Genome-Wide Characterization of B-Box Gene Family and Its Roles in Responses to Light Quality and Cold Stress in Tomato

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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. In this study, 31 SlBBX genes were identified from the newly released tomato (Solanum lycopersicum) genome sequences and were clustered into five subgroups. 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. Promoter cis-regulatory elements prediction and gene expression indicated that SlBBX genes were highly responsive to light, hormones, and stress conditions. Reverse genetic approaches revealed that disruption of SlBBX7, SlBBX9, and SlBBX20 largely suppressed the cold tolerance of tomato plants. Furthermore, the impairment of SlBBX7, SlBBX9, and SlBBX20 suppressed the photosynthetic response immediately after cold stress. Due to the impairment of non-photochemical quenching (NPQ), the excess photon energy and electron flow excited by low temperature were not consumed in SlBBX7-, SlBBX9-, and SlBBX20- silenced plants, leading to the over reduction of electron carriers and damage of the photosystem. Our study emphasized the positive roles of light signaling transcription factors SlBBXs in cold tolerance in tomato plants, which may improve the current understanding of how plants integrate light and temperature signals to adapt to adverse environments.

Keywords: BBX, light, cold stress, Solanum lycopersicum, photoinhibition
HIGHLIGHT
- SlBBXs function as a signal integrator between light and temperature in tomato.

INTRODUCTION

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 (Gangappa and Botto, 2014). The B-box domains are of two classes, and each of them coordinates two zinc atoms (Khanna et al., 2009). The dissimilarities in the consensus sequences of the two B-box domains are the results of evolution through the segmental duplication and deletion events (Crocco and Botto, 2013). Studies suggest that the highly conserved CCT domain is important for transcriptional regulation and nuclear transport (Gendron et al., 2012). 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) (Holm et al., 2001; Datta et al., 2006). Based on the domain structures, 32 BBX proteins are divided into five subfamilies in Arabidopsis (Crocco and Botto, 2013; Gangappa and Botto, 2014).

A variety of wavelength-specific photoreceptors are involved in perceiving the light signals in plants, including phytochromes (phy), cryptochromes (CRYs), phototropins (PHOTs), ZEITLUPE family members, and UV-B resistance locus 8 (UVR8) (Galvao and Fankhauser, 2015; Paik and Huq, 2019). 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 (Galvao and Fankhauser, 2015; Paik and Huq, 2019). Notably, HY5, a target of COP1-mediated protein degradation, plays a vital role in light-regulated plant growth and development (Osterlund et al., 2000; Ahammed et al., 2020). Thus, the light-dependent regulation of COP1–HY5 mediates the plant developmental transition from dark to light.

Upon light irradiation, BBX21 directly binds to BBX22, HY5 and its own promoter regions and activates its transcription (Xu et al., 2016, 2018; Xu, 2020). Moreover, both BBX21 and HY5 can associate with the BBX11 promoter to promote its transcription, while BBX11 binds to the HY5 promoter to activate its transcription (Zhao et al., 2020). Thus, these three TFs (BBX21, HY5, and BBX11) form a positive feedback loop that precisely regulates plant photomorphogenesis. OsBBX14 induces OsHY5L1 gene expression to stimulate photomorphogenesis in rice (Bai et al., 2019). MdBBX37 associates with MdHY5 promoter to inhibit its expression in apple (An et al., 2019a). Additionally, MdBBX22 and MdBBX25/MdCOL4 bind to the MdHY5 promoter to increase and decrease the transcriptional activation of MdHY5, respectively (An et al., 2019b). Both PpBBX16 and PpBBX18 interact with PpHY5 to increase the biochemical activity of PpHY5, while PpHY5 binds to the promoter region of PpBBX18 to promote the transcription of PpBBX18 in pear (Bai et al., 2019a,b). Furthermore, the interaction of PpBBX21 with PpHY5 and PpBBX18 affects the biochemical heterodimer formation of PpHY5–PpBBX18 (Bai et al., 2019b). Tomato RIPENING INHIBITOR (SIRIN) binds to the ripening-induced SlBBXs (i.e., SlBBX19, SlBBX20, and SlBBX26) promoter (Lira et al., 2020). SlBBX20 modulates carotenoid biosynthesis by directly activating PHYTOENE SYNTHASE 1, and is targeted for 26S proteasome-mediated degradation in tomato (Xiong et al., 2019). Therefore, specific BBXs and HY5 constitute an important regulatory network to precisely control normal plant growth and development.

