Genome-Wide Characterization of B-BOX Gene Family and Their Responses to Light Quality and Cold Stress in Tomato

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Abstract

Background: Perceiving incoming environmental information is critical for optimizing plant growth and development. Multiple B-box proteins (BBXs) play essential roles in light-dependent developmental processes in plants. However, whether BBXs function as a signal integrator between light and temperature in tomato plants remains elusive.

Results: In this study, 31 *SlBBX* genes were identified from the newly released tomato (*Solanum lycopersicum*) genome sequences, and were clustered into five subgroups with phylogenetic analysis. Gene structure and protein motif analyses showed relatively high conservation of closely clustered *SlBBX* genes within each subgroup; however, genome mapping analysis indicated the uneven distribution of the *SlBBX* genes on tomato chromosomes. Synteny analysis indicated that segmental duplication events happened in the expansion of the *SlBBX* genes in tomato. Promoter cis-regulatory elements prediction indicated that *SlBBX* genes were highly responsive to light, hormones, and stress conditions. Furthermore, the transcript analysis revealed that various *SlBBX* genes differed significantly in expression after exposure to different light quality and low temperatures, while 61.3% of *SlBBX* genes were responsive to both light and low temperatures.

Conclusions: Our study presented a genome-wide survey of *SlBBX* gene family in tomato, and emphasized their functions in perceiving light quality and low temperature, which may improve the current understanding of *SlBBX* gene functions in integrating light and temperature signals for plant adaptation to adverse environments.

Background

The B-box (BBX) proteins represent a unique class of zinc-finger transcription factors (TFs) that possess single or double B-box domains in their N termini and a CCT (CO, CO-like and TOC1) domain in their C termini in some cases [1]. The B-box domains are of two classes, and each of them coordinates two zinc atoms [2]. The dissimilarities in the consensus sequences of the two B-box domains are the results of evolution through the segmental duplication and deletion events[3]. Studies suggest that the highly conserved CCT domain is important for transcriptional regulation and nuclear transport [4]. Furthermore, the valine-proline (VP) motif of six amino acids (G-I/V-V-P-S/T-F) contained by some BBX proteins, plays a crucial role in the interaction with CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) [5, 6]. Based on the domain structures, 32 BBX proteins are divided into five subfamilies in Arabidopsis [1, 3].

A variety of wavelength-specific photoreceptors are involved in perceiving the light signals in plants, including phytochromes (phys), cryptochromes (CRYs), phototropins (PHOTs), ZEITLUPE family members, and UV-B resistance locus 8 (UVR8) [7, 8]. Light-activated photoreceptors inhibit the COP1-SUPPRESSOR OF PHYA-105 (SPA) E3 ubiquitin ligase complex, which functions for the degradation of the positive regulators of photomorphogenesis [7, 8]. Notably, HY5, a target of COP1-mediated protein degradation, plays a vital role in light-regulated plant growth and development [9, 10]. Thus, the light-
dependent regulation of COP1–HY5 mediates the plant developmental transition from dark to light. Recent studies have demonstrated that numerous BBX proteins, along with COP1 and HY5, play critical roles in light-dependent development in plants.

Upon light irradiation, BBX21 directly binds to $BBX22$, $HY5$ and its own promoter regions and activates their transcription [11–13]. Moreover, both BBX21 and HY5 can associates with the $BBX11$ promoter to promote its transcription, while BBX11 binds to the $HY5$ promoter to activate its transcription [14]. Thus, these three TFs (BBX21, HY5 and BBX11), regulate the transcription, forming a positive feedback loop that precisely regulates plants photomorphogenesis. Moreover, BBX20, BBX21, BBX22 and BBX23 interact with HY5 to increase its transcriptional activity toward the target genes [15–17], whereas BBX24, BBX25, BBX28 and BBX29 suppress photomorphogenesis by reducing the activity of HY5 [17–20]. The HY5 positively controls $BBX22$ at the transcriptional level [21], whereas it represses the transcription of $BBX30$ and $BBX31$ by binding to the promoters of these two genes [22, 23]. Meanwhile, BBX30 and BBX31 promote the expression of $BBX28$ and $BBX29$ by directly binding to the promoter regions of these genes [20]. In addition, direct interactions between BBX32 and BBX21 lead to inhibition in the BBX21-HY5 [24]. Interestingly, BBX4, accumulated after exposure to red light, directly interacts with phyB to promote photomorphogenesis in Arabidopsis [25]. These mechanisms are also found in other plant species. For example, OsBBX14 induces Os$HY5L1$ gene expression to stimulate photomorphogenesis in rice [26]. MdBBX37 associates with Md$HY5$ promoter to inhibit its expression in apple [27]. Additionally, MdBBX22 and MdBBX25/MdCOL4 bind to the Md$HY5$ promoter to increase and decrease the transcriptional activation of Md$HY5$, respectively [28]. Both PpBBX16 and PpBBX18 interact with Pp$HY5$ to increase the biochemical activity of Pp$HY5$, while Pp$HY5$ binds to the promoter region of $PpBBX18$ to promote the transcription of $PpBBX18$ in pear [29, 30]. Furthermore, the interaction of PpBBX21 with Pp$HY5$ and PpBBX18 affects the bioactive heterodimers formation of Pp$HY5$-PpBBX18 [30]. Therefore, specific BBXs and HY5 constitute an important regulatory network to precisely control normal plant growth and development.

