2.9. Statistical Analyses

The values presented are the means ± standard deviation (SD) of three replicates. Significance analysis was conducted using Stst 1.0 software with the one-way ANOVA method and Duncan's test.

3. Results

3.1. Identification and Characterization of Cucumber TDC Genes

In this study, only two members of the *TDC* gene family were identified in cucumber, namely *CsaV3_1G036910* and *CsaV3_3G028450*, which were designated as *CsTDC1* and *CsTDC2*, respectively, indicating that the *TDC* gene family is relatively small in plants (Table 1 and Figure S1). Of the two identified genes, *CsaV3_1G036910* (*CsTDC1*) was detected on chromosome 1 and encodes a protein comprising 499 amino acids, with a molecular weight of 55.7 kDa. In contrast, *CsaV3_3G028450* (*CsTDC2*) was localized to chromosome 3 and encodes a protein consisting of 486 amino acids, with a molecular weight of 54.6 kDa. For both of the encoded proteins, the isoelectric point (pI) was below 7, reflecting their acidic nature. These proteins were predicted to be primarily localized in the cytoplasm and plasma membrane.

Table 1. Characteristics of the tryptophan decarboxylases in cucumber.

Gene ID	Length (aa)	Molecular Weight (KD)	Chromosome	Location	pI	Strand Direction	Subcellular Location
CsaV3_1G036910	499	55.7	1	22,824,145–22,827,250	6.34	–	Cytoplasm and plasma membrane
CsaV3_3G028450	486	54.6	3	24,862,649–24,886,753	5.79	–	Cytoplasm and plasma membrane

3.2. Identification of Cis-Elements in CsTDC Promoters

Cis-elements play important roles in the regulation of gene expression. To identify the putative cis-elements in the *CsTDC* promoters, a 2 kb sequence of the promoter region of each gene was analyzed using the PlantCARE database. Some of the identified cis-elements were related to development as well as responses to stress, hormones, and light (Table 2). The most abundant cis-elements (11 types) were related to light responses. Hormone-responsive cis-elements were also common among the *CsTDC* promoters. More specifically, six types of cis-elements responsive to various phytohormones, including ethylene, abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA), and auxin, were identified. The cis-elements in the *CsTDC1* promoter were related to ethylene, ABA, and SA responses. The cis-elements in the *CsTDC2* promoter were associated with ethylene, ABA, MeJA, and auxin responses. The considerable abundance of hormone-responsive elements suggested the importance of the proteins encoded by the *CsTDC* genes for hormone signal perception and transduction. In addition, cis-elements involved in environmental stress responses were also detected. For example, anaerobic induction (ARE), wound-responsive (WRE3), stress-responsive (STRE and MYB), and drought- and cold-responsive (MYC) cis-elements were identified in the *CsTDC1* and *CsTDC2* promoters. The *CsTDC2* promoter also contained the LTR cis-element related to the response to low temperatures. Thus, we hypothesized that the *CsTDC* genes may affect plant resistance to environmental stresses. Moreover, we identified four types of development-related cis-elements in the *CsTDC* promoters, implying that *CsTDC* genes may encode proteins with key roles related to plant growth and development.

Table 2. Analysis of cis-elements in *CsTDC* promoters. The numbers represent the number of repeats of each cis-element.

Cis-Element	Function	*CsTDC1*	*CsTDC2*
Stress related			
ARE	cis-element essential for the anaerobic induction	2	5
WRE3	wound response elements	1	1
LTR	cis-element involved in low-temperature responsiveness	0	1
STRE	stress response elements	4	2
MYB	stress response elements	1	3
MYC	drought- and cold-responsive elements	1	3
Hormone related			
ERE	ethylene-responsive element	1	5
ABRE	cis-element involved in abscisic acid responsiveness	1	1
CGTCA-motif	cis-element involved in MeJA-responsiveness	0	2
TCA-element	cis-element involved in salicylic acid responsiveness	1	0
TGA-element	auxin-responsive element	0	1
TGACG-motif	cis-element involved in MeJA-responsiveness	0	2
Development related			
AACA_motif	involved in endosperm-specific negative expression	1	0
circadian	cis-acting regulatory element involved in circadian control	1	0
as-1	cis-element involved in root-specific expression	0	2
CAT-box	cis-acting regulatory element related to meristem expression	0	2

Table 2. *Cont.*

Cis-Element	Function	*CsTDC1*	*CsTDC2*
Light related			
AE-box	part of a module for light response	0	1
Box 4	a conserved DNA module involved in light responsiveness	4	7
GATA-motif	part of a light-responsive element	1	0
GT1-motif	light responsive element	0	3
TCCC-motif	part of a light-responsive element	1	0
TCT-motif	part of a light-responsive element	1	0
G-Box	cis-element involved in light responsiveness	1	2
G-box	cis-element involved in light responsiveness	0	1
GA-motif	part of a light-responsive element	0	1
MRE	MYB binding site involved in light responsiveness	4	1
AAAC-motif	light responsive element	1	0

3.3. CsTDC Expression Profiles in Different Tissues

To investigate whether *CsTDC1* and *CsTDC2* influence cucumber growth and development, we analyzed the expression of these two genes in various cucumber tissues. The *CsTDC1* and *CsTDC2* expression levels varied among the examined tissues (Figure 1). The genes were most highly expressed in the seeds, followed by the roots. The lowest expression levels were detected in the old leaves. Accordingly, CsTDC1 and CsTDC2 may regulate cucumber seed development and seedling growth. The analysis of the reproductive organs, such as the male flower (MF), female flower petal (FF), ovary (O), and fruit (F), indicated that the *CsTDC1* expression level was highest in FF, followed by MF, and lowest in F. However, the *CsTDC2* expression level was highest in O, followed by F, and lowest in MF. Hence, CsTDC1 and CsTDC2 may help regulate vegetative growth and reproductive growth, with distinct but partially overlapping functions. Additional research is required to clarify their specific regulatory functions.