In the 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 (Fan et al., 2012; Gangappa et al., 2013; Wang et al., 2015; Xu et al., 2016; Zhang et al., 2017; Lin et al., 2018; Ordoñez-Herrera et al., 2018; Heng et al., 2019a; Song et al., 2020). Moreover, BBX2 to 9 and BBX13 to 16 interacts with COP1 in vitro, indicating a role for COP1 in controlling the stability of these proteins in darkness (Ordoñez-Herrera et al., 2018). Nevertheless, COP1 preferentially stabilizes BBX11 instead of promoting its degradation (Zhao et al., 2020), which suggests that COP1 likely regulates a yet unidentified protein degrading BBX11. These studies suggest that numerous BBX proteins, along with COP1 and HY5, play critical roles in light-dependent development in plants.

BBX proteins also play vital roles in regulatory networks that control plant adaption to abiotic stress. Previous studies show that both BBX5 and BBX21 positively regulate plant tolerance to drought and salt stress in Arabidopsis (Nagaoka and Takano, 2003; Min et al., 2015). BBX24/STO directly interacts with H-protein promoter binding factor 1 (HPPBF-1), which is a salt-responsive MYB transcription factor, to enhance the root growth and salt tolerance in Arabidopsis (Nagaoka and Takano, 2003). CmBBX22 also positively regulates the plant drought tolerance (Liu Y. N. et al., 2019). In addition, MdBBX10 enhances tolerance to salt and drought by modulating ABA signaling and ROS accumulation (Liu X. et al., 2019). In Arabidopsis, BBX18 and BBX23 control thermomorphogenesis (Ding et al., 2018). Both MdBBX20 and MaCOL1 are responsive to low temperature in apple and banana, respectively (Chen et al., 2012; Fang et al., 2019). Furthermore, ZFPL, a homologous gene of AtBBX32, enhances cold tolerance in the grapevine (Takihara et al., 2011). CmBBX24 also increases plant cold tolerance in Chrysanthemum (Yang et al., 2014). However, whether SlBBXs are involved in light and cold response in tomato remains to be explored.

In the present study, 31 SlBBX genes were identified and characterized in tomato. Gene distribution, synteny analyses, the architecture of exon-intron and motifs differences were investigated. Promoter and gene expression analysis showed that SlBBXs played important roles in plant response to light and low temperature signaling. Meanwhile, we found that the impairment of SlBBX7, SlBBX9, and SlBBX20 suppressed the photosynthetic response and non-photochemical quenching (NPQ) immediately after cold stress, which resulted in excess photon energy and electron flow in SlBBX7-, SlBBX9-, and SlBBX20- silenced plants, leading to the overreduction of electron carriers and damage of photosystem. Our study indicated that light signaling...
transcription factors SIBBX7, SIBBX9, and SIBBX20 play positive roles in cold tolerance in tomato plants, which may improve the current understanding of plant integrated light and temperature signals to adapt the adverse environments.

**MATERIALS AND METHODS**

**Genome-Wide Identification of SIBBX Genes in Tomato**

The BBXs proteins in tomato were identified based on protein homology searches from Arabidopsis using the Hidden Markov Model (HMM) as previously described (Upadhyay and Matteo, 2018). 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 Phytozome 13.0 database (https://phytozome.jgi.doe.gov/pz/portal.html), and the Sol Genomics Network tomato database (version ITAG 4.0, https://solgenomics.net/). Tomato BBX proteins resulting from both searches (E-value, 10^-5) were pooled and redundant sequences were removed. 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/) were used to further confirm the existence of B-box domain in retrieved BBX sequences.

**Protein Properties, Multiple Sequence Alignment, Phylogenetic, and Conserved Motifs 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/) (Yu et al., 2006) and pSORT prediction software (http://www.genscript.com/wolf-psort.html) (Horton et al., 2007). Open Reading Frame (ORF) numbers were calculated using the NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/).

A multiple sequence alignment of the identified tomato BBX proteins and the known BBX families from Arabidopsis, rice, and potato, was performed with the MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar, 2004) and DNASAN software (Version 5.2.2). We constructed phylogenetic tree using MEGA 7.0 with 1,000 bootstrap value and the maximum likelihood method (Kumar et al., 2016), and the phylogenetic tree was displayed with an online website Evolview (http://www.evolgenius.info/evolveview/#mytrees/) (Zhang et al., 2012).

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) (Ren et al., 2009). We performed the protein structural motif annotation using the Meme program (http://meme-suite.org/tools/meme) (Bailey et al., 2009; Upadhyay and Matteo, 2018) and limited our search to a maximum of 20 motifs.

**Chromosomal Location, Gene Structure, and Synteny Analysis**

SIBBX genes were mapped to tomato chromosomes according to the Phytozome 13.0 database with the MapChart software. The chromosome distribution diagram was drawn by the online website MG2C (http://mg2c.iask.in/mg2c_v2.1/) with the information from Sol Genomics Network (http://www.solgenomics.net).

