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Comprehensive genomic analysis and expression profiling of the BTB and TAZ (*BT*) genes in cucumber (*Cucumis sativus* L.)

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Abstract: BTB-TAZ (BT) proteins are plant-specific transcription factors containing a BTB domain and a TAZ domain. They play vital roles in various biological processes and stress responses. In this study, a total of three *BT* genes (*CsBT1–3*) were identified from cucumber genome, and they were unevenly distributed in two of the seven chromosomes. Phylogenetic analysis of the BT proteins from cucumber, *Arabidopsis*, apple, tomato, and rice revealed that these proteins could be distinctly divided into two groups in accordance with their motif distributions. We also determined the structures of *BT* genes from cucumber, *Arabidopsis*, and rice to demonstrate their differences. The quantitative real-time PCR (qRT-PCR) results showed that the *CsBT* genes displayed differential expression patterns in cucumber tissues, and their expression was regulated by cold, salt, and drought stresses. These findings suggest that *CsBT* genes may participate in cucumber development and responses to various abiotic stresses.

Keywords: abiotic stress; BTB domain; CaMBD domain; gene expression; TAZ domain

Plants have a number of transcription factor families in their genomes, which play important roles in regulating plant growth and development by binding the promoters of target genes to activate or repress their expression (Chu *et al.* 2016; Wang *et al.* 2018b). The BTB (bric-à-brac, tramtrack and broad complex) family comprises a type of transcription factors characterized by the presence of a highly conserved BTB domain, which is also referred to as the POZ (Pox virus and zinc finger) domain (Chaharbakhshi & Jemc 2016; Li *et al.* 2018). The core BTB fold consists of five α-helices (A1 to A5) made up in part of two α-helical hairpins, and three β-strands (B1 to B3) to form a β-sheet (Stogios *et al.* 2005), which contribute to BTB proteins possess the function of protein–protein interaction, including components of an E3 ubiquitin ligase complex (Figueroa *et al.* 2005; Gingerich *et al.* 2005). Besides the BTB domain, several other domains are also present in BTB proteins, such as transcriptional adapter zinc finger (TAZ), Kelch, BTB and C-terminal Kelch (BACK), meprin and TRAF homology (MATH), and Ankyrin repeats

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(ANK), resulting in different subfamilies and diverse roles of BTB family proteins (Chaharbakhshi & Jemc 2016; Li *et al.* 2018). Among these subfamilies, BTB-TAZ (BT) proteins are only present in plants and possess an N-terminal BTB domain, a central TAZ domain and a C-terminal calmodulin binding domain (CaMBD) (Du & Poovaiah 2004; Robert *et al.* 2009).

In recent years, BT subfamily members have been identified in various plant species, including *Arabidopsis* (Du & Poovaiah 2004), rice (Gingerich *et al.* 2007), tomato (Li *et al.* 2018), and apple (Zhao *et al.* 2016; Wang *et al.* 2018a). However, only a few *BT* genes have been functionally characterised. For example, five *BT* genes (*AtBT1–AtBT5*) were identified in *Arabidopsis*, and *AtBT2* was found to be essential for female and male gametophyte development, and functions downstream of TELOMERASE ACTIVATOR1 (TAC1) to mediate the telomerase activation pathway (Ren *et al.* 2007; Robert *et al.* 2009). In apple, MdBT2 can regulate anthocyanin biosynthesis by interacting with other important transcription factors, such as MdMYB1 (Wang *et al.* 2018a), MdMYB9 (An *et al.* 2018a), MdMYB23 (An *et al.* 2018b), and MdbZIP44 (An *et al.* 2018c). Moreover, some BT members were demonstrated to play vital roles in gametophyte development (Robert *et al.* 2009), hormone and sugar signaling (Ren *et al.* 2007; Misra *et al.* 2018), leaf senescence (An *et al.* 2019), defense response (Hao *et al.* 2013; Zheng *et al.* 2019), and abiotic stress response (Mandadi *et al.* 2009; An *et al.* 2018b), as well as nutrition including iron homeostasis (Zhao *et al.* 2016), and nitrate uptake (Araus *et al.* 2016; Sato *et al.* 2017). These findings demonstrate that BT proteins have diverse roles in plant growth and developmental processes.

In the present study, we conducted a genome-wide analysis of *BT* genes in cucumber, including chromosomal localization, phylogenetic analysis, conserved domain analysis, gene structure analysis, and *cis*-element analysis in their promoters. In addition, quantitative real-time PCR (qRT-PCR) was carried out to examine the expression patterns of *BT* genes in different tissues and in response to various abiotic stresses. The results are expected to pave the way for future functional characterization of these genes in cucumber.