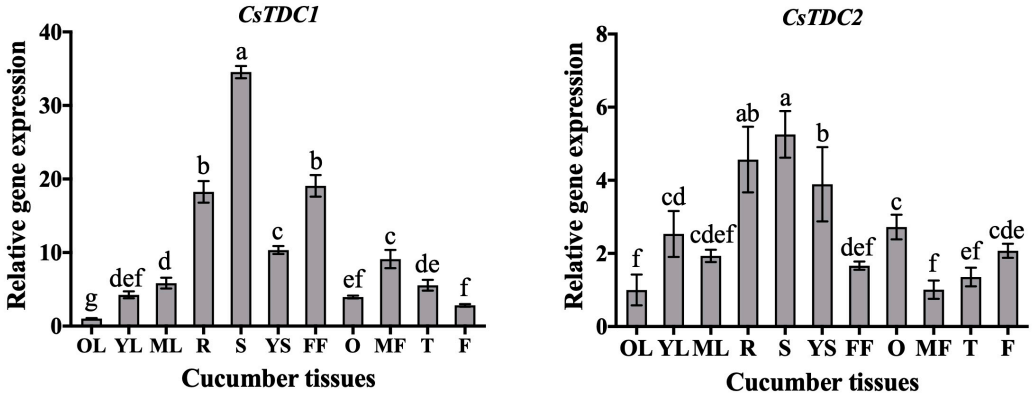

Figure 1. *CsTDC* expression levels in different tissues. A qRT-PCR analysis was performed to analyze *CsTDC* expression levels in the basal old leaves (OL), upper young leaves (YL), middle mature leaves (ML), roots (R), isolated seeds (S), young stems (YS), blooming female flower petals (FF), ovaries (O), blooming male flowers (MF), tendrils (T), and fruits (F). The expression levels in OL were set as 1. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

3.4. CsTDC Expression Profiles in Response to Various Abiotic Stresses and Exogenous Phytohormones

To determine whether *CsTDC1* and *CsTDC2* are involved in cucumber stress responses, we analyzed their expression patterns after different abiotic stress treatments, including salt (NaCl), alkali ($NaHCO_3$), heat (40 °C), cold (5 °C), and drought (polyethylene glycol; PEG) (Figure 2). The abiotic stress treatments significantly induced the expression of *CsTDC1*

and *CsTDC2*, although the expression levels varied considerably among the treatments. Notably, *CsTDC1* and *CsTDC2* expression levels were highest in the leaves and roots exposed to salt stress. In the leaves, the expression levels peaked at 12 h, with 22-fold and 32-fold increases in the *CsTDC1* and *CsTDC2* expression levels, respectively (compared with the corresponding levels at 0 h). Similarly, in the roots, the *CsTDC1* and *CsTDC2* expression levels were highest at 24 h (57-fold and 20.6-fold increases, respectively). The alkali treatment significantly increased the expression of *CsTDC2* in the leaves (up to a 13-fold increase). However, the alkali treatment had a relatively weak inductive effect on *CsTDC1* expression. Conversely, the low-temperature treatment strongly induced the expression of *CsTDC1* in the leaves (up to a 14.9-fold increase), whereas it had a weaker inductive effect on *CsTDC2* expression. The high-temperature and drought treatments substantially increased the *CsTDC1* expression levels in the leaves and roots as well as the *CsTDC2* expression level in the leaves, but they only weakly induced the transcription of *CsTDC2* in the roots. These findings suggest that *CsTDC1* and *CsTDC2* are responsive to these five abiotic stresses and may have a significant role in regulating cucumber stress tolerance. However, their specific regulatory functions remain to be precisely characterized.

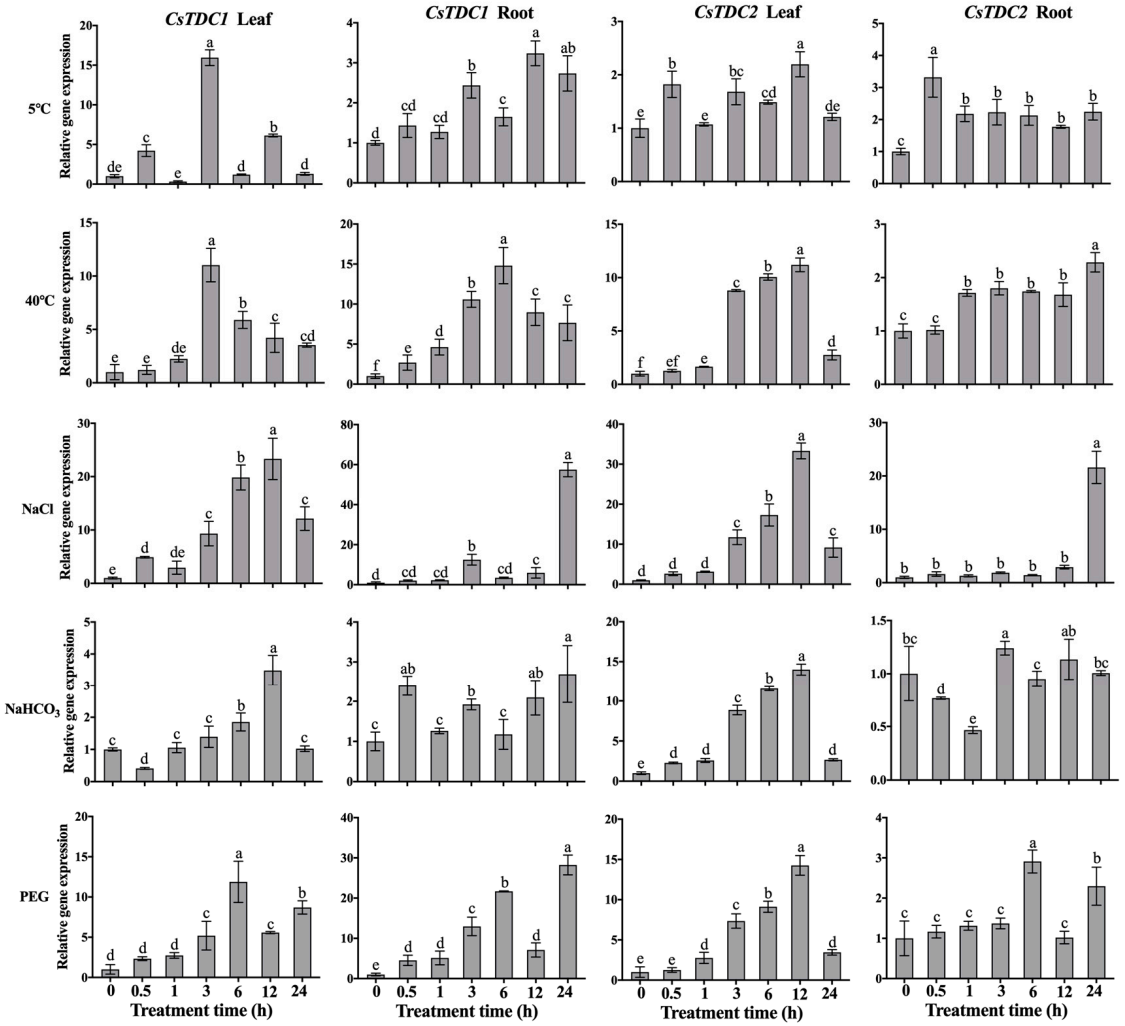

Figure 2. Analysis of *CsTDC* expression following abiotic stress treatments. The gene expression levels under non-stressed conditions were set as 1. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