Genomic DNA sequence and CDS corresponding to each identified SIBBX gene were retrieved from the tomato genome database. Intron-exon were displayed by comparing CDS to genomic sequences with the gene structure display server (GSDS, http://gds.cbi.pku.edu.cn/) (Hu et al., 2015).

The syntenic blocks were designed from the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/). 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).

**cis-Elements of Promoters Analysis**

To identify potential light-, stress-, hormone-, and development-related cis-elements, the 2,000-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://bioinfomatics.psb.ugent.be/webtools/plantcare/html/) (Lescoat et al., 2002).

**Plant Material and Growth Conditions**

Seeds of 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 (Wang et al., 2016). 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, light intensity of 600 μmol m^-2 s^-1, and 65% relative humidity. Tobacco rattle virus-based vectors (pTRV1/2) were used for the VIGS of SIBBX genes in tomato with the specific PCR-amplified primers listed in **Supplementary Table 3**. The PCR-amplified fragment was cloned into the pTRV2 vector. The empty vector of pTRV2 was used as the control. All constructs were confirmed by sequencing and subsequently transformed into Agrobacterium tumefaciens strain GV3101. VIGS was performed as described previously (Wang et al., 2016, 2019). The inoculated plants were grown under a 12 h photoperiod at 22/20°C (day/night).

**Light and Cold Treatments**

The six-leaf stage plants were used for all experiments. Plants grown under white light were exposed to a temperature of 25 or 4°C for the control or cold treatment, respectively, in environment-controlled growth chambers (Ningbo Jiangnan instrument factory, Ningbo, China). For different light quality treatments, plants were exposed to dark (D), white light (W),
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 SIBBX genes were identified. The detailed information of each SIBBX is presented in Table 1. The lengths of amino acids (AA) of 31 SIBBXs range from 88 aa (SIBBX18) to 475 aa (SIBBX27). Thus, varied molecular weight (MW) and theoretical isoelectric point (pl) were observed among SIBBX proteins. The MW of SIBBX proteins varies from 9.57 (SIBBX18) to 53.14 kDa (SIBBX27). The pl ranged from 4.25 (SIBBX5 and SIBBX7) to 9.28 (SIBBX26), with 74.2% SIBBXs with a pl lower than seven, which indicated that most of the SIBBX proteins were acidic in nature. The pl ranged from 4 to 9 in SIBBX proteins contained one (single) or two (double) B-box domains; however, the pl ranged from 4 to 7 when SIBBX proteins contained a CCT domain (Supplementary Figure 1), suggesting that the CCT domain in SIBBX proteins may decrease its pl. The majority of SIBBXs were grouped into unstable proteins because their instability index was greater than 40, except for 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 23 SIBBX proteins are located in nuclei. Among the rest 8 tomato BBXs, five (SIBBX5, SIBBX6, SIBBX17, SIBBX25, and SIBBX31), two (SIBBX16 and SIBBX18), and one (SIBBX19) SIBBX proteins are located in chloroplast, cytoplasm, and peroxisome, respectively (Table 1).

Protein Sequences and Phylogenetic Analysis of SIBBXS

The domains logos and the sequences of the B-box1, B-box2, and CCT domains of the SIBBX proteins are shown in Figure 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 10 SIBBXSs, 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 ~40 residues with the consensus sequence C-X2-C-X8-C-X2-D-X4-C-X2-C-D-X3-H-X8-H (Figure 1). The conserved C, C, D, and H residues ligated two zinc ions (Khanna et al., 2009). 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-G-R-F-X-K (Figure 1). To better reveal the evolutionary relationships, we generated a phylogenetic tree with the known BBX families from Arabidopsis, rice and potato, and the identified tomato BBX protein sequences (Figure 2. Supplementary Figure 2). All sequences of tomato BBX proteins were clustered into five subfamilies (Figure 2). The BBX genes in 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 2 were characterized by two B-box domains and also a
CCT domain with the exception for SIBBX7 and SIBBX27, which contained two B-box domains only, and SIBBX8 and SIBBX10, which only had one B-box domain and a CCT domain. In group 3, all the members contained one B-box domain as well as a CCT domain. Group 4 and 5 possessed two and one B-box domain, respectively. Additionally, BBX proteins from two species showed dissimilar exon-intron arrangements. For instance, SlBBX7 and SlBBX27, which only had one B-box domain and a CCT domain. In group 4 and 5 possessed two and one B-box domain, respectively. Additionally, BBX proteins from two species showed dissimilar exon-intron arrangements. For instance, SlBBX7 and SlBBX27, which only had one B-box domain and a CCT domain.