MATERIAL AND METHODS

Identification of *BT* genes from cucumber. The BT protein sequences of various plant species, including rice (Gingerich *et al.* 2007), *Arabidopsis* (Robert *et al.* 2009), apple (Zhao *et al.* 2016; Wang *et al.* 2018a), and tomato (Li *et al.* 2018), were used as queries to search against the cucumber genome database (http://cucurbitgenomics.org/organism/2). The resulting sequences were submitted into SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) to check for the presence of both the BTB and TAZ domains. Each candidate CsBT sequence was loaded into the online ProtParam program (http://web.expasy.org/protparam/) to analyze the molecular weight (MW), grand average of hydropathicity (GRAVY), and theoretical isoelectric point (pI).

Sequence alignment and phylogenetic analysis. The Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) was employed for the multiple sequence alignments of BT protein sequences from cucumber and other plant species, such as rice, *Arabidopsis*, apple, and tomato. A phylogenetic tree for these BT proteins was created by MEGA software (Ver. 7.0, 2016) with the neighbor-joining (NJ) method, and the bootstrap replicates were set as 1000. The information of BT proteins used to create the phylogenetic tree is listed in Table S1 in Electronic Supplementary Material (ESM).

Gene structure and motif analysis of *CsBT* genes. The sequences of coding sequence (CDS) and corresponding genomic DNA (gDNA) of *BT* genes from cucumber, rice, and *Arabidopsis* were retrieved from the cucumber genome database, the Arabidopsis Information Resource (http://www.arabidopsis.org/), and the Rice Genome Annotation Project Database (https://rice.plantbiology.msu.edu/), respectively. Then, these sequences were submitted to Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) to examine the gene structures. Motif analysis of BT proteins from cucumber, rice, and *Arabidopsis* was conducted using the online software MEME (http://meme-suite.org/tools/meme) with the following settings: number of motifs, 10; minimum width ≥ 6; and maximum width ≤ 50.

Chromosomal location and *cis*-element analysis of *CsBT* genes. The chromosomal location of each *CsBT* gene was obtained from the cucumber genome database and visualized by the MapInspect software as previously described (Zhou *et al.* 2018). The 1000 bp promoter sequences of *CsBT* genes were downloaded from the cucumber genome database and the *cis*-elements were examined by using the PlantCARE program (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Plant materials and growth conditions. A cucumber inbred line (*Cucumis sativus* var. *sativus* line 9930) was grown in soil within a plant growth chamber under the conditions of 16/8 h (day/night) at 22–24°C. For

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tissue expression profiling, a total of six tissues were collected from cucumber plants on day 7 after flowering, including roots, stems, leaves, male flowers, female flowers, and fruits. For abiotic stress study, 2-week-old cucumber seedlings were exposed to different abiotic stresses including cold, salt, and drought treatments as described in a previous report (Zhou *et al.* 2018). The leaf tissues were sampled at continuous intervals of 0, 3, 6, and 12 h with three biological triplicates, and all samples were immediately frozen in liquid nitrogen and kept at –80°C until use.

RNA isolation and quantitative real-time PCR (qRT-PCR). Total RNA was isolated with RNA prep Pure Plant Kit (TransGen, China), and the concentration was quantified by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, USA). After the removal of genomic DNA contamination, about 3 μg RNA was then reverse-transcribed into cDNA with the TransScript First-Strand cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). qRT-PCR was conducted in triplicate on an ABI 7500 Real-Time PCR System using a FastStart Universal SYBR Green Master (ROX) kit (Roche Diagnostics). The reaction program was as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. The *CsAct3* gene was used as an internal control, and the relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001). Gene specific primers are presented in Table S2 in ESM. The statistical analysis was performed using SPSS software, and data were statistically analyzed using analysis of variance (ANOVA) and a *P*-value < 0.05 was considered as significant with Tukey's test.

RESULTS

Identification of *BT* genes in cucumber. A genome-wide search against the cucumber genome database using the *Arabidopsis* and rice BT proteins revealed that the cucumber genome contained three BT members that possess both the BTB and TAZ domains, and they were designated as *CsBT1*–3 (Table 1). The Protparam tool analysis showed that the three putative *CsBT* genes harbored open reading frame (ORF) ranging from 1101 bp (*CsBT1*) to 1392 bp (*CsBT3*), which encoded proteins ranging from 366 to 463 amino acids in length, with MWs ranging from 42.21 to 53.69 kDa, GRAVYs from –0.343 to –0.322, and theoretical pIs from 9.03 (CsBT2) to 9.66 (CsBT3) (Table 1).