Phytohormones are essential regulators of plant growth, development, and stress resistance. To investigate the regulatory effects of phytohormones on *CsTDCs*, we examined

the *CsTDC1* and *CsTDC2* expression profiles in response to six different phytohormones (Figure 3). All of the hormone treatments significantly stimulated the expression of *CsTDC1* and *CsTDC2*, but the jasmonic acid (JA) treatment had the strongest inductive effect, increasing the *CsTDC1* expression level in the roots by 1277 times. The ABA treatment considerably increased the expression of *CsTDC1* and *CsTDC2* in the leaves (by up to 184 fold and 159 fold, respectively). Similarly, the SA and ethephon (ETH) treatments strongly induced the expression of *CsTDC1* in the leaves and roots as well as the expression of *CsTDC2* in the leaves. However, they did not result in substantial increases in *CsTDC2* expression in the roots. Moreover, the gibberellin (GA) and 2,4-dichlorophenoxyacetic acid (2,4-D) treatments increased the expression of *CsTDC1* in the roots (by up to 34.6 times and 14.6 times, respectively) and the expression of *CsTDC2* in the leaves (by up to 62 times and 22 times, respectively). These results suggest that CsTDCs may be crucial for sensing hormones as well as for the subsequent hormone-related responses in cucumber.

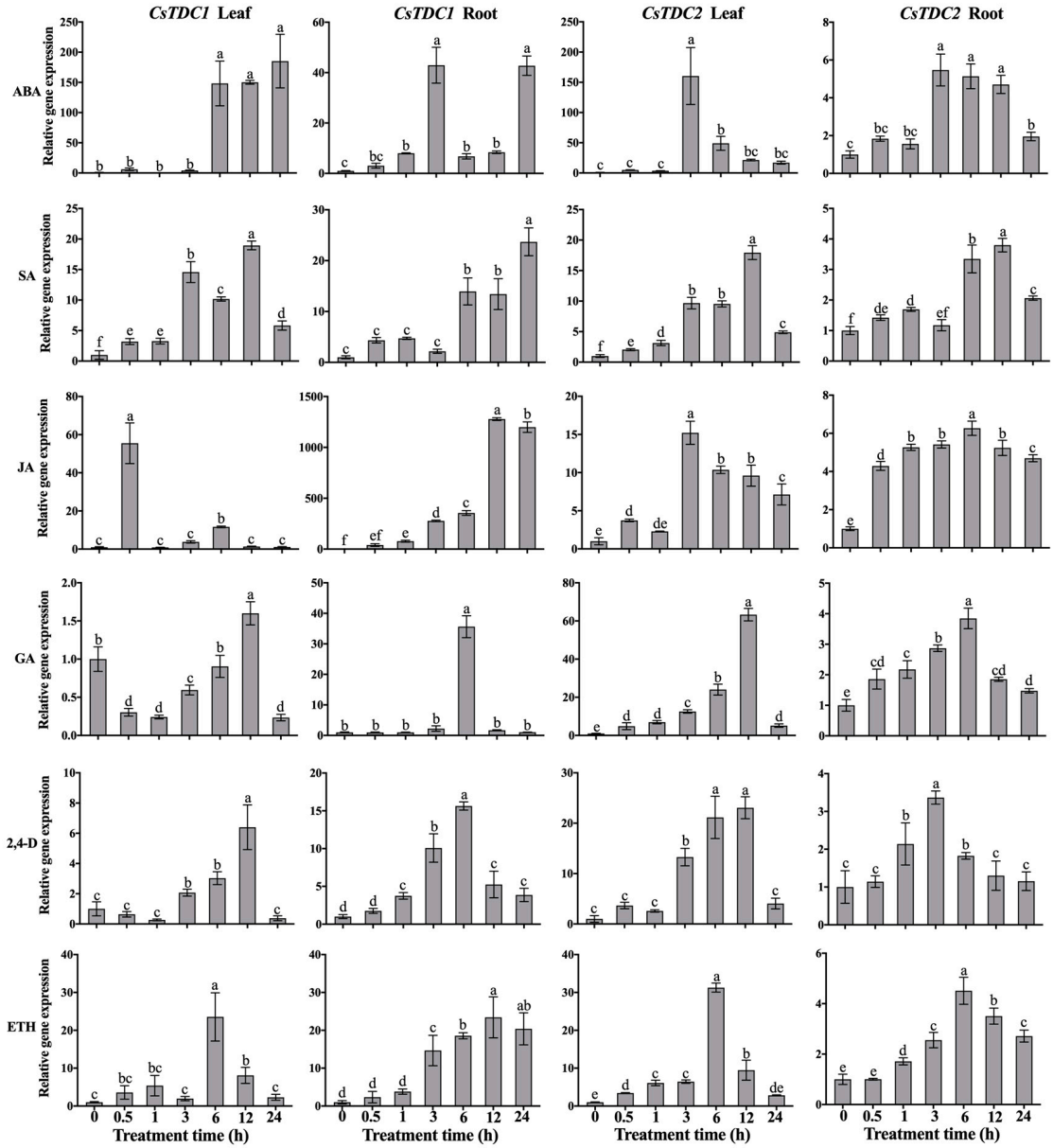

Figure 3. Analysis of *CsTDC* expression following exogenous phytohormone treatments. The gene expression levels before the treatments (0 h) were set as 1. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

3.5. Subcellular Localization of CsTDC1 and CsTDC2

Both CsTDC1 and CsTDC2 were predicted to be primarily located in the cytoplasm and plasma membrane. To verify this localization, the *CsTDC1* and *CsTDC2* coding sequences were fused with the *GFP* sequence (Figure 4A) and then transiently expressed in tobacco leaves. The green fluorescence emitted by the CsTDC1-GFP and CsTDC2-GFP fusion proteins overlapped with the red fluorescence of the plasma membrane marker protein AtPIP2-mCherry (Figure 4B). Additionally, fluorescence was also observed in the cytoplasm, confirming the localization of CsTDC1 and CsTDC2 in both the plasma membrane and cytoplasm, which was in accordance with the predicted subcellular localization of CsTDC1 and CsTDC2. Therefore, it is possible that CsTDC1 and CsTDC2 contribute to signal perception and transduction.