### 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, SIBBX17, and SIBBX30 had no introns (Figure 3B, Table 1, Supplementary Figure 3). Among them, nine SIBBXs had one intron, followed by 10 SIBBXs with two introns, five SIBBXs with three introns, three SIBBXs with four introns, and one SIBBXs with five introns. Generally, members of each subclass, which are most closely related, exhibited analogous exon-intron structures. For instance, the members in groups 1 and 4 had one to two, and zero to one intron, respectively (Figures 3A,B, Supplementary Figure 3). However, a few SIBBX genes showed dissimilar exon-intron arrangements. For instance, SIBBX18 and SIBBX19 had high sequence similarity, but SIBBX18 and SIBBX19 contained two and five introns, respectively (Figures 3A,B, Supplementary Figure 3). These divergences indicated that both the gain and loss of introns during
evolution, may better explain the functional diversity of \textit{SIBBX} homologous genes.

To further examine the structural features of \textit{SIBBX} proteins, the conserved motif distributions were analyzed. Twenty conserved motifs were predicted (Figure 3C), while multilevel consensus sequences and the E-value of them were shown in Supplementary Table 1. The results showed that motif 17 was the largest motif depending on the width, followed by motif 8 and motif 2 (Supplementary Table 1). Motif 1 was found in all the SIBBXs (Figure 3C). Notably, 74.2% and 70.1% SIBBXs contained motif 4 and motif 3, respectively. Motif 2 was unique to the group 1, 2, and 3 of SIBBXs, while motif 5 was unique to group 2 except for the SIBBX27. Motif 10 was found only in group 3 of SIBBXs. Our results showed
### TABLE 2 | Structure of the tomato BBX proteins

| Name   | Gene locus ID   | AA(aa) | Domains       | B-box1   | B-box2   | CCT       | VP        | Protein structure |
|--------|----------------|--------|---------------|----------|----------|-----------|-----------|------------------|
| SlBBX1 | Solyc02g089520 | 409    | 2B-box+CCT    | 19–63    | 59–106   | 340–382   |           |                  |
| SlBBX2 | Solyc02g089500 | 142    | 2B-box        | 19–63    | 62–106   |           |           |                  |
| SlBBX3 | Solyc02g089540 | 391    | 2B-box+CCT    | 12–56    | 52–99    | 322–364   | 386–391   |                  |
| SlBBX4 | Solyc08g006530 | 349    | 2B-box+CCT    | 12–55    | 51–98    | 285–327   | 344–349   |                  |
| SlBBX5 | Solyc12g096500 | 358    | 2B-box+CCT    | 12–55    | 51–98    | 295–337   | 353–358   |                  |
| SlBBX6 | Solyc07g006630 | 386    | 2B-box+CCT    | 22–63    | 59–106   | 307–349   | 379–384   |                  |
| SlBBX7 | Solyc12g006240 | 269    | 2B-box        | 4–47     | 47–73    |           |           |                  |
| SlBBX8 | Solyc05g020020 | 410    | 1B-box+CCT    | 1–44     |          | 356–396   |           |                  |
| SlBBX9 | Solyc07g045180 | 418    | 2B-box+CCT    | 4–47     | 47–90    | 361–404   |           |                  |
| SlBBX10| Solyc05g046040 | 419    | 1B-box+CCT    | 3–47     |          | 363–406   |           |                  |
| SlBBX11| Solyc09g074560 | 373    | 2B-box+CCT    | 15–58    | 58–99    | 322–365   |           |                  |
| SlBBX12| Solyc05g024010 | 452    | 2B-box+CCT    | 7–39     | 51–94    | 404–447   |           |                  |
| SlBBX13| Solyc04g007210 | 428    | 1B-box+CCT    | 17–61    |          | 373–415   |           |                  |
| SlBBX14| Solyc03g119540 | 408    | 1B-box+CCT    | 18–62    |          | 349–392   |           |                  |
| SlBBX15| Solyc05g009310 | 437    | 1B-box+CCT    | 17–61    |          | 380–423   |           |                  |
| SlBBX16| Solyc12g005750 | 110    | 1B-box        | 21–50    |          |           |           |                  |
| SlBBX17| Solyc07g052620 | 130    | 1B-box        | 35–76    |          |           |           |                  |
| SlBBX18| Solyc02g084420 | 88     | 2B-box        | 2–33     | 52–84    |           |           |                  |
| SlBBX19| Solyc01g110370 | 241    | 2B-box        | 2–45     | 54–96    |           |           |                  |
| SlBBX20| Solyc12g089240 | 329    | 2B-box        | 5–47     | 53–100   |           |           |                  |
| SlBBX21| Solyc04g081020 | 299    | 2B-box        | 5–47     | 56–100   |           |           |                  |
| SlBBX22| Solyc07g062160 | 311    | 2B-box        | 5–47     | 53–99    |           |           |                  |
| SlBBX23| Solyc12g005420 | 282    | 2B-box        | 5–47     | 53–99    |           |           |                  |
| SlBBX24| Solyc06g073180 | 233    | 2B-box        | 5–44     | 53–98    |           |           |                  |
| SlBBX25| Solyc01g110180 | 203    | 2B-box        | 3–33     | 56–100   |           |           |                  |
| SlBBX26| Solyc10g006750 | 104    | 1B-box        | 4–34     |          |           |           |                  |
| SlBBX27| Solyc04g007470 | 475    | 2B-box        | 7–49     | 49–87    |           |           |                  |
| SlBBX28| Solyc12g005660 | 202    | 1B-box        | 4–45     |          |           |           |                  |
| SlBBX29| Solyc02g079430 | 185    | 1B-box        | 1–45     |          |           |           |                  |