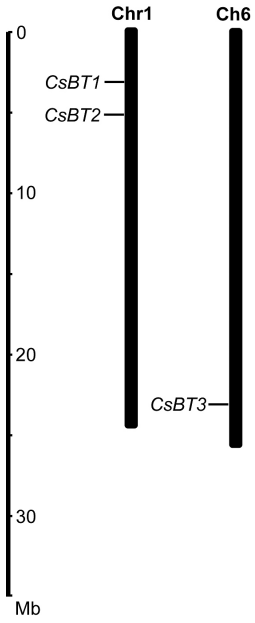

Figure 1. Locations of the three *CsBT* genes on cucumber chromosomes

The three *CsBT* genes were distributed in two of the seven chromosomes in the cucumber genome, with two (*CsBT1* and *CsBT2*) on chromosome 3 and one (*CsBT3*) on chromosome 6, respectively (Figure 1).

Phylogenetic relationships of BT proteins. To study the phylogenetic relationships of CsBT proteins, a phylogenetic tree was created by a multiple sequence alignment of 21 BT proteins from cucumber and other plant species including *Arabidopsis*, apple, tomato, and rice. As shown in Figure 2, these BT proteins could be divided into two phylogenetic groups named as Group I and II, which contained 12 and 9 proteins, respectively. As for the three CsBT proteins, CsBT1 and CsBT2 were grouped in Group I,

Table 1. The basic characterizations of all identified *BT* genes in cucumber

Gene	Gene ID	Genomic position	gDNA (bp)	ORF (bp)	length (aa)	pI	MW (kDa)	GRAVY
CsBT1	Csa1G032450.1	Chr1: 3451337 .. 3453508 (–)	2172	1101	366	9.32	42.21	–0.343
CsBT2	Csa1G049460.1	Chr1: 5650939 .. 5653299 (–)	2361	1182	393	9.03	44.71	–0.333
CsBT3	Csa6G495100.1	Chr6: 23975583 .. 23978468 (–)	2886	1392	463	9.66	53.69	–0.322

gDNA – genomic DNA; bp – base pair; ORF – open reading frame; aa – amino acid; MW – molecular weight; pI – isoelectric point; GRAVY – grand average of hydropathicity

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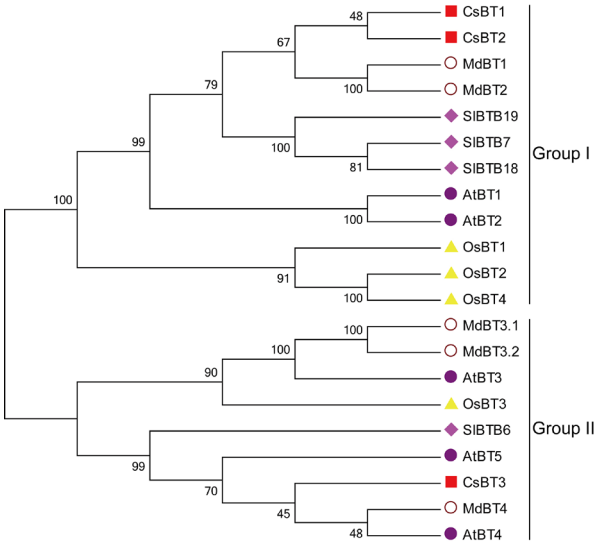

Figure 2. Phylogenetic analysis of BT proteins from cucumber and other plant species

The phylogenetic tree was constructed by the NJ method using MEGA 7.0 software with 1000 replicates on each node; the information of proteins used to create the phylogenetic tree is listed in Table S1 in ESM

while CsBT3 fell into Group II. All the CsBT proteins were clustered with members of dicots, especially apple BT proteins (Figure 2), suggesting that BT proteins are evolutionarily conserved.

Conserved domain analysis of cucumber BT proteins. The amino acid sequence alignment showed that the BT proteins from cucumber, *Arabidopsis* and rice had sequence identities of 32.59–73.58% (Figure 3). Besides, all the BT proteins contained an N-terminal BTB domain, a C-terminal TAZ and a CaMBD domain, which were highly conserved (Figure 3).

To obtain further insights into the structures of the cucumber BT proteins, the MEME online tool was used to identify the conserved motifs of BT proteins from cucumber, *Arabidopsis* and rice. A total of 10 motifs were obtained (designated as motif 1–10, Figure 4). Motifs 5, 2 and 1 composed the BTB domain, and motifs 8, 4, 6, 3, and 7 were annotated as the TAZ domain (Figure 4). These motifs were widely distributed in all the BT proteins, except for OsBT2, which was lack of motif 4, and CsBT3, which harbored an additional motif 4 in its N-terminus. It