In darkness, CO/BBX1, BBX4, BBX10, BBX19, BBX20, BBX21, BBX22, BBX23, BBX24, BBX25, BBX28 and BBX29 are ubiquitinated by COP1 and subsequently degraded by the 26S proteasome system [11, 16, 18–20, 22, 31–33]. Moreover, BBX2-9 and BBX13-16 interacts with COP1 in vitro, indicating a role for COP1 in controlling the stability of these proteins in darkness [33]. Nevertheless, COP1 preferentially stabilizes BBX11 instead of promoting its degradation [14], which suggests that COP1 likely regulates a yet unidentified protein degrading BBX11.

BBX proteins also act vital roles in regulatory networks that control plant adaption to abiotic stress. Previous studies show that both BBX5 and BBX21 positively regulate plants tolerance to drought and salt stress in Arabidopsis [34, 35]. BBX24/STO directly interacts with H-protein promoter binding factor 1 (HPPBF-1), which is a salt-responsive MYB TF, to enhance the root growth and salt tolerance in Arabidopsis [34]. CmBBX22 also positively regulates the plant drought tolerance [36]. In addition, MdBBX10 enhances tolerance to salt and drought by modulating ABA signaling and ROS accumulation [37]. In Arabidopsis, BBX18 and BBX23 control thermomorphogenesis [38]. Both Md$BBX20$ and Ma$COL1$
are responsive to low temperatures in apple and banana, respectively [39, 40]. Furthermore, ZFPL, a homologous gene of \textit{AtBBX32}, enhances cold tolerance in the grapevine [41]. \textit{CmBBX24} also increases plant cold tolerance in \textit{Chrysanthemum} [42]. However, whether SIBBXs are involved in light and cold response in tomato remains to be explored.

In the present study, 31 \textit{SIBBX} genes were identified and characterized in tomato. Gene distribution, synteny analyses, the architecture of exon-intron and motifs differences were investigated. Furthermore, the three-dimensional structure and evolution of SIBBX proteins were performed. Promoter analysis showed that the \textit{cis}-elements in light signaling, hormones and stress response were the main elements of \textit{SIBBXs} promoters. Meanwhile, we found that multiple \textit{SIBBX} genes were either up-regulated or down-regulated in response to different light quality and low temperatures, and 61.3\% of \textit{SIBBX} genes were responsive to both light and low temperatures. Therefore, our results suggest that SIBBXs are an essential component of light and temperature cues, which function to integrate environmental stimuli and plant hormones to coordinate plant growth under low temperatures.