(Continued)
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 (Figure 4A). A maximum number of SlBBX genes were found on chromosome 12 (Chr12), comprising 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.

Thirty-six pairs of SlBBXs were identified as segmental duplication in the tomato genome (Figure 4B). 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 (Figure 4A), and their sequence identity was 83% (Supplementary Figure 4), they were not tandem duplication. To further examine the evolutionary relationships between SlBBXs and AtBBXs, a synteny analysis was performed. A total of 16 of SlBBX-AtBBX orthologous pairs were identified (Figure 4C), 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**

A total of 61 major cis-elements were predicted in promoters of SlBBX genes (Figure 5A), 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 SlBBX genes (Figure 5B). The number of cis-elements in the promoters of SlBBX17 and SlBBX2 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 SlBBXs promoters, respectively (Figure 5C). The most common motifs 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 SlBBX 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 findings suggest that the promoter regions of SlBBX genes that contained the cis-elements played a critical role in the light and stress responses.

**SlBBX Genes Expression in Response to Different Light Quality**

To assess whether light signaling regulates SlBBXs, we investigated the gene expression of SlBBXs 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 SlBBX1, SlBBX4, SlBBX10, and SlBBX12, while it increased the transcripts of SlBBX7, SlBBX13, and SlBBX15 (Figure 6). Plants grown at R light conditions showed higher expression of SlBBX4, SlBBX14, SlBBX23, SlBBX24, and SlBBX29 than those grown at other light qualities. Whereas, FR light significantly up-regulated the transcripts of SlBBX7, SlBBX13, SlBBX15, SlBBX21, SlBBX25, SlBBX26, and SlBBX27, it obviously down-regulated the transcripts of SlBBX14, SlBBX16, SlBBX18, SlBBX24, SlBBX28, SlBBX30, and SlBBX31 (Figure 6). Transcripts of SlBBX16, SlBBX17, SlBBX18, SlBBX30, and SlBBX31 were induced, while transcripts of SlBBX5, SlBBX6, SlBBX19, and SlBBX20 were inhibited in plants when grown at B light conditions. SlBBX15 was induced by G light irradiation at 6h, whereas SlBBX9 and SlBBX28 were repressed (Figure 6). Y light led to an obvious reduction in expression of SlBBX9 and SlBBX31. Obviously, the P light increased the expression of SlBBX3, SlBBX5, SlBBX6, SlBBX15, SlBBX19, SlBBX20, SlBBX21, SlBBX26, and SlBBX27, but decreased the expression of SlBBX10 and SlBBX16. Interestingly, SlBBX4, SlBBX23, and SlBBX29 were only responsive to R light, while SlBBX7, SlBBX13, and SlBBX25 were induced just in response to FR light. Meanwhile, R light induced the expression of SlBBX14 and SlBBX24, but FR light inhibited their expression (Figure 6). In general, the results showed that SlBBX genes might act a critical role in response to light quality signaling.
**SIBBXs Act Critical Roles in Regulation of Cold Tolerance in Tomato**