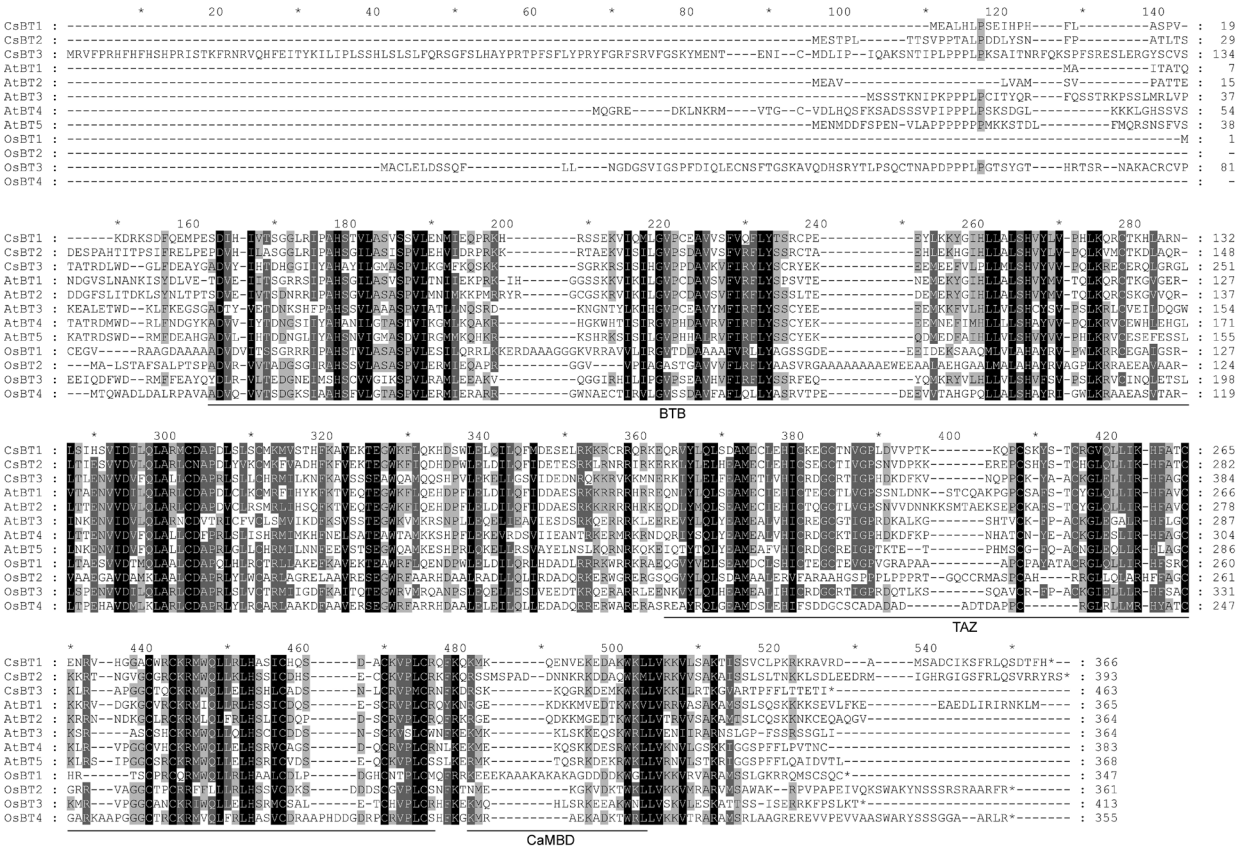

Figure 3. Alignment of BT protein sequences from cucumber, *Arabidopsis* and rice

The BTB, TAZ and CaMBD conserved domains are underlined

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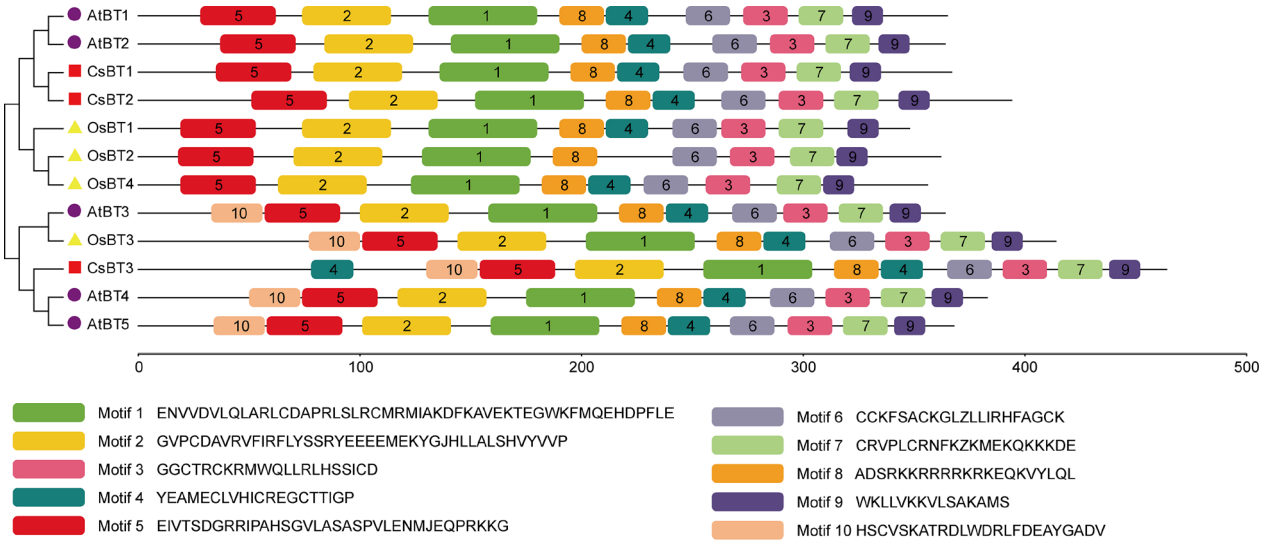