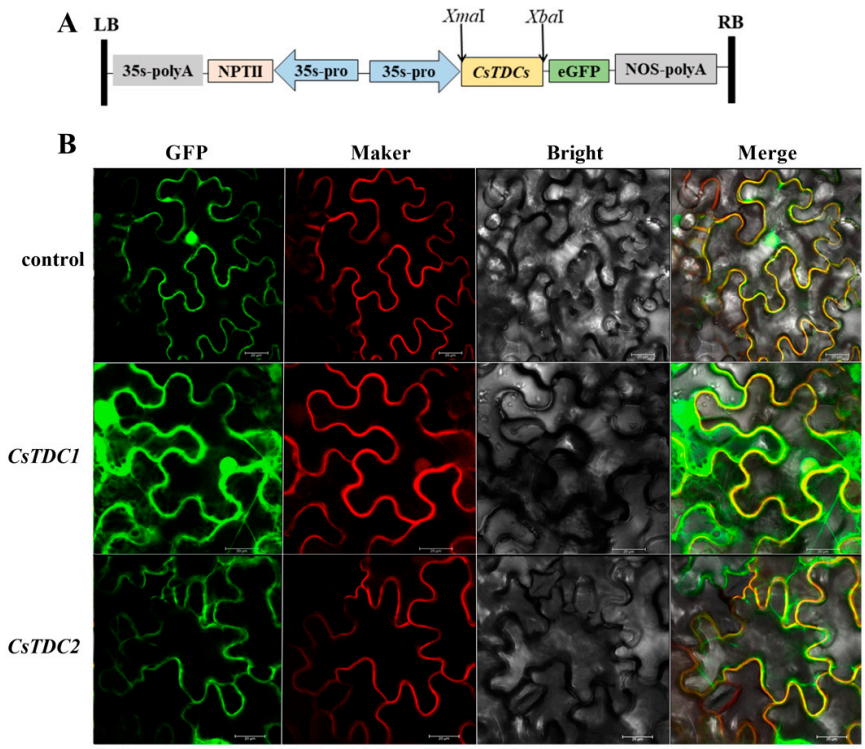

Figure 4. Subcellular localization of CsTDC1 and CsTDC2. (**A**) Plasmid used for the subcellular localization assay. (**B**) Subcellular localization of CsTDC1 and CsTDC2 in tobacco leaf epidermal cells. Bars = 20 μm.

3.6. Transient Overexpression of CsTDC Genes in Tobacco Leaves Promoted Melatonin Biosynthesis

To investigate the potential roles of CsTDC1 and CsTDC2 in the melatonin synthesis pathway and their other biochemical functions, we transiently overexpressed *CsTDC1* and *CsTDC2* (Figure 5A) in tobacco leaves and then analyzed the TDC activity and melatonin content. The tobacco leaves transformed with the empty pCAMBIA 1305.4 vector served as the negative control. The transient overexpression of *CsTDC1* and *CsTDC2* increased the TDC activity and melatonin content in the tobacco leaves (Figure 5B,C), with significantly greater increases in the leaves overexpressing *CsTDC2* than in the leaves overexpressing *CsTDC1*. Hence, both CsTDC1 and CsTDC2 may promote the synthesis of melatonin.

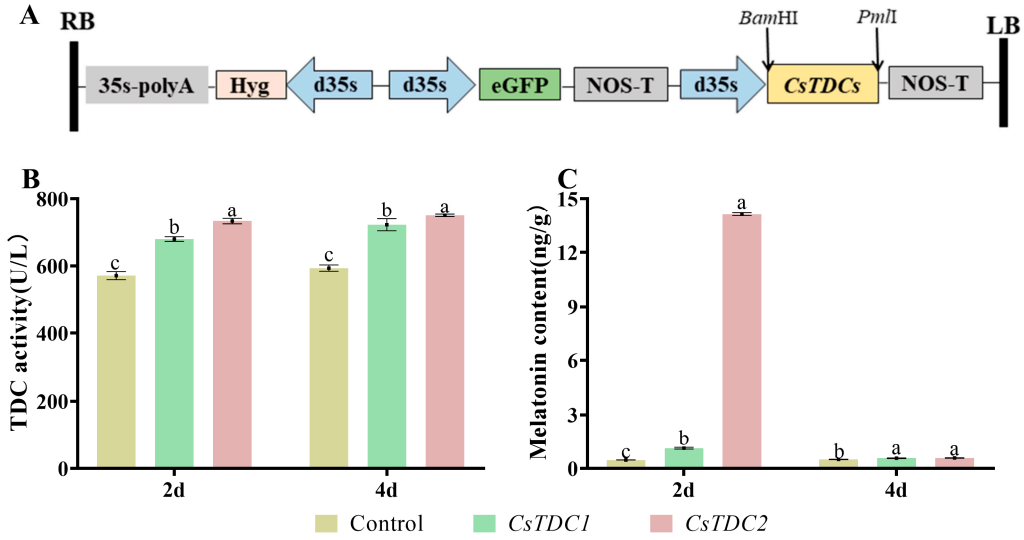

Figure 5. Effects of the transient overexpression of *CsTDC* genes in tobacco leaves on the TDC activity and melatonin content. (**A**) Plasmid used for the transient overexpression assay. The empty pCAMBIA 1305.4 vector (control) and the recombinant plasmids carrying *CsTDC1* and *CsTDC2* were used for the transient expression analysis involving tobacco leaves. The injected leaf tissues were collected after 2 and 4 days to determine the TDC activity (**B**) and melatonin content (**C**). Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

3.7. Transient Overexpression of CsTDC Genes Enhanced Abiotic Stress Tolerance in Tobacco

Because of the observed increases in *CsTDC1* and *CsTDC2* expression under abiotic stress conditions, tobacco leaves transiently overexpressing *CsTDC1* or *CsTDC2* were subjected to different abiotic stresses. The leaves infiltrated with the empty pCAMBIA 1305.4 vector served as the negative control. When the detached leaves were placed in distilled water (i.e., stress-free treatment), the control leaves were wilted and shrunken after 24 h (Figure 6A). Similarly, the leaves transiently overexpressing *CsTDC1* (OE1) were wilted after 26 h. However, after 36 h, the leaves transiently overexpressing *CsTDC2* (OE2) were only slightly wilted, whereas the control and OE1 leaves were severely wilted, especially the control leaves, which also had significantly shrunken edges. In addition, all of the detached leaves initially absorbed water, but the control leaves started losing water after 12 h, while the OE1 leaves began losing water after 24 h. Surprisingly, the OE2 leaves were still actively absorbing water even at 36 h (Figure 6B). After 36 h, the electrolyte leakage rate (Figure 6C) and malondialdehyde (MDA) content (Figure 6D) were significantly lower for the OE2 leaves than for the control leaves. The electrolyte leakage rate of the OE1 leaves was also significantly lower than that of the control leaves, but there was no significant difference in the MDA content. These findings indicate that the overexpression of *CsTDC1* and *CsTDC2* in tobacco leaves can lead to increases in water absorption and retention, but the overexpression of *CsTDC2* may have a greater effect than the overexpression of *CsTDC1*.