To investigate whether *SIBBX* genes participated in cold stress, we analyzed the relative expression data of *SIBBX* genes in tomato plants (Chu et al., 2016), chose five genes, including *SIBBX4, SIBBX7, SIBBX9, SIBBX18*, and *SIBBX20*, and performed virus-induced gene silencing (VIGS) experiments to study their function under cold stress. After cold stress, the levels of relative electrolyte leakage (REL) in *SIBBX7*-silenced plants (pTRV-BBX7), *SIBBX9*-silenced plants (pTRV-BBX9) and *SIBBX20*-silenced plants (pTRV-BBX20) were higher than wild-type (pTRV) (Figure 7A), meanwhile, these silenced plants showed an increased sensitivity to cold stress compared with pTRV, as evidenced by a decrease in the maximum photosystem II efficiency (Fv/Fm) (Figure 7B, Supplementary Figure 5). In addition, the transcription of COLD RESPONSIVE (COR) genes, including *COR47*-like *COR413*-like was markedly lower in *SIBBX*-silenced plants than those in control plants (pTRV) under cold stress (Figure 7C), which indicated that *SIBBX7*, *SIBBX9*, and *SIBBX20* induced cold responsive genes under cold stress. Together, these results indicated that *SIBBX7*, *SIBBX9*, and *SIBBX20* play positive roles in cold tolerance in tomato plants.

**FIGURE 2** Molecular phylogenetic analysis of SlBBX genes in tomato. All SlBBX proteins were divided into five subclasses represented by different colored clusters. Red, orange, blue, purple, and green clusters represent subclasses I, II, III, IV, and V, respectively. The evolutionary history was inferred by using the Maximum Likelihood method using MEGA7 software with 1,000 bootstrap replicates. The red circles and blue triangles represent Arabidopsis and tomato, respectively.
Roles of *SIBBX* in Alleviation of Photoinhibition Under Cold Stress in Tomato

We analyzed the roles of *SIBBX* in cold-induced photoinhibition. The maximum quantum yield of PSII (Fv/Fm) and the maximum level of the P700 signal (Pm, full oxidation of P700) in the dark were measured before and after cold treatment. Before treatment, Fv/Fm and Pm were similar among *SIBBX*-silenced, *SIBBX*-silenced, and WT (pTRV) plants (Figures 8A,F). Fv/Fm was decreased by 27% in the pTRV plants after cold stress, while it was decreased by 51%, 48%, and 52% in *SIBBX*-silenced, *SIBBX*-silenced, and *SIBBX*-silenced plants, respectively, after cold stress, which indicated that disruption of these three *SIBBX* caused photoinhibition of PSII during cold stress. Furthermore, the Pm was decreased by 25% after cold stress, whereas it was decreased by 51%, 48%, and 33%, respectively, in *SIBBX*-silenced, *SIBBX*-silenced, and *SIBBX*-silenced plants after cold stress, which suggested that the disruption of *SIBBX* and *SIBBX* caused obvious photoinhibition of PSI after cold stress, but the photoinhibition of PSI was slight in *SIBBX*-silenced plants after cold stress. Together, these results indicated that these three *SIBBXs* play important roles in alleviating the photoinhibition of both photosystems during cold stress.

In order to obtain a more detailed insight into the processes affecting photoinhibition in the *SIBBX*-silenced plants after cold stress, some electron transport parameters of photosystems I and II were measured. Both ETR II (Figures 8E, 9D) and ETR I (Figures 8J, 9H) were significantly reduced after cold stress in tomato plants, showing a decrease of 50%–55% over those in plants grown at normal temperature. The ETR II and ETR I in *SIBBX*-silenced plants were lower than those in control plants (pTRV) after cold stress, except *SIBBX*-silenced plants, whose ETR I is close to the values of pTRV. To further explore the decrease in the ETRs, we measured Y(II), Y(NPQ), and Y(NO) for PSII and Y(I), Y(ND), and Y(NA) for PSI. We found that Y(II) values were significantly lower in pTRV-*SIBBX*, pTRV-*SIBBX*, and pTRV-*SIBBX*-silenced plants than those in pTRV plants under cold stress (Figures 8B–D, 9A–C). Like Y(II), Y(I) was also decreased in *SIBBX*-silenced plants, except for in pTRV-*SIBBX*-silenced plants (Figures 8G, 9E). These decrease seemed to be due to obvious donor side limitation of PSI (due to lower ETR II in...
PSII) as reflected by the elevated Y(ND), which were 29% and 47% higher in pTRV-BBX9 and pTRV-BBX20 plants, respectively, than in pTRV plants (Figures 8H, 9F). Although Y(ND) was 14% higher in pTRV-BBX7 plants than in pTRV plants, its Y(I) was similar to the control plants (pTRV), which indicated that disruption of SlBBX7 damaged the PSII rather than PSI during cold stress. Therefore, cold stress seriously reduced the capacity for photochemical energy conversion, electron transport rate and photoprotection in SlBBX7-, SlBBX9-, SlBBX20- silenced plants, suggesting that these three SlBBXs play critical roles in alleviating photoinhibition under cold stress.