Figure 4. Conserved motifs of BT proteins from cucumber, *Arabidopsis* and rice based on their evolutionary relationships
The relative positions of the 10 identified motifs are indicated by boxes with different colours

is noteworthy that motif 10 was specifically found in Group II BT proteins (Figure 4).

Structural analysis of cucumber *BT* genes. GSDS was employed to investigate the structures of *BT* genes from cucumber, *Arabidopsis* and rice. As shown in Figure 5, the numbers of introns and exons in the *BT* gene family were highly conserved, as all *BT* genes possessed 3–4 introns, with the exception of CsBT3, which contained six introns. In addition, some genes clustered together had similar numbers and lengths of CDSs, although they had variable lengths of introns and untranslated regions (UTRs), such as *AtBT1*/*AtBT2*, *OsBT2*/*OsBT4*, and *AtBT4*/*AtBT5* (Figure 5).

Promoter region analysis of cucumber *BT* genes. To understand the putative functions of cucumber *BT* genes, the promoter regions (1 kb upstream of ATG site) of *CsBT* genes were used to identify *cis*-elements by using PlantCARE program. A total of 25 types of *cis*-elements were detected in the promoters of *CsBT* genes, many of which were related to development, stress, and hormone (Table S3 in ESM, Figure 6). Seven stress-related *cis*-elements were detected, including heat stress responsive elements (HSE), MYB binding site involved in drought and stress (MBS), defense- and stress-responsive elements (TC-rich repeats), WRKY binding site involved in abiotic stress and defense response (W-box), elicitor-responsive element (EIRE), wound-responsive element (WUN-motif), and anaerobic induction element (ARE), suggesting that *CsBT* genes may be

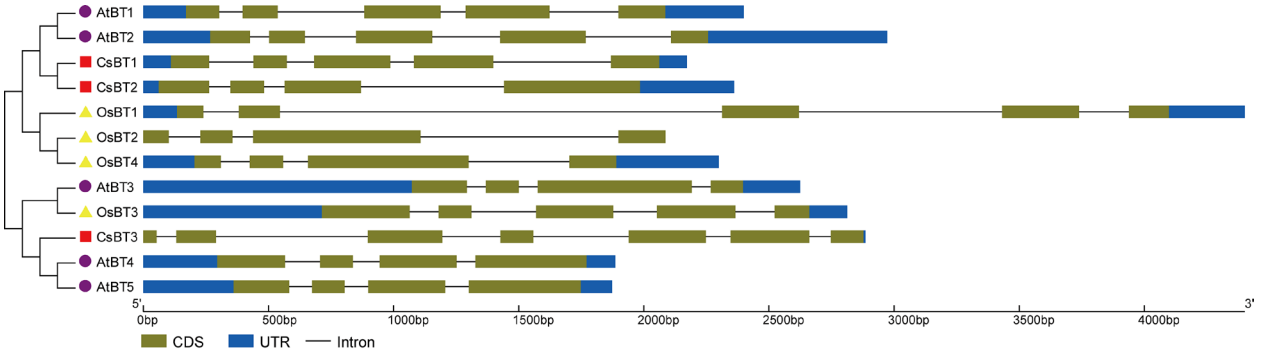

Figure 5. Gene structures of *BT* genes from cucumber, *Arabidopsis* and rice according to phylogenetic analysis
The untranslated regions (UTRs) and coding sequences (CDSs) are indicated by blue and brown boxes, respectively; the introns are represented by black lines

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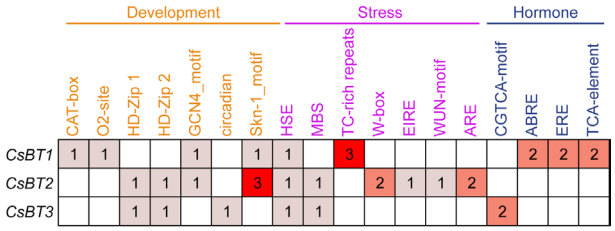