When the detached leaves were placed in the NaCl solution, the control and OE1 leaves were slightly wilted after 1 h, whereas the OE2 leaves were slightly wilted after 12 h (Figure 7A). Moreover, after 7 h, the water loss rate of the detached leaves tended to increase as the duration of the treatment increased. However, the water loss rate was significantly lower for the OE2 leaves than for the control and OE1 leaves (Figure 7B). After 36 h, the electrolyte leakage rate (Figure 7C) and MDA content (Figure 7D) of the control and OE1 leaves did not differ significantly, but both were significantly higher than those of the OE2 leaves. Hence, the overexpression of *CsTDC2* may enhance the salt stress tolerance of tobacco leaves.

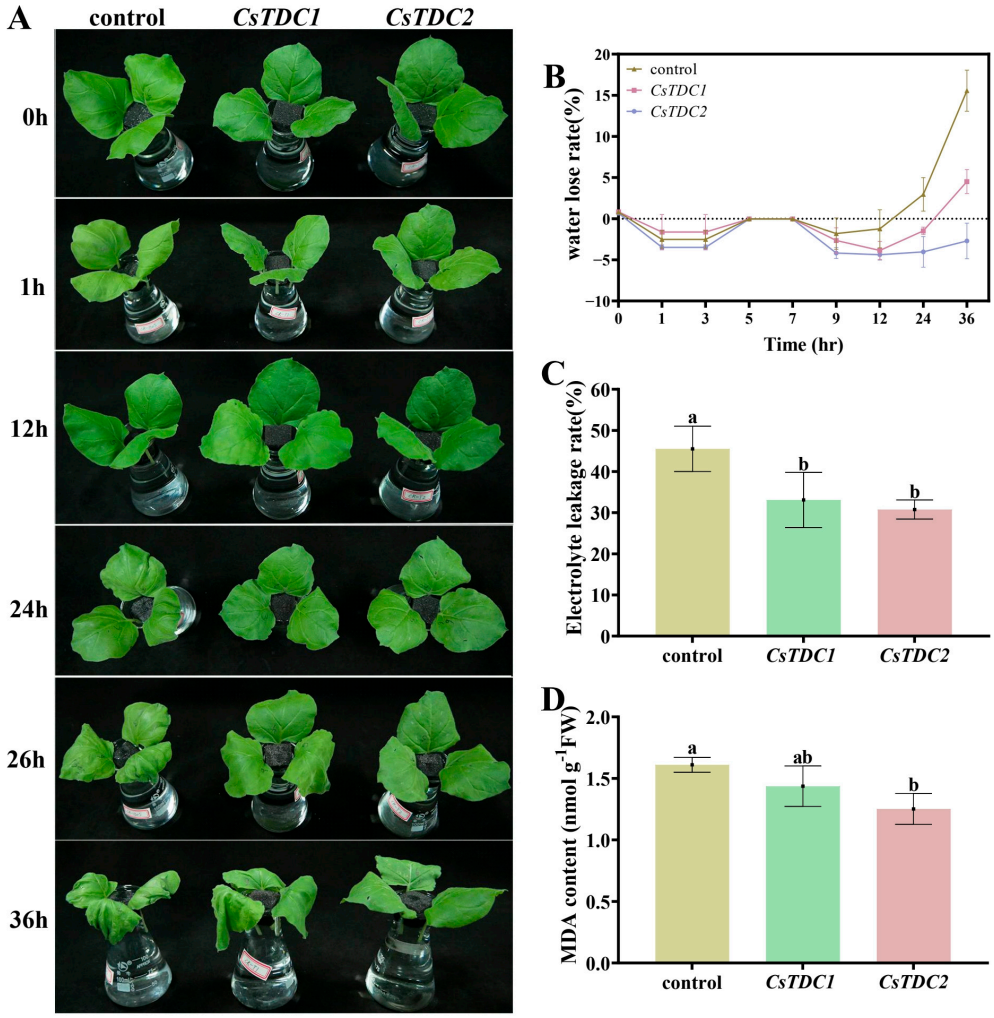

Figure 6. Physiological analysis of transgenic plants transiently overexpressing *CsTDC1* or *CsTDC2* and negative control (pCAMBIA 1305.4) plants under normal conditions. (**A**) Phenotype of detached leaves placed in a 100 mL Erlenmeyer flask containing distilled water. (**B**) Water loss rate of detached leaves. The electrolyte leakage rate (**C**) and MDA content (**D**) were measured when the detached leaves were maintained in distilled water for 36 h. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

Among the leaves placed in the PEG solution, the control leaves were slightly wilted after 1 h, whereas the OE1 and OE2 leaves wilted after 3 h (Figure 8A). However, the wilting was considerably less extensive for the OE2 leaves than for the OE1 leaves. After 24 h, the control leaves were more severely dehydrated and shrunken than the OE1 and OE2 leaves. The water loss rate of the detached leaves increased as the treatment time increased. At each treatment time-point, the water loss rate was highest for the control leaves, followed by the OE1 leaves and then the OE2 leaves (Figure 8B). The electrolyte leakage rate (Figure 8C) and MDA content (Figure 8D) were significantly lower for the OE2 leaves than for the control leaves after 24 h. Conversely, the relative water content (Figure 8E) was significantly higher for the OE2 leaves than for the control leaves. Additionally, compared with the control leaves, the OE1 leaves had a significantly lower electrolyte leakage rate, but there were no significant differences in the MDA content and relative water content. These findings indicate that the overexpression of *CsTDC1* and *CsTDC2* can improve the drought stress tolerance of tobacco leaves, but the improvement may be greater for the overexpression of *CsTDC2*.