**Results**

**Identification and characterization of SIBBX Genes in tomato**

Based on the gene annotation as well as the conserved B-box motif characteristic of the BBX members, a total of 31 \textit{SIBBX} genes were identified. The detailed information [gene name, gene identifiers, chromosome location, theoretical isoelectric point (pI), molecular weight (MW), genomic, coding sequences (CDS), peptide length, number of exon and intron, instability and aliphatic index, the grand average of hydropathicity (GRAVY) values and subcellular localization] of each \textit{SIBBX} was presented in Table 1. The lengths of CDS and amino acids (AA) of 31 SIBBXs range from 267 bp and 88 aa (\textit{SIBBX18}) to 1428 bp and 475 aa (\textit{SIBBX27}), respectively. Thus, varied MW and pI were observed among SIBBX proteins. The MW of SIBBXs varies from 9.57 (SIBBX18) to 53.14 kDa (SIBBX27). The pI ranged from 4.25 (SIBBX5 and SIBBX7) to 9.28 (SIBBX26), with 74.2\% SIBBXs with a pI lower than seven, which indicated that most of the SIBBX proteins were acidic in nature. The pI ranged from 4 to 9 in SIBBX proteins contained one (single) or two (double) B-box domains, while it decreased when plus a CCT domain, especially in the SIBBX proteins with a B-BOX domain plus a CCT domain (Additional file 1: Figure S1), which suggested that the CCT domain in SIBBX proteins may decrease their pI. Majority of SIBBXs were grouped into unstable proteins because their instability index was greater than 40, except for \textit{SIBBX6} in this family (Table 1). The predicted aliphatic index ranged from 50.05 to 97.43 in SIBBX proteins. All SIBBX proteins, with the exception of SIBBX18, were predicted to be hydrophilic due to the GRAVY value (< 0). Subcellular localization predicted that most SIBBXs (23 of 31) were localized in the nuclear region, five of them, including SIBBX5, SIBBX6, SIBBX17, SIBBX25 and SIBBX31 in the chloroplast, while SIBBX16 and SIBBX18 in the cytoplasm, SIBBX19 in the peroxisome (Table 1). In
addition, none of the 31 BBX proteins have a transmembrane domain, which indicated that these SIBBX proteins were not located on the cell membrane (Additional file 1: Figure S2).

### Protein sequences, phylogenetic analysis and three-dimensional structure of SIBBXs

The domains logos and the sequences of the B-box1, B-box2 and CCT domain of the SIBBX proteins are shown in Fig. 1. Eight members out of the 31 SIBBXs, were characterized by the occurrence of two B-box domains and also a conserved CCT domain, whereas four members of them had a valine-proline (VP) motif (Table 2). Only two B-BOX domains were found in ten SIBBXs, whereas five members had one B-box domain and also a CCT domain, and eight members had only one B-box domain (Table 2). Among the three domains, we found that each tomato B-box motif contained approximately 40 residues with the consensus sequence C-X2-C-X8-C-X2-D-X4-C-X2-C-D-X3-H-X8-H (Fig. 1). The conserved C, C, D and H residues ligated two zinc ions [2]. Additionally, the consensus sequence of the conserved CCT domain was R-X5-R-Y-X-E-K-X3-R-X3-K-X2-R-Y-X2-R-K-X2-A-X2-R-X-R-X-K-G-R-F-X-K (Fig. 1).

To better reveal the evolutionary relationships, we generated a phylogenetic tree based on the 32 AtBBXs and 31 SIBBXs (Fig. 2). All sequences were clustered into five subfamilies according to the phylogenetic analysis and previous article [2]. The BBX genes in clade I (group 1) had two concatenated B-box domains, a CCT domain and a VP motif except for SIBBX1 and SIBBX2, which did not have a VP motif and a CCT domain. The members of group II were characterized by two B-box domains and also a CCT domain with the only exception for SIBBX7, which contained two B-box domains only, and SIBBX8 and SIBBX10, which only had one B-box domain and a CCT domain. In the group III, all the members contained one B-box domain as well as a CCT domain. The group IV and V possessed two and one B-box domain, respectively; nonetheless, SIBBX27 that contained two B-box was also grouped into V. Additionally, BBX proteins from two species showed scattered distribution across the branches of the evolutionary tree, which implies that the duplication events occurred after the lineages diverged.

Protein structural features are crucial for understanding the biological properties as well as the evolutionary origins of proteins. Here, we found that most members of SIBBX proteins in a subfamily had a similar three-dimensional structure (Fig. 3). In addition, we found physical connections in each protein sector in the tertiary structure. Moreover, a distinct functional role, and an independent mode of sequence divergence in the protein family, reflected the evolutionary histories of the conserved biological properties of BBX proteins.