Figure 6. Numbers of development-, stress- and hormone-related *cis*-elements in the promoter regions of cucumber *BT* genes

regulated by various stresses. In addition, seven types of development-related *cis*-elements were found to be widely distributed in the promoters of *CsBT* genes, implying that *CsBT* genes may also participate in regulating plant growth and development. Moreover, the promoter region of *CsBT1* harbored three kinds of hormone-related *cis*-elements, including ABRE, ERE, and TCA elements, and that of *CsBT3* carried two CGTCA motifs. These four *cis*-elements were suggested to be responsive to abscisic acid (ABA), ethylene, salicylic acid (SA) and methyl jasmonate (MeJA), respectively (Cao *et al.* 2016).

Expression analysis of *CsBT* genes in different tissues of cucumber. qRT-PCR analysis was performed to evaluate the transcript levels of *CsBT* genes in six different tissues of cucumber, including roots, stems, leaves, male flowers, female flowers, and fruits. As a result, *CsBT1* was highly expressed in roots, followed by leaves and fruits, and had the lowest expression in stems (Figure 7A). *CsBT2* showed the highest transcript level in stems, while a very low transcript level in roots (Figure 7B). *CsBT3* showed the highest and lowest transcript levels in fruits and stems, respectively (Figuer 7C). These results indicated that the three *CsBT* genes have differential transcript levels in different tissues of cucumber.

Expression patterns of *CsBT* genes under various abiotic stresses. To elucidate the expression patterns of *CsBT* genes in response to various abiotic stresses, qRT-PCR analysis was performed to examine their transcript levels in leaves of cucumber seedlings under cold, salt, and drought treatments. Upon cold treatment, all the *CsBT* genes exhibited obviously decreased expression levels (Figure 8A). The expression of *CsBT2* and *CsBT3* showed no obvious changes under salt treatment, but that of *CsBT1* notably declined at 3 h, followed by an observable increase at 6 h (Figure 8B). Under drought treatment, the expression levels of all *CsBT* genes

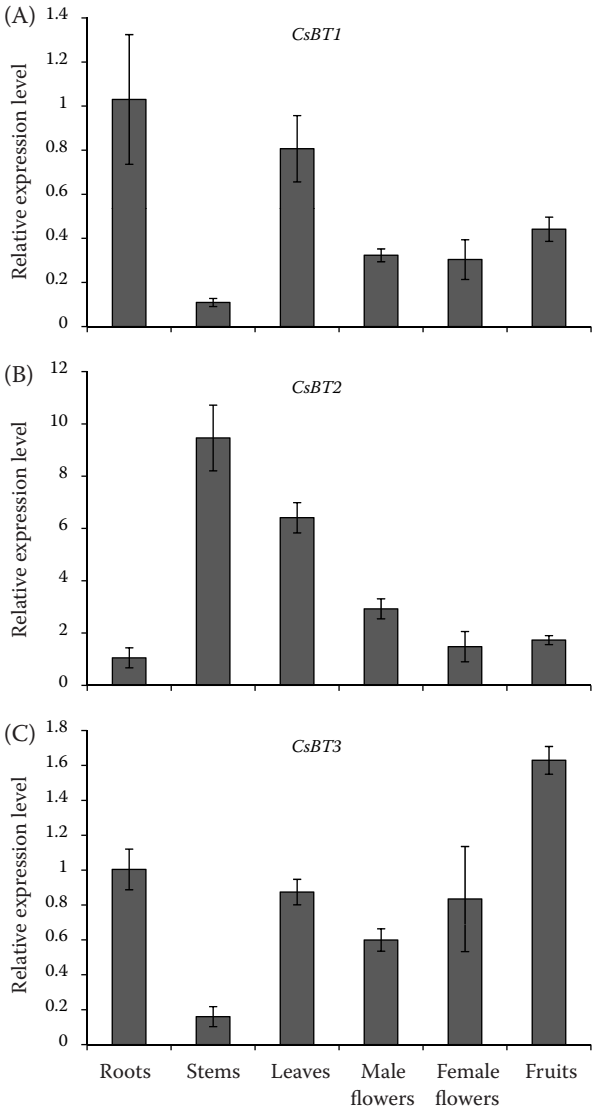

Figure 7. Tissue-specific expression levels of *CsBT1* (A), *CsBT2* (B) and *CsBT3* (C) genes in cucumber based on qRT-PCR analysis; RNA samples were collected from roots, stems, leaves, male flowers, female flowers, and fruits
The error bars represent standard deviation of three biological replicates

showed significant decreases at certain time points (Figure 8C). These results suggested that *CsBT* genes may play important roles in response to various abiotic stresses.