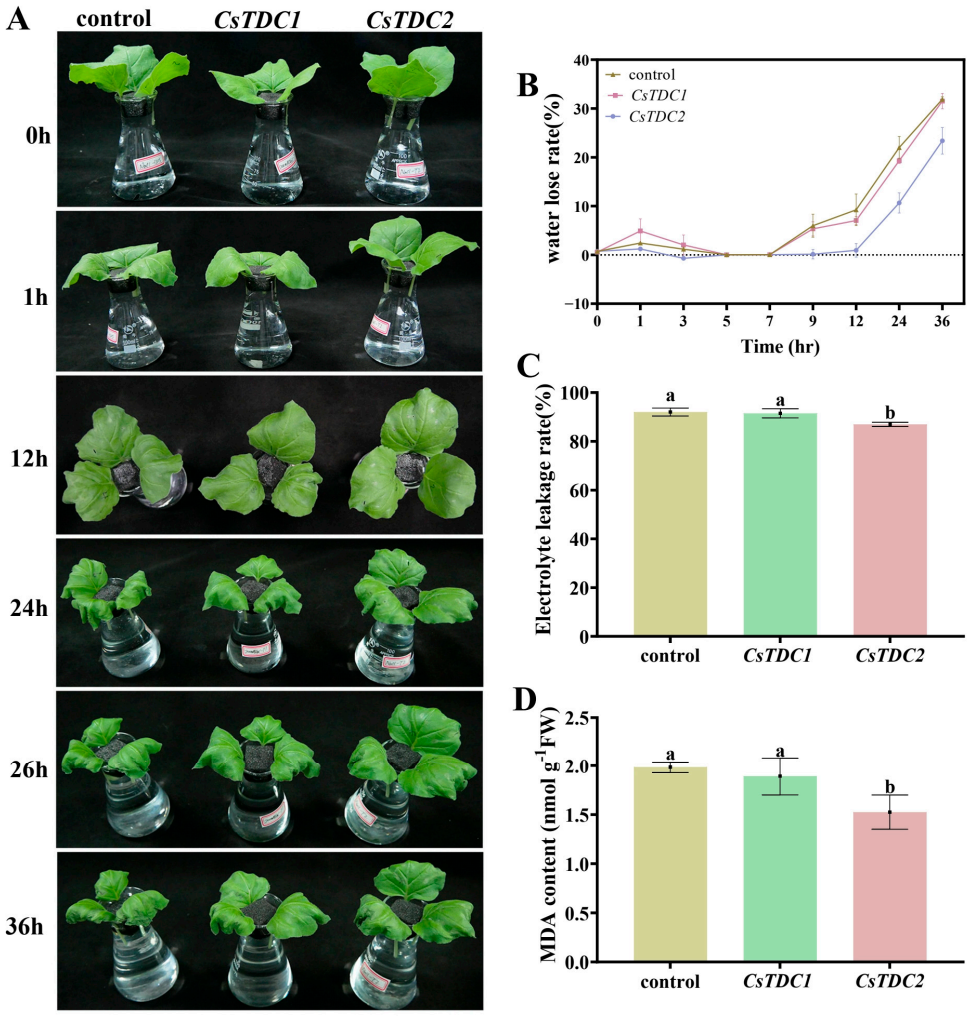

Figure 7. Transient overexpression of *CsTDC1* and *CsTDC2* increased the tolerance of tobacco to salt stress. (**A**) Phenotype of detached leaves after the NaCl treatment. (**B**) Water loss rate of detached leaves. The electrolyte leakage rate (**C**) and MDA content (**D**) were measured when the detached leaves were maintained in the NaCl solution for 36 h. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

When the detached leaves were exposed to low-temperature stress (0 °C), the control leaves started to wilt after 12 h (Figure 9A). In contrast, the OE1 leaves were curled after 24 h, but the OE2 leaves were curled only after 163 h. Notably, by 163 h, the control leaves had already lost a significant amount of water and were shrunken. During the first 36 h of the treatment period, the control leaves absorbed water. However, after 36 h, they started losing water, with an increase in the water loss rate as the treatment time increased. In contrast, the OE1 and OE2 leaves continued to absorb water throughout the treatment period (Figure 9B). After 163 h, the electrolyte leakage rate was significantly higher for the control leaves than for the OE1 and OE2 leaves (Figure 9C). Furthermore, the MDA content of the control leaves was significantly higher than that of the OE2 leaves, but it did not differ significantly from that of the OE1 leaves (Figure 9D). Accordingly, the overexpression of *CsTDC1* and *CsTDC2* can enhance the tolerance of tobacco leaves to cold conditions, but the effect of the overexpression of *CsTDC2* may be greater than that of the overexpression of *CsTDC1*.