**DISCUSSION**

In this study, we identified and characterized 31 SlBBX genes in tomato (Figure 1, Tables 1, 2), which contained two additional loci encoding BBX proteins in the tomato genome, that were named SlBBX30 and SlBBX31, in comparison with the previous studies (Chu et al., 2016). 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 (Gangappa and Botto, 2014). Here, we found both the newly retrieved SlBBX proteins (SlBBX30 and SlBBX31) contain a B-box domain at the N-terminal (Figure 1, Table 2), and they were also clustered in group 5 (Figure 2, Supplementary Figure 2), which further indicated these two proteins were new SlBBX proteins. The release of new tomato genomes and database updates may be the primary causes of this phenomenon. There were five subfamilies in the 32 members of Arabidopsis BBXs according to the combination of different conserved domains (Khanna et al., 2009). However, the conserved domain-based classification of BBX proteins in tomato was rather complex. As shown in Figure 2, SlBBX1 to SlBBX6 were classified into group 1, 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.
SlBBXs Regulate Cold-Induced Photoinhibition

FIGURE 5 | Inspection of cis-acting elements in tomato BBX genes. (A) The numbers of different promoter elements in these SlBBX genes were indicated by different colors and numbers of the grid. (B) The sum of the cis-acting elements in each category was represented by different colored histograms. (C) Pie charts with different sizes represented the ratio of each promoter element in each category.

(Table 2). Meanwhile, SlBBX7 to SlBBX12 and SlBBX27 were clustered into group 2, which possessed two B-boxes and a CCT domains; however, SlBBX8 and SlBBX10 had one B-box and a CCT domains, while SlBBX7 and SlBBX27 contained two B-boxes. Group 4 contained only one B-box. We investigated the detail of sequence alignment in SlBBXs (Figure 1), and found a high degree of conservation of the B-box1 domain among SlBBX7 to SlBBX12, 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.

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 SlBBX genes (Figure 5), which indicated that SlBBX genes were regulated by light signaling. Thus, we examined the gene expression of all the SlBBXs in response to different light quality. Results showed that light decreased the transcripts of SlBBX1, SlBBX8, SlBBX10, and SlBBX12, while increased the transcripts of SlBBX7, SlBBX13, and SlBBX15 compared with dark (Figure 6). 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 (Osterlund et al., 2000; Han et al., 2020). For example, COP1 interacts with multiple BBXs, such as CO/BBX1 and BBX10, and subsequently degrades them by the 26S proteasome system (Liu et al., 2008; Ordoñez-Herrera et al., 2018). Nevertheless, COP1 stabilizes BBX11 rather than degrading it (Zhao et al., 2020), which suggests that COP1 likely degrades a yet unidentified component(s) targeting BBX11. Thus, COP1 may also control the stability of SlBBX proteins, including SlBBX1, SlBBX7, SlBBX8, SlBBX10, SlBBX12, SlBBX13, and SlBBX15, in the transition from dark to light. Interestingly, we found that SlBBX4, SlBBX23, and SlBBX29 were expressed only in response to R light, while SlBBX7, SlBBX13, and SlBBX25 were expressed just in response to FR light (Figure 6). These results indicate that these SlBBX 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 (Heng et al., 2019a), which demonstrates that photoreceptors may directly control some BBX proteins. In addition, the results showed that R light induced the expression of SlBBX14 and SlBBX24, whereas FR light inhibited their expression (Figure 6),
FIGURE 6 | Gene expression of SiBBXs in tomato leaves after the exposure of plants to different light quality for 6 h from the dark. Light quality treatments include dark (D), white light (W) or purple (P), blue (B), green (G), yellow (Y), red (R), and far-red (FR) light. The light intensity was 100 µmol m⁻² s⁻¹. Data are presented as the means of three biological replicates (±SD). Different letters indicate significant differences (P < 0.05) according to Tukey's test.
which implied that these two SlBBX proteins might function antagonistically to regulate some plant physiological processes, such as shade avoidance and the elongation of hypocotyls. Here, we observed that B light induced the gene expression of SlBBX16, SlBBX17, SlBBX18, SlBBX30, and SlBBX31, whereas inhibited the transcripts of SlBBX5, SlBBX6, SlBBX19, and SlBBX20 (Figure 6). Previous work demonstrated that BBX28/BBX29 and BBX30/BBX31 could precisely control each other by forming a feedback loop in Arabidopsis (Lin et al., 2018; Heng et al., 2019b; Yadav et al., 2019; Song et al., 2020). Thus, these SlBBX 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 (Wang et al., 2016). Here, we observed that the disruption of SlBBX7, SlBBX9, and SlBBX20 largely reduced the cold tolerance in tomato plants as evidenced by phenotypes, REL, Fv/Fm, and cold responsive genes (Figure 7, Supplementary Figure 5), which indicated that SlBBX7, SlBBX9, and SlBBX20 positively regulate cold tolerance in tomato plants. In addition, far-red light (FR) induced the transcription of SlBBX7 and SlBBX9 (Figure 6), and enhanced the cold tolerance in tomato plants (Wang et al., 2016, 2018, 2019, 2020a,b), which indicate that SlBBXs may play critical roles in the links between cold response and light signaling. Recent studies also showed that BBX18 and BBX23 are involved in the thermomorphogenesis in Arabidopsis (Ding et al., 2018).
MdBBX20 and MaCOL1 are responsive to low temperature in apple and banana, respectively (Chen et al., 2012; Fang et al., 2019). ZFPL, a homologous gene of AtBBX32, enhances cold tolerance in the grapevine (Takukara et al., 2011). CmBBX24 also increases plant cold tolerance in *Chrysanthemum* (Yang et al., 2014). Furthermore, MdBBX37 positively regulates JA-mediated cold-stress resistance in apples (An et al., 2021). However, whether BBXs are involved in cold stress–induced photoinhibition and the regulation of photoprotection during cold stress remains elusive.