### Gene structure, conserved motifs, chromosomal localization and synteny analysis of SIBBXs

The evolution of multigene families can be driven by gene structural diversity. Examination of the genomic DNA sequences revealed that most SIBBXs contained one to five introns, while SIBBX16,
**SlBBX17** and **SlBBX30** had no introns (Fig. 4b and Table 1; Additional file 1: Figure S3). Among them, nine SlBBXs had one intron, followed by ten SlBBXs with two introns, five SlBBXs with three exons, four SlBBXs with four exons, and one SlBBXs with five introns. Generally, members of each subclass, which are most closely related, exhibited analogous exon-intron structures. For instance, the members in group I and V had one to two, and zero to one intron, respectively (Fig. 4a and 4b; Additional file 1: Figure S3). However, a few SlBBX genes showed dissimilar exon-intron arrangements. For instance, SlBBX18 and SlBBX19 had high sequence similarity, but SlBBX18 and SlBBX19 contained two and five introns, respectively (Fig. 4a and 4b; Additional file 1: Figure S3). These divergences indicated that both the gain and loss of introns during evolution, may better explain the functional diversity of SlBBX homologous genes.

To further examine the structural features of SlBBXs, the conserved motif distributions were analyzed. Twenty conserved motifs were predicted (Fig. 4c), while multilevel consensus sequences and the E-value of them were shown in Additional file 2: Table S1. The results showed that motif 17 was the largest motif depending on the width, followed by motif 8 and motif 2 (Additional file 2: Table S1). Motif 1 was found in all the SlBBXs (Fig. 4c). Notably, 74.2% and 70.1% SlBBXs contained motif 4 and motif 3, respectively. Motif 2 was unique to the group I, II and III of SlBBXs, while motif 5 was unique to group II except for the SlBBX27. Motif 10 was found only in group III of SlBBXs. Our results showed that members that are most closely related in the phylogenetic tree contained common motifs on the basis of alignment and position, which indicated that they may have a similar biological function.

Chromosomal locations showed that 31 SlBBX genes were unevenly distributed on the 12 chromosomes (Fig. 5a). A maximum number of SlBBX genes were found on chromosome 12 (Chr12), comprising of six genes. Five genes were located on Chr2 and Chr7. Four and three SlBBX genes were located on Chr5 and Chr4, respectively. Both Chr1 and Chr6 contained two members of SlBBX genes, whereas only one gene was detected on Chr3, 8, 9 and 10. Additionally, no SlBBX genes were found on Chr11.

To examine the duplication of SlBBX genes, the MCScanX program was used. Thirty-six pairs of SlBBXs were identified as segmental duplication in the tomato genome (Fig. 5b). Chr2, 7 and 12 had more duplication regions, which partially explain the greater numbers of SlBBX genes that were located on these three chromosomes. Although SlBBX1 and SlBBX3 were located on the same chromosome (Fig. 5a), and their sequence identity was 83% (Additional file 1: Figure S4), they were not tandem duplication. To further examine the evolutionary relationships between SlBBXs and AtBBXs, a synteny analysis was performed with MCScanX software. A total of 16 of SlBBX-AtBBX orthologous pairs were identified (Fig. 5c), which indicated the existence of numerous SlBBX genes prior to the divergence of Arabidopsis and tomato. Some members of SlBBXs were not localized in the syntenic block, suggesting that these genes might have certain specificity due to their evolution time.

**Analysis of cis-elements in the promoter region of SlBBXs**

Transcription factors directly bind the cis-elements in regulatory networks controlling gene expression; therefore, analysis of the putative cis-elements is critical to examine the expression of SlBBX genes. A
total of 61 major cis-elements were predicted from the PlantCARE database (Fig. 6a), including 22 light responsive, 12 hormone responsive, 11 stress responsive and 16 development. The number of light responsive cis-elements was the largest in the promoters of 31 SIBBX genes (Fig. 6b). The number of cis-elements in the promoters of SIBBX17 and SIBBX2 was the largest and least, respectively. The major light responsive elements contained box4 (21%), G-box (17.9%) and CMA1a/2a/2b (14.3%), which were located on 87.1% (27/31), 83.9% (26/31) and 96.8% (30/31) of SIBBXs promoters, respectively (Fig. 6c). The most common motif were the JA-responsive elements (MYC), abscisic acid (ABA)-responsive element (ABRE), and ethylene-responsive element (ERE), accounting for 24.8%, 21.5% and 17.2% of the scanned hormone responsive motifs, respectively. The stress responsive elements MYB, STRE (stress-related elements) and WUN were located on 96.8% (30/31), 90.3% (28/31) and 77.4% (24/31) of 31 SIBBX genes promoters, respectively. In the development category, various growth and development related elements, such as AT-rich element (19.2%), O₂-site for zein metabolism regulation (13.7%), CAT-box for meristem expression (12.3%), GCN4_motif required for endosperm expression (9.6%), were found. Our ndings suggest that the promoter regions of SIBBX genes that contained the cis-elements played a critical role in the light and stress responses.