DISCUSSION

In the present study, a comprehensive set of three *BT* genes were identified in cucumber, which were unevenly distributed in two of the seven chromosomes in cucumber genome (Table 1 and Figure 1).

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The number of *BT* genes in cucumber is smaller than that of *Arabidopsis* (five genes) (Robert *et al.* 2009), rice (four genes) (Gingerich *et al.* 2007), apple (five genes) (Zhao *et al.* 2016), and tomato (four genes) (Li *et al.* 2018), indicating that *BT* gene family has not been expanded in cucumber.

A phylogenetic analysis of BT proteins from cucumber, *Arabidopsis*, apple, tomato, and rice revealed that these BT proteins can be divided into two groups (Group I and II), and members from the same species tended to cluster together because of their high degrees of similarity, especially in Group I (Figure 2), implying that BT proteins in Group I from the same species have overlapping or redundant functions. For example, AtBT1 and AtBT2 act as conserved negative regulators to influence nitrate uptake under low nitrate conditions by down-regulating the major components of the high affinity nitrate transport system in *Arabidopsis* (Araus *et al.* 2016). In addition, although the overall amino acid sequence identities were not high among BT proteins from cucumber, *Arabidopsis*, and rice, a much higher sequence identity was observed in BTB and TAZ domains of them (Figure 3), and MEME analysis revealed that all of these BT proteins possess the conserved BTB and TAZ domains (Figure 4), indicating that the two conserved domains have important functions for BT proteins. BTB and TAZ were known as two versatile protein-protein binding domains involved in interaction with various transcription factors. Previous studies have shown that apple MdBT2 can directly interact with its target proteins (such as MdCUL3, MdbHLH104, MdMYB1, MdMYB9, MdMYB23, MdbHLH93, and MdbZIP44) by using the BTB or TAZ domain, and therefore mediate the degradation of the target proteins through the 26S proteasome pathway (Zhao *et al.* 2016; An *et al.* 2018a, b, c, 2019; Wang *et al.* 2018a). In view of the high sequence identities of CsBT1 and CsBT2 to MdBT2, it can be speculated that CsBT1 and CsBT2 can also recruit and degrade the target proteins to regulate their expression.

The exon-intron distribution can be considered as an imprint of evolution in a gene family, which can provide extra evidence to reveal the phylogenetic relationship of the gene family from different organisms (Xu *et al.* 2012; Bai *et al.* 2016). In this study, the majority of *BT* genes from cucumber, *Arabidopsis* and rice had 3–4 introns (Figure 5), indicating a low structural diversity of *BT* genes in these plants. In line with the results of phylogenetic analysis, *BT* genes clustered together usually exhibited similar gene structures, such as intron number and exon length (Figure 5). However, some *BT* genes have a variable number of introns, especially *CsBT3*. We thus speculate that both exon loss and gain occurred during the evolution of the *BT* gene family, such as *CsBT1*/