Interestingly, the impairment of *SIBBX7*, *SIBBX9*, and *SIBBX20* significantly suppressed the photochemical efficiencies and energy conversion in tomato plants under cold stress, leading to the overreduction of electron carriers and damage of photosystem in tomato plants (Figures 8, 9). More recently, it has been demonstrated that heterologous expression of Arabidopsis *BBX21* in potato plants increases photosynthetic efficiency and reduces photoinhibition (Crocco et al., 2018). Therefore, BBXs play critical roles in photosynthesis and photoinhibition, however, the regulation mechanism is poorly understood. Previous works have revealed that BBX20, BBX21, BBX22, and BBX23 interact with HY5 to increase its transcriptional activity toward the target genes (Datta et al., 2008; Zhang et al., 2017; Job et al., 2018), whereas BBX24, BBX25, BBX28, and BBX29 suppress HY5 activity in Arabidopsis (Gangappa et al., 2013; Job et al., 2018; Lin et al., 2018; Song et al., 2020). HY5 also positively controls *BBX22* at the transcriptional level (Chang et al., 2008), while repressing *BBX30* and *BBX31* gene expression by binding to the promoters of these two genes (Heng et al., 2019b; Yadav et al., 2019). In addition, direct interactions between BBX32 and BBX21 lead to inhibition in the BBX21-HY5 (Holten et al., 2011). Therefore, SIBBXs might alleviate the photoinhibition in tomato plants during cold stress through an HY5-dependent photoprotection pathway. Our previous results demonstrated that SfHY5 alleviated photoinhibition in tomato plants under cold stress by induction of photoprotection, including increased NPQ, cyclic electron flux (CEF) around PSI and the activities of Forrey-Halliwell-Asada cycle enzymes (Wang et al., 2018). Here, we showed that the over-reduction in the flow of electrons from PSII to PSI and considerably low levels of NPQ caused a high excitation pressure in SIBBXs-silenced plants against cold stress and considerably low levels of NPQ caused a high excitation pressure in SIBBXs-silenced plants against cold stress, leading to a severe photoinhibition (Figures 8, 9). Thus, NPQ might function as a protective measure to prevent damage from high excitation pressure against photosynthesis and plant development (Upadhyay et al., 2013).

**CONCLUSIONS**

In this study, SIBBX family genes were identified and characterized in tomato by systematic analysis of conserved domains, phylogenetic relationship, gene structure, chromosome location. Two new members, *SIBBX30* and *SIBBX31*, were identified from the newly released tomato genome sequences. The promoter responsive cis-acting regulatory elements and gene expression analysis indicated that multiple SIBBX genes were highly responsive to light quality and low temperature. Furthermore, we found that *SIBBX7*, *SIBBX9*, and *SIBBX20* positively regulate cold tolerance in tomato plants via the prevention of photoinhibition and enhancing photoprotection. Our study emphasized the positive roles of light signaling transcription factors SIBBXs in cold tolerance in tomato plants, which may improve the current understanding of the integration of light and temperature signals by plants to adapt to adverse environments.
DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FW and TL designed the research. FW, XB, XW, JY, YZ, SZ, and XS performed the experiments. FW, YL, MQ, and GA analyzed the data. FW, GA, and TL wrote and revised the paper. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.698525/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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