SIBBX20 interacts with the COP9 signalosome subunit SICSNS5-2 to regulate anthocyanin biosynthesis by activating SIDLFR expression in tomato

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Abstract
Anthocyanins play vital roles in plant stress tolerance and growth regulation. Previously, we reported that the photomorphogenesis-related transcription factor SIBBX20 regulates anthocyanin accumulation in tomato. However, the underlying mechanism remains unclear. Here, we showed that SIBBX20 promotes anthocyanin biosynthesis by binding the promoter of the anthocyanin biosynthesis gene SIDLFR, suggesting that SIBBX20 directly activates anthocyanin biosynthesis genes. Furthermore, we found by yeast two-hybrid screening that SIBBX20 interacts with the COP9 signalosome subunit SICSNS5-2, and the interaction was confirmed by bimolecular fluorescence complementation and coimmunoprecipitation assays. SICSNS5 gene silencing led to anthocyanin hyperaccumulation in the transgenic tomato calli and shoots, and SICSNS5-2 overexpression decreased anthocyanin accumulation, suggesting that SIBBX20-SICSNS5-2 module may represent a novel regulatory pathway in light-induced anthocyanin biosynthesis.

Introduction
Anthocyanins are pigments synthesized by the flavonoid pathway involved in the coloring of various organs, such as leaves, fruits, and flowers. Anthocyanins accumulate in response to plant hormones, low temperature, high temperature, strong light, UV-B radiation, and other environmental factors. Moreover, fruit color is a significant indicator of quality. Anthocyanins are the main pigments responsible for determining color in a broad variety of fruits. Anthocyanins also play significant roles as antioxidants in plant biotic and abiotic stress tolerance and thereby facilitate plant resistance to pathogens and insects.

Aft and Atv are two important loci that regulate anthocyanin biosynthesis in tomato. The Aft gene from tomato was mapped to chromosome 10 and found to encode a SIAN2-like R2R3-MYB protein that promotes anthocyanin biosynthesis. Atv is located on chromosome 7 and encodes the SIMYBATV protein, which negatively regulates the synthesis of anthocyanins. In addition to positively regulating anthocyanin biosynthesis, Aft was reported to directly activate SIMYBATV expression. Additionally, SIMYBATV competes with Aft for interaction with the transcription factor SIIJAF13, thereby downregulating the accumulation of anthocyanins in tomato fruit. Mutation of SIMYBATV results in the release of SIIJAF13, which interacts with Aft, further
leading to the upregulation of SLAN1 and SLAN11 expression and accumulation of anthocyanins in tomato fruit\(^9,11\).

Anthocyanin accumulation is mostly regulated by transcription factors and structural genes, including CHS, CHI, F3H, F3\(^3\)H, DFR, ANS, and UFGT\(^6,12\). The biosynthesis of anthocyanins is regulated by different transcription factors. The MYB-bHLH-WD40 (MBW) complex plays vital roles in regulating the biosynthesis of anthocyanins. The molecular mechanism by which the MBW complex regulates anthocyanin biosynthesis has been extensively studied. The WD40 protein likely plays a more general role in the regulation of the complex\(^8,13\). The activation of particular genes is determined by the expression pattern and DNA-binding specificity of the MYB and bHLH proteins.

SLAN2 was reported to promote anthocyanin biosynthesis when plants were grown in strong light and under low-temperature conditions\(^14\). A recent study showed that the overexpression of SLMYB75 induced the accumulation of anthocyanins\(^15\). Overexpression of SLANT1 was reported to increase the expression levels of structural genes in the anthocyanin biosynthesis pathway\(^16,17\). The MdBHLH10 gene was found to be involved in regulating the accumulation of anthocyanins in apple\(^17,18\). The MYB-TF gene Cs6g17570 was identified as a vital player in the regulation of anthocyanin biosynthesis in blood oranges\(^19\). MdMYB16 and MdbHLH33 were also reported to be involved in anthocyanin metabolism\(^20\). In addition, MdbHLH3 was found to promote anthocyanin accumulation and fruit coloring under low-temperature conditions in apple\(^21\). The WD40 protein MdTTG1 was reported to interact with bHLH to regulate anthocyanin synthesis in apple\(^22\).

B-box (BBX) proteins are a class of zinc finger protein transcription factors that contain one or two B-box domains. Many studies in Arabidopsis have revealed that BBX family proteins play an important role in photomorphogenesis\(^23\). This particular group of BBX factors includes AtBBX1, AtBBX4, AtBBX19, AtBBX20, AtBBX21, AtBBX22, AtBBX23, AtBBX24, AtBBX25, AtBBX28, AtBBX30, AtBBX31 and AtBBX32\(^24\)-\(^31\). The accumulation of anthocyanin is a general phenomenon in photomorphogenesis, and BBX proteins were also found to regulate anthocyanin synthesis. In pear, the BBX proteins PpBBX16, PpBBX18, PpBBX21, and PpBBX24 are involved in anthocyanin accumulation\(^5,6,32\). In apple, MdCOL4, MdBBX20, MdBBX22, and MdBBX37 were found to participate in the regulation of anthocyanin accumulation\(^2\)-\(^4,33\).