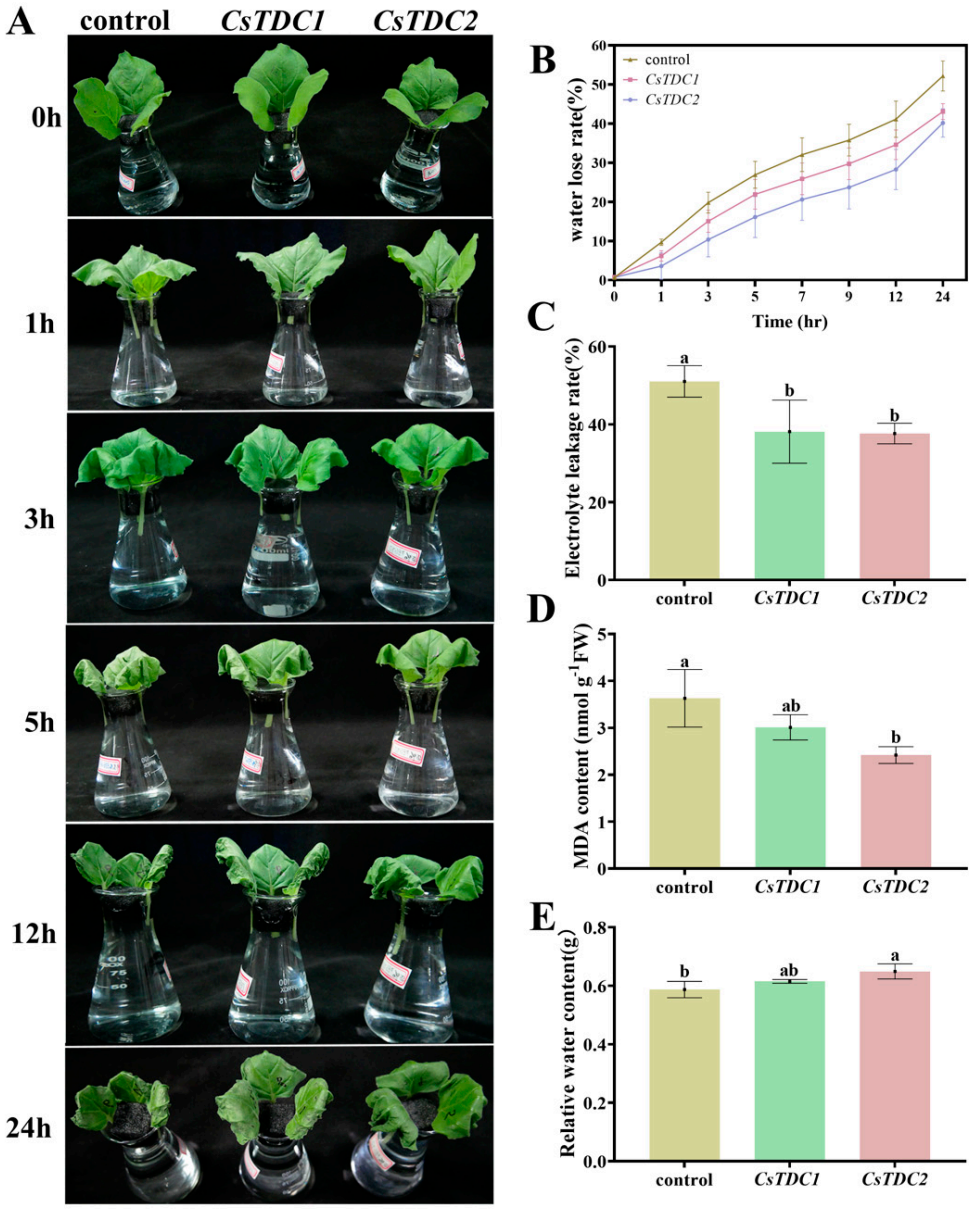

Figure 8. Transient overexpression of *CsTDC1* and *CsTDC2* increased the tolerance of tobacco to drought stress. (**A**) Phenotype of detached leaves after the PEG treatment. (**B**) Water loss rate of detached leaves. The electrolyte leakage rate (**C**), MDA content (**D**), and relative water content (**E**) were measured when the detached leaves were maintained in the PEG solution for 24 h. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

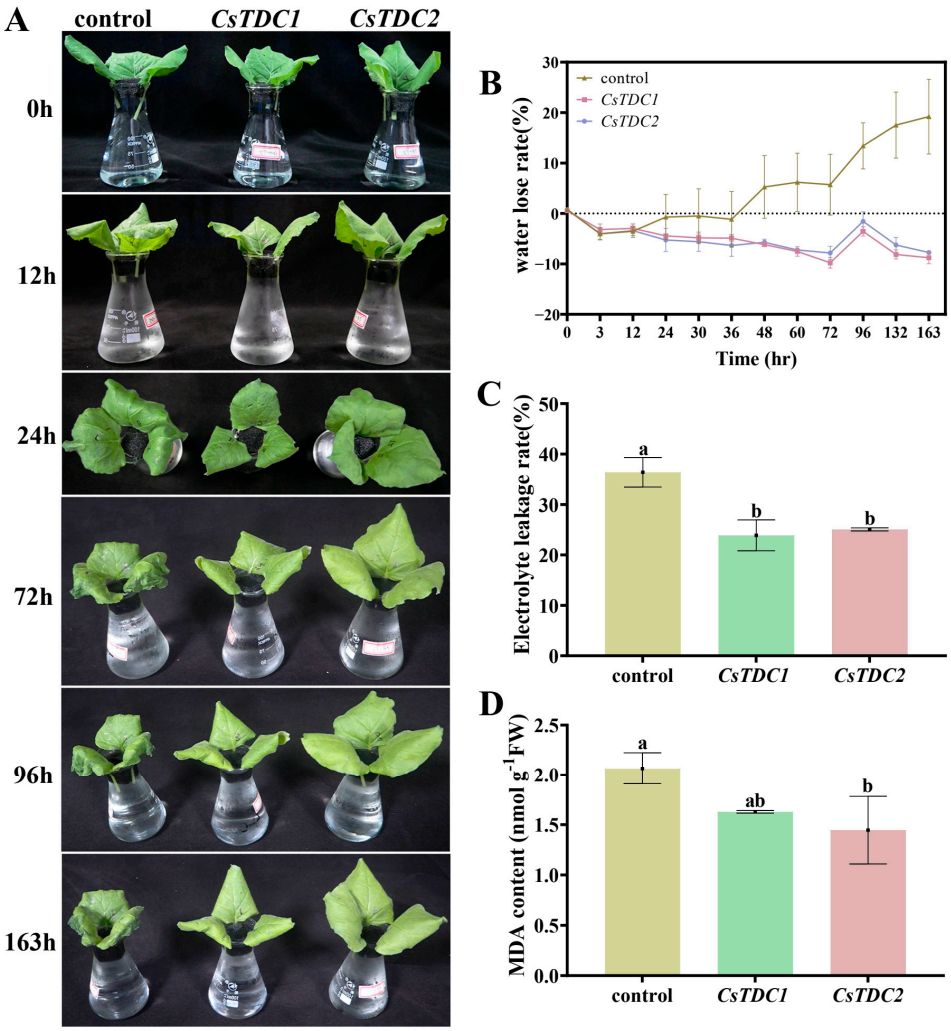

Figure 9. Transient overexpression of *CsTDC1* and *CsTDC2* increased the tolerance of tobacco to cold stress. (**A**) Phenotype of detached leaves after the cold treatment. (**B**) Water loss rate of detached leaves. The electrolyte leakage rate (**C**) and MDA content (**D**) were measured when the detached leaves were maintained at 0 °C for 163 h. Data are presented as the mean ± SD (n = 3). Different letters indicate significant differences ($p < 0.05$).

4. Discussion

Melatonin, which is a novel plant growth regulator, plays a critical role in regulating various growth and developmental processes, while also enhancing plant stress tolerance, making it a promising candidate for future agricultural applications [31]. However, in terms of its functions, endogenous melatonin has not been as thoroughly analyzed as exogenous melatonin. Therefore, the melatonin synthase-encoding genes in diverse species must be comprehensively identified and characterized regarding their regulatory effects. The biosynthesis of plant melatonin involves four main pathways; the initial reactions in all of these pathways involve tryptophan [8,9]. In most cases, tryptophan is decarboxylated by TDC to form tryptamine. Thus, TDC is considered to be the initial rate-limiting enzyme in the melatonin synthesis pathway. The final two rate-limiting steps in this pathway are mediated by SNAT and COMT. However, in plants, the *TDC* gene family is smaller than the *ASMT* [32] and *COMT* [33] gene families. Most plants contain only one *TDC* gene, but some plants, including *C. acuminata* and *Capsicum annuum*, have two *TDC* genes, while others, such as *O. sativa* and *Solanum lycopersicum* L., have three *TDC* genes [34]. A previous study showed that *CsSNAT* enhances salt tolerance and promotes growth in cucumber

seedlings because it promotes melatonin synthesis [35]. To the best of our knowledge, there are no reports regarding the cloning and characterization of the cucumber *TDC* gene(s).