**SIBBX genes expression in response to different light quality**

To assess whether light signaling regulates SIBBXs, we investigated the gene expression of SIBBXs in tomato plants grown at dark (D), white (W) and different light quality [purple (P), blue (B), green (G), yellow (Y), red (R), and far-red (FR)] conditions. In comparison with D, light decreased the transcripts of SIBBX1, SIBBX8, SIBBX10 and SIBBX12, while it increased the transcripts of SIBBX7, SIBBX13 and SIBBX15 (Fig. 7). Plants grown at R light conditions showed higher expression of SIBBX4, SIBBX14, SIBBX23, SIBBX24 and SIBBX29 than those grown at other light qualities. Whereas FR light significantly up-regulated the transcripts of SIBBX7, SIBBX13, SIBBX15, SIBBX21, SIBBX25, SIBBX26 and SIBBX27, it obviously down-regulated the transcripts of SIBBX14, SIBBX16, SIBBX18, SIBBX24, SIBBX28, SIBBX30 and SIBBX31 (Fig. 7). Transcripts of SIBBX16, SIBBX17, SIBBX18, SIBBX30 and SIBBX31 were induced, while transcripts of SIBBX5, SIBBX6, SIBBX19, and SIBBX20 were inhibited in plants when grown at B light conditions. SIBBX15 was induced by G light irradiation at 6 h, whereas SIBBX9 and SIBBX28 were repressed (Fig. 7). Y light led to an obvious reduction in expression of SIBBX9 and SIBBX31. Obviously, the P light increased the expression of SIBBX3, SIBBX5, SIBBX6, SIBBX15, SIBBX19, SIBBX20, SIBBX21, SIBBX26 and SIBBX27, but decreased the expression of SIBBX10 and SIBBX16. Interestingly, SIBBX4, SIBBX23 and SIBBX29 were only responsive to R light, while SIBBX7, SIBBX13 and SIBBX25 were induced just in response to FR light. Meanwhile, R light induced the expression of SIBBX14 and SIBBX24, but FR light inhibited their expression (Fig. 7). In general, the results showed that SIBBX genes might act a critical role in response to light quality signaling.
Expression pattern of the SlBBX genes in response to chilling stress

To investigate whether SlBBX genes participated in chilling stress, we analyzed the transcriptome data of tomato plants after chilling stress [43]. Results revealed that the expression levels of ten members of SlBBX family genes, including SlBBX3, SlBBX16, SlBBX17, SlBBX19, SlBBX24, SlBBX26, SlBBX28, SlBBX29, SlBBX30 and SlBBX31, were higher in tomato plants after chilling stress than those grown at optimal temperature conditions (Fig. 8). Furthermore, we found the transcripts of SlBBX1, SlBBX7, SlBBX9, SlBBX12, SlBBX13, SlBBX15, SlBBX18, SlBBX21, and SlBBX27 were significantly decreased after chilling stress. These findings suggest that SlBBX genes might have an important role in response to chilling stress, whereas further studies are essential to investigate the mechanism.