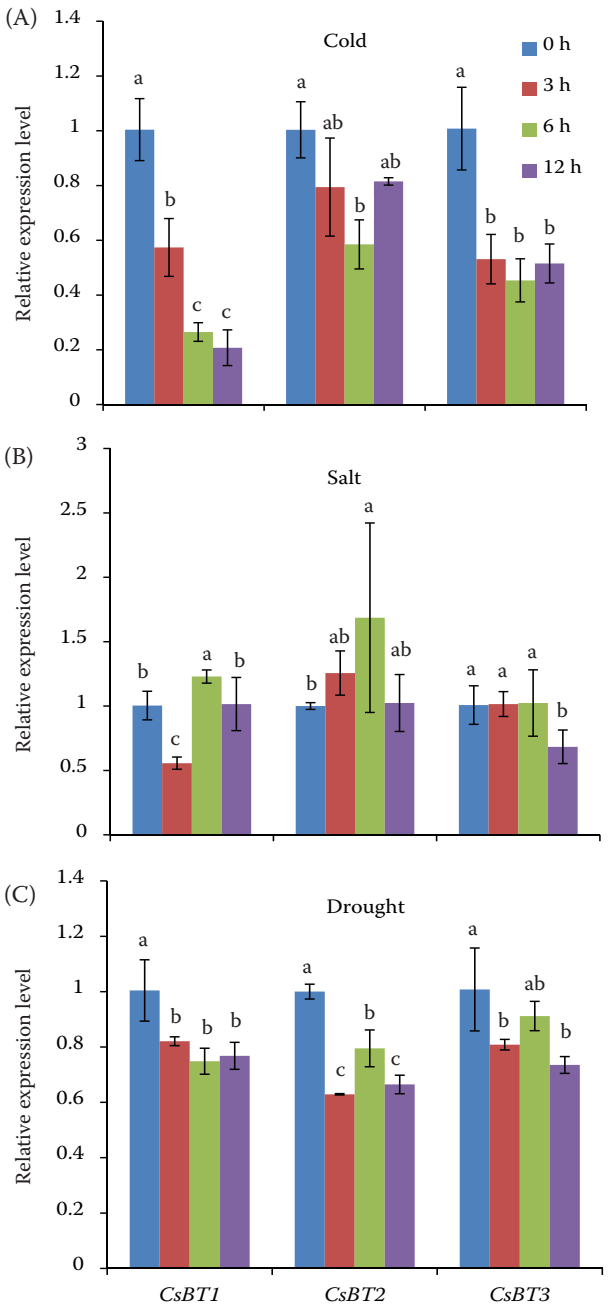

Figure 8. Transcript levels of *CsBT* genes under various abiotic stresses of cold (A), salt (B), and drought (C)
Bars represent the mean values ± SD based on three biological replicates; different letters above the bars indicate significant differences using Tukey's test ($P < 0.05$)

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CsBT2, *OsBT1*/*OsBT2*/*OsBT4*, and *AtBT3*/*OsBT3*/*CsBT3* (Figure 5).

Previous reports have shown that plant *BT* genes display various expression patterns in different tissues and can play crucial roles in plant growth and development (Robert *et al.* 2009; Li *et al.* 2018). We thus investigated the expression of the three *CsBT* genes in various tissues of cucumber. The results showed that the *CsBT* genes displayed differential expression patterns among cucumber tissues. For example, *CsBT1* and *CsBT3* displayed relatively higher transcript levels in roots (Figure 7), suggesting that they may play a role in root development of cucumber. Tomato *SlBTB6* and *SlBTB18* are also highly expressed in roots (Li *et al.* 2018). In *Arabidopsis*, simultaneous disruption of *AtBT1* and *AtBT2* can affect nitrate-dependent lateral root development (Araus *et al.* 2016; Sato *et al.* 2017). In addition, all the *CsBT* genes were expressed in leaves (Figure 7), which is consistent with the expression of *AtBT* genes, whose expression was found to be the highest in rosette leaves (Robert *et al.* 2009). Additionally, the *CsBT* genes were also expressed in male and female flowers (Figure 7). Similarly, all tomato *BT* genes (*SlBTB6*, *SlBTB7*, *SlBTB18* and *SlBTB19*) are highly expressed in flowers (Li *et al.* 2018), implying their similar roles in flower development. The transcripts of *AtBT4* and *AtBT5* were found to be the most abundant in flowers, and *AtBT2* was essential for female and male gametophyte development (Ren *et al.* 2007; Robert *et al.* 2009). Moreover, the promoter of *CsBT3* possesses a *cis*-element involved in circadian rhythm (Figure 6), suggesting that its expression may be controlled by circadian clock. Similarly, the expression of *AtBT2* was regulated diurnally and controlled by the circadian clock (Mandadi *et al.* 2009). The differential expression patterns indicated different functions of *CsBT* genes in various tissues of cucumber.

In recent years, increasing reports have revealed that *BT* genes are involved in stress responses (Araus *et al.* 2016; Mandadi *et al.* 2009; An *et al.* 2018b). Our analysis of *cis*-elements revealed that a number of hormone- and stress-related *cis*-elements are present in the promoter regions of *CsBT* genes (Figure 6), implying that *CsBT* genes may be involved in multiple hormone and stress responses. However, the expression levels of *CsBT* genes were inhibited in response to cold, salt, and drought stress (Figure 8), indicating that they may act as negative regulators of plant response to abiotic stresses. Similarly, the expression of *AtBT2* was induced by H_2O_2, while was decreased by ABA and cold treatments (Mandadi *et al.* 2009). In apple, MdBT2 is also repressed by ABA and cold stress, and it can negatively modulate cold tolerance by promoting the degradation of MdMYB23 protein (An *et al.* 2018b). In addition, the expression of some *BT* members from *Arabidopsis*, rice and apple is significantly regulated by carbon and nitrogen nutrients (Araus *et al.* 2016; An *et al.* 2018c). Therefore, it is likely that *CsBT* genes may also participate in the regulation of plant response to abiotic stresses.

CONCLUSIONS

In summary, three *CsBT* genes were identified from the cucumber genome. Based on the results of phylogenetic analysis, the *BT* genes from different plant species were classified into two groups. The qRT-PCR results showed that the *CsBT* genes were differentially expressed in cucumber tissues, implying that they play specific roles in cucumber development. In addition, the *CsBT* genes were highly responsive to abiotic stresses such as cold, salt, and drought. The results of our study reveal that the *CsBT* genes are involved in cucumber growth and development, as well as abiotic stress response, which can lay a basic foundation for further functional characterization of *CsBT* genes under cold, salt, and drought stresses in cucumber.

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