In a previous study, we found that the SIBBX20 protein is modified by the CRL4 E3 ubiquitin ligase to regulate the biosynthesis of carotenoids in tomato fruit\(^34\). In addition to the carotenoid content, we found that overexpression of the SIBBX20 gene led to a significant increase in the anthocyanin content. Here, SIBBX20 was found to target the DFR promoter and activate its expression. To further uncover the mechanism by which anthocyanin biosynthesis is regulated, we screened a yeast two-hybrid library and found that SICSN5-2 interacts with SIBBX20. The downregulation of SICSN5-2 resulted in the accumulation of anthocyanin in tomato. Furthermore, when we interfered with the expression of a SICSN5-2 homolog in tobacco, the expression level of the SIBBX20 protein was significantly increased, indicating that SICSN5-2 regulates the accumulation of the SIBBX20 protein.

**Results**

**Overexpression of SIBBX20 led to increased anthocyanin accumulation**

To study the role of SIBBX20 in regulating the accumulation of anthocyanins, we overexpressed full-length SIBBX20 in tomato with the 35S promoter. In the transformation process, we found that the partial calli growing on the medium had become purple (Fig. 1a). After rooting, the seedlings and roots of the SIBBX20-overexpressing plants were also purple (Fig. 1b, c). Purple sepals in the flowers of SIBBX20-overexpressing plants were also observed (Fig. 1d). Thus, anthocyanins accumulated in diverse tissues with the overexpression of SIBBX20.

We also obtained three homozygous SIBBX20-knockout plants using CRISPR-Cas9 technology. No obvious difference in anthocyanin content was observed between the knockout plants and control plants, which may be due to redundant gene functions. Recent studies have shown that Arabidopsis BBX20, 21 and 22 are functionally redundant and regulate hypocotyl elongation and anthocyanin accumulation as rate-limiting cofactors of HY5\(^35\). Therefore, we selected SIBBX20-overexpressing plants to perform follow-up experiments.

We quantified the expression of SIBBX20 and measured the anthocyanin content in 14 independent transgenic lines (Fig. 2a–c). The level of SIBBX20 transcription was highly correlated with anthocyanin content. We further used the Pearson correlation coefficient to assess the correlation between these two parameters (Fig. 2d). The scatter plot showed the Pearson correlation coefficient between the relative SIBBX20 expression level and level of accumulated anthocyanin to be 0.84, indicating a strong positive correlation between them.

**The expression of flavonoid biosynthesis genes was upregulated in SIBBX20-overexpressing lines**

To explore the molecular mechanism by which SIBBX20 regulates anthocyanin accumulation, we analyzed the transcriptomes of the SIBBX20-overexpressing plants and controls by RNA sequencing (RNA-Seq).
Anthocyanins are synthesized by the flavonoid pathway. The expression levels of some genes in the pigment, flavonoid, and anthocyanin biosynthesis pathways (DFR, ANS, CHS1, CHS2, F3H, F3’5’H, and FLS) were found to be upregulated in the SIBBX20-overexpressing plants (Fig. 3a). To independently validate these results, we quantified the expression of these anthocyanin-related structural genes in two SIBBX20-overexpressing lines, OE-20 and OE-40 (Fig. 3b). The expression levels of CHS1, CHS2, F3H, F3’5’H, DFR, and FLS were found to be upregulated in the SIBBX20-overexpressing lines relative to the control line. Among these genes, DFR, ANS, and F3’5’H were elevated more than twofold. This result is consistent with the transcriptome data, and these data imply that SIBBX20 promotes the accumulation of anthocyanins by regulating the expression of anthocyanin biosynthesis genes.

**SIBBX20 directly regulates SDFR**

We hypothesized that SIBBX20 directly regulates transcription of the anthocyanin biosynthesis genes that were upregulated in the SIBBX20-overexpressing lines. BBX proteins were reported to regulate their target genes by binding G-boxes in their promoters. The cis-elements in the promoters of DFR, ANS, CHS1, CHS2, F3H, F3’5’H, and FLS were analyzed by using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The promoter of ANS does not contain a G-box. Therefore, we speculated that SIBBX20 cannot bind the ANS promoter. Yeast one-hybrid assays were conducted to test whether SIBBX20 regulates the other genes in this group. The yeast strain Y1GOLD was cotransformed with AD-SIBBX20 and pAbAi-CHS1, pAbAi-CHS2, pAbAi-F3H, pAbAi-F3’5’H, pAbAi-FLS or a negative control. None of these transformants survived on the selective medium, which lacked Leu and Ura and contained AbA. These data indicate that SIBBX20 could not interact with the promoters of CHS1, CHS2, F3H, F3’5’H, or FLS, although these promoters contain G-boxes (data not shown). The promoter of SDFR contains three G-boxes (Fig. 4a). We designed the G-box1, 2 and 3 sequences to confirm their interaction. We found that Y1GOLD yeast cells cotransformed with AD-SIBBX20 and pAbAi-SDFR (including G-box1, 2) could survive on the selective medium. However, pAbAi-SDFR including G-box3 could not survive on the medium (data not shown). These data indicate that SIBBX20 can bind G-box1 or 2 in the promoter of SDFR (Fig. 4b).

Next, we performed an EMSA to determine which G-box (G-box1 or 2) is targeted by SIBBX20. In the EMSAs, the SIBBX20 protein bound