Discussion

Here, we identified and characterized 31 SlBBX genes in tomato (Fig. 1; Tables 1 and 2), which contained two new members (SlBBX30 and SlBBX31) in comparison with the previous studies [44]. BBX proteins are characterized by one or two B-box domains at the N-terminal and, in some cases, a CCT domain at the C-terminal [1]. Here, we found both the newly retrieved SlBBX proteins (SlBBX30 and SlBBX31) contain a B-box domain at the N-terminal (Fig. 1 and Table 2), and they were also clustered in group V (Fig. 2). In addition, as shown in Fig. 3, the three-dimensional structures of SlBBX30 and SlBBX31 were similar to the other members of SlBBXs, which further indicated these two proteins were new SlBBX proteins. There were five subfamilies in the 32 members of Arabidopsis BBXs according to the combination of different conserved domains [2]. However, the conserved domain-based classification of tomato BBX proteins was rather complex. As shown in Fig. 2, SlBBX1-6 were classified into group I, which had two B-boxes and a CCT plus a VP domains, whereas SlBBX1 lacked a VP domain, and SlBBX2 only contained two B-boxes (Table 2). Meanwhile, SlBBX7-12 were clustered into group II, which possessed two B-boxes and a CCT domains; however, SlBBX8 and SlBBX10 had one B-box and a CCT domains, while SlBBX7 contained two B-boxes. Group V contained only one B-box, except for SlBBX27, which contained two B-boxes. We investigated the detail of sequence alignment in SlBBXs (Fig. 1), and found a high degree of conservation of the B-box1 domain among SlBBX7-12, thus the clustering results of these proteins were similar to that based on B-box1. These results indicated that during the process of evolution, some SlBBX proteins lost the B-box2 domain. Since gene duplications play a vital role in genomic rapid expansions during evolution [45], we speculated the new two genes (SlBBX30 and SlBBX31) were retrieved because of genes duplication events. The identified SlBBX genes were distributed unevenly (Fig. 5a). There were no SlBBXs located in Chr11, while SlBBX30 and SlBBX31 were located in the Chr6 and Chr7, respectively. As shown in Fig. 5b, almost SlBBX genes were located within syntenic blocks. Among them, SlBBX30 on Chr6 and SlBBX31 on Chr7 had similarities with SlBBX28 on Chr12. These results revealed that segmental duplication events happened in the expansion of the SlBBX genes family in tomato.
Accumulating evidence showed that some BBX proteins act as central players in a variety of light-regulated physiological processes in plants. Here, we found that the number of light responsive cis-elements was the largest in the promoters of 31 SIBBX genes (Fig. 6), which indicated that SIBBX genes were regulated by light signaling. Thus, we examined the gene expression of all the SIBBXs in response to different light quality. Results showed that light decreased the transcripts of SIBBX1, SIBBX8, SIBBX10 and SIBBX12, while increased the transcripts of SIBBX7, SIBBX13 and SIBBX15 compared with dark (Fig. 7). Previous studies had demonstrated that COP1, which is degraded after illumination, works as an E3 ubiquitin ligase that targets a variety of light signaling factors for ubiquitination and degradation in darkness [9, 46]. For example, COP1 interacts with multiple BBXs, such as CO/BBX1 and BBX10, and subsequently degrades them by the 26S proteasome system [33, 47]. Nevertheless, COP1 stabilizes BBX11 rather than degradating it [14], which suggests that COP1 likely degrades a yet unidentified component(s) targeting BBX11. Thus, COP1 may also control the stability of SIBBX proteins, including SIBBX1, SIBBX7, SIBBX8, SIBBX10, SIBBX12, SIBBX13 and SIBBX15, in the transition from dark to light. Interestingly, we found that SIBBX4, SIBBX23 and SIBBX29 were only in response to R light, while SIBBX7, SIBBX13 and SIBBX25 were just in response to FR light (Fig. 7). These results indicated that these SIBBX proteins might directly interact with the photoreceptors, which sense R and FR light signals. Similarly, recent work has revealed that phyB directly interacts with BBX4 and positively regulates its accumulation in red light in Arabidopsis [25], which demonstrates that photoreceptors may directly control some BBX proteins. In addition, the results showed that R light induced the expression of SIBBX14 and SIBBX24, whereas FR light inhibited their expression (Fig. 7), which implied that these two SIBBX proteins might function antagonistically to regulate some plant physiological processes, such as shade avoidance and the elongated of hypocotyls. Here, we observed that B light induced the gene expression of SIBBX16, SIBBX17, SIBBX18, SIBBX30 and SIBBX31, whereas inhibited the transcripts of SIBBX5, SIBBX6, SIBBX19 and SIBBX20 (Fig. 7). Previous work demonstrated that BBX28/BBX29 and BBX30/BBX31 could precisely control each other by forming a feedback loop in Arabidopsis [19, 20, 22, 23]. Thus, these SIBBX proteins may work in concert with each other and some unidentified factors to regulate the plant growth in response to light signaling.