In this study, we identified two homologous *TDC* genes (*CsTDC1* and *CsTDC2*) in the cucumber genome (Table 1). The subcellular localization results indicated that the CsTDC proteins are primarily located in the cytoplasm and plasma membrane (Figure 4). Tissue-specific expression analyses revealed differences in the *CsTDC1* and *CsTDC2* expression levels among the 11 examined cucumber tissues (Figure 1). The expression levels were highest in the seeds, followed by the roots and stems. The genes were expressed at relatively low levels in the leaves and reproductive organs. Hence, CsTDC1 and CsTDC2 may be important for regulating cucumber vegetative and reproductive growth processes, especially seed development and root growth. Earlier research confirmed that melatonin plays a vital role in strengthening plant stems [36]. For example, melatonin treatments of peony leaves can significantly strengthen the pedicels. In accordance with this finding, silencing the peony melatonin synthesis-related gene *PlTDC* results in a decrease in the endogenous melatonin content and weakens the pedicels. Furthermore, melatonin reportedly regulates the germination of Arabidopsis seeds by modulating the levels of endogenous hormones (e.g., ABA, GA, and IAA) [37]. Additionally, exogenous melatonin promotes the germination of cucumber seeds and stimulates lateral root growth [38]. Numerous studies have demonstrated that the application of exogenous melatonin increases the endogenous melatonin content, while the synthesis of endogenous melatonin is mediated by various enzymes, including TDC. Interestingly, the current study revealed that *CsTDC1* and *CsTDC2* are highly expressed in the seeds, roots, and stems, suggestive of the importance of TDC for multiple melatonin-mediated processes (e.g., seed germination, lateral root growth, and strengthening of the stem). However, the associated regulatory mechanisms remain to be more thoroughly investigated.

Cis-elements are crucial for regulating gene expression. In this study, we identified four types of cis-elements (i.e., related to stress, hormone, and light responses as well as development) in the *CsTDC1* and *CsTDC2* promoters (Table 2). Intriguingly, the number and diversity of the cis-elements were greater for the *CsTDC2* promoter than for the *CsTDC1* promoter. According to the qRT-PCR data, *CsTDC1* and *CsTDC2* expression levels increased substantially in response to various abiotic stresses (NaCl, $NaHCO_3$, PEG, cold, and heat) (Figure 2) as well as exogenous hormones (ABA, SA, JA, ETH, 2,4-D, and GA) (Figure 3). Notably, the JA treatment resulted in a 1277 fold increase in the *CsTDC1* expression level in the roots. The ABA treatment also significantly increased the expression of *CsTDC1* and *CsTDC2* by 184 fold and 159 fold, respectively. These results suggest that *CsTDC1* and *CsTDC2* are responsive to abiotic stresses and exogenous hormones. The encoded proteins may have distinct but partially overlapping regulatory effects on cucumber stress tolerance as well as the perception and transduction of hormone signals, particularly JA and ABA signals.

To further explore how CsTDC1 and CsTDC2 contribute to cucumber stress responses, we transiently overexpressed *CsTDC1* and *CsTDC2* in tobacco leaves, which underwent various abiotic stress treatments. On the basis of the results, the overexpression of *CsTDC1* and *CsTDC2* apparently leads to increases in the TDC activity and melatonin content in tobacco leaves (Figure 5). Moreover, it can enhance the tolerance of tobacco leaves to salt (Figure 7), drought (Figure 8), and low-temperature stresses (Figure 9). The overexpression of *CsTDC2* appears to have a more pronounced effect than the overexpression of *CsTDC1*. In addition, in the absence of stress, the detached leaves overexpressing *CsTDC1* and *CsTDC2* had a significantly lower water loss rate and were less wilted than the control leaves, implying that CsTDC1 and CsTDC2 can enhance the absorption and retention of water by tobacco leaves (Figure 6). A previous study demonstrated that ABA induces stomatal closure, thereby limiting transpiration [39]. In the current study, *CsTDC1* and *CsTDC2* were highly expressed in response to ABA, suggestive of a regulatory role in ABA-mediated stomatal closure. Earlier research indicated that low temperatures induce *TDC* gene expression in cucumber plants [40,41]. Moreover, the addition of exogenous melatonin promotes

melatonin production in cucumber and enhances seedling cold tolerance [40,41]. Similarly, the application of exogenous melatonin also increases melatonin production in loquat leaves and improves the ability of loquat seedlings to withstand drought conditions [42]. Furthermore, exogenous melatonin helps regulate the salinity–alkalinity tolerance of certain horticultural crops (e.g., grapes and tomatoes) [43,44]. Its antioxidant and free radical scavenging properties may be the primary reasons for the positive effects of melatonin on plant stress tolerance [45]. Plant growth, development, and stress tolerance are controlled by a complex process involving numerous genes, proteins, and regulatory mechanisms. Therefore, to further functionally characterize melatonin and clarify the associated gene regulatory network, additional research on endogenous melatonin is required. The present study provides preliminary evidence that *CsTDC1* and *CsTDC2* encode positive regulators of cucumber stress tolerance and establishes a theoretical foundation for future research on the roles of TDC and endogenous melatonin in cucumber and related crops.

5. Conclusions

The current study identified two *CsTDC* genes that are expressed in the vegetative and reproductive organs of cucumber, implying that they may encode regulators of cucumber growth and development. Moreover, *CsTDC1* and *CsTDC2* were highly expressed following treatments with various abiotic stressors (NaCl, $NaHCO_3$, PEG, cold, and heat) as well as exogenous hormones (ABA, SA, JA, ETH, 2,4-D, and GA), indicative of their non-specific responses to abiotic stresses and phytohormones. The transient overexpression of *CsTDC1* and *CsTDC2* in tobacco leaves enhanced the tolerance to salt, drought, and low-temperature stresses, with the overexpression of *CsTDC2* having a more significant effect than the overexpression of *CsTDC1*. These findings provide evidence of the positive regulatory effects of CsTDC1 and CsTDC2 on cucumber tolerance to abiotic stress. However, the specific regulatory mechanism(s) will need to be more precisely characterized using a cucumber genetic transformation system.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10040307/s1. Figure S1: Nucleic acid and protein sequences of CsTDC genes; Table S1: Primers used for qRT-PCR analysis, subcellular localization vector construction, and transient overexpression vector construction.

Author Contributions: Conceptualization, S.L. and S.W.; data curation, Y.Z., Q.L. and Y.L.; formal analysis, Q.L. and S.L.; writing—original draft preparation, Y.Z. and Q.L.; writing—review and editing, S.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data are contained within the article and the Supplementary Materials.

Conflicts of Interest: The authors declare no conflicts of interest.

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