Light and temperature are more or less inter-related during plant growth and stress response [48]. Studies previously showed that BBX18 and BBX23 are involved in the thermomorphogenesis in Arabidopsis [38]. Both MdBBX20 and MaCOL1 are responsive to low temperatures in apple and banana, respectively [39, 40]. ZFPL, a homologous gene of AtBBX32, enhances cold tolerance in grapevine [41]. Furthermore, CmBBX24 also increases plant cold tolerance in Chrysanthemum [42]. However, whether SIBBXs regulate plant cold response in tomato remains elusive. Here, we observed that there were numerous hormones and stress responsive cis-elements in the promoters of SIBBX genes (Fig. 7). Furthermore, the results showed that low temperatures induced the expression of SIBBX3, SIBBX16, SIBBX17, SIBBX19, SIBBX24, SIBBX26, SIBBX28, SIBBX29, SIBBX30 and SIBBX31, whereas inhibited the transcripts of SIBBX1, SIBBX7, SIBBX9, SIBBX12, SIBBX13, SIBBX15, SIBBX18, SIBBX21 and SIBBX27 (Fig. 8). We have demonstrated that SIHY5 positively regulates plant cold tolerance [49, 50], and a variety of BBX proteins interact with HY5 [19, 20, 22, 23]; thus, it indicates that SIBBXs may play critical roles in plant cold tolerance in tomato.
Conclusions

In conclusion, this investigation found two new members (SlBBX30 and SlBBX31) of the SlBBX gene family. We made a systematic analysis of the 31 SlBBX genes, including their conserved domain, phylogenetic relationship, three-dimensional structure, gene structure, chromosome location, gene duplication and cis-elements analysis. The results suggested that 31 members of SlBBXs were distributed unevenly in the whole genome, and no SlBBXs were located on chromosome 11. Gene duplication analysis indicated that segmental duplications had driven the expansion of the tomato BBX genes. Gene expression analysis revealed that multiple SlBBX genes highly responsive to light quality and low temperatures, which lay a foundation for understanding their biological functions in response to the crosstalk between light and temperature.

Methods

Plant material and growth conditions

Seeds of wild-type tomato (Solanum lycopersicum) in the cv ‘Ailsa Craig’ (Accession: LA2838A) background were obtained from the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu) as previously reported [48]. Seedlings, which grown in pots with a mixture of one part vermiculite to three parts peat, receive Hoagland nutrient solution. The growth conditions for tomato seedlings were 25/20 °C (day/night) temperature with a 12 h photoperiod, the light intensity of 600 µmol m⁻² s⁻¹, and 65% relative humidity.

Light and cold treatments

The six-leaf stage plants were used for all experiments. Plants were grown at white light conditions with an aerial temperature of 25°C or 4°C for the cold treatment in controlled environment growth chambers (Ningbo Jiangnan instrument factory, Ningbo, China). For light quality treatments, plants were exposed to dark (D), white light (W) or different wavelength [purple (P), 394 nm; blue (B), 450 nm; green (G), 522 nm; yellow (Y), 594 nm; red (R), 660 nm and far-red (FR), 735 nm, Philips] light from 6:00 AM to 6:00 PM. The light intensity was 100 µmol m⁻² s⁻¹. The Lighting Passport (Asenseket, Model No. ALP-01, Taiwan) was used to measure light intensity and light quality as a previous study [51].

Genome-wide identification of SlBBX genes in tomato

The protein sequence of Arabidopsis BBXs were downloaded from the TAIR database (https://www.arabidopsis.org/). Tomato BBX proteins were searched and downloaded from three public databases, including the NCBI database (http://www.ncbi.nlm.nih.gov/), the Phytozone 11.0 database (https://phytozone.jgi.doe.gov/pz/portal.html) and the Sol Genomics Network tomato database (https://solgenomics.net/). We chose the candidate BBX accroding to the E-value (1e⁻⁵) and the highest
similarity scores. All the putative BBX genes were submitting to the InterProScan database (http://www.ebi.ac.uk/interpro/), SMART (http://smart.embl-heidelberg.de/) and Conserved Domains Database (http://www.ncbi.nlm.nih.gov/cdd/) to further confirm their completeness and existence of the core domains. The proteins without the B-Box domain and duplicate proteins were removed.

**Protein properties, multiple sequence alignment and phylogenetic analysis**

The various physiochemical properties of tomato BBX proteins, such as MW, polypeptide length, pl, instability index, aliphatic index and GRAVY were investigated using the ExPASy online tool (http://web.expasy.org/protparam/). To estimate the subcellular localization of tomato BBX proteins, we used CELLO v.2.5: sub-cellular localization predictor (http://cello.life.nctu.edu.tw/) [52] and pSORT prediction software (http://www.genscript.com/wolf-psort.html) [53]. ORF numbers were calculated using the NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/).

Multiple sequence alignments of all BBX proteins, including 31 SIBBX and 32 AtBBX proteins, were performed with the MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) [54] and DNAMAN software (Version 5.2.2). We constructed a neighbor-joining phylogenetic tree using MEGA 7.0 with 1000 bootstrap value and Jones-Taylor-Thornton (+ G) method [55], and embellish the phylogenetic tree with an online website Evolview (http://www.evolgenius.info/evolview/#mytrees/)[56].

**Conserved motifs analysis and three-dimensional structure prediction**

The presence of conserved BBX_N and CCT_C – domains were identified by NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), and drawn with IBS software (Illustrator for Biological Sequences, http://ibs.biocuckoo.org/online.php) [57]. We identified the conserved motifs using the Meme program (http://meme-suite.org/index.html) [58], which was run with the maximum number of motifs that were defined as 10, the maximum width was set to 300, and the e-values < 1e⁻³⁰. The three-dimensional structure of tomato BBX proteins were generated with a 3D-PSSM online website (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index).

**Chromosomal location, gene structure, tandem duplication and synteny analysis**

SIBBX genes were mapped to tomato chromosomes according to the Phytozome 11.0 database with the MapChart software. The chromosome distribution diagram was drawn by the online website MG2C.
Exon and intron structures of the SIBBXs were determined according to their corresponding full-length gene sequences in Phytozome 11.0 database. We performed gene structure analysis of the SIBBX genes by using the gene structure display server (GSDS, http://gsds.cbi.pku.edu.cn/) [59].

The syntenic blocks were designed from the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/). MCscanX (http://chibba.pgml.uga.edu/mcscan2/) was used to investigate duplication types and gene synteny [60]. The synteny figures were drawn by Circos-0.69 (http://circos.ca/) with E-value setting to 1e-10 and output format as tabular (-m 8). We used the physical location of a gene on the chromosome to find out the tandem duplication of SIBBX genes in tomato.

Cis-elements of promoters analysis

To identify potential light-, stress-, hormone- and development-related cis-elements, the 2000-bp genomic DNA sequence upstream of the start codon (ATG) of SIBBX genes were obtained from the tomato genome database. The cis-elements in these SIBBX genes promoter were identified by using the Plant Cis-Acting Regulatory Element (PlantCARE; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [61].

Gene expression analysis

Total RNA was extracted from tomato leaves as previously [62, 63]. RNA-seq data of the tomato plants after exposure to 25°C or 4°C for 6 h were obtained and performed as described previously [43, 49]. Three biological replicates for various samples were prepared. For the gene transcript analysis, the cDNA template for real-time RT-PCR was synthesized using a Rever-Tra Ace qPCR RT Kit with a genomic DNA-removing enzyme (Toyobo). qRT-PCR experiments were carried out with an SYBR Green PCR Master Mix Kit (TaKaRa) using an Applied Biosystems 7500 Real-Time PCR System (qTOWER³G, Germany). The PCR was run at 95°C for 3 min, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C. The Tomato ACTIN2 gene was used as an internal control to normalize small differences in template amounts. The relative gene expression was calculated as described previously [64]. The primers sequence was in Additional file 2: Table S2.

Abbreviations

BBXs: B-box proteins; COP1: Constitutively photomorphogenic 1; HY5: Elongated hypocotyl 5; VP: Valine-proline; ZFPL: Zinc-finger protein like; CCT: Constans, CO-like, and Timing of CAB; CDS: Coding sequence; pI: Theoretical isoelectric point; AA: Amino acids; Chr: Chromosome; Gravy: Grand average of hydropathicity; MW: Molecular weight; qRT-PCR: Real-Time PCR; TFs: Transcription factors.
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Authors’ contributions

FW and TLL designed the research, FW, XB, XJW, JRY, YZ, SYZ and XS performed the experiments. FW, YFL, TX, HYQ, MFQ, YXY and GJA analyzed the data. FW, GJA and TLL wrote and revised the paper. All authors have read and approved the manuscript.

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Availability of data and materials

The RNA-seq datasets for expression profiles used in this study were downloaded from NCBI (https://pubmed.ncbi.nlm.nih.gov/31189657/). The datasets are available in the published article Additional file (Supplemental Tables S2 to S4) [43].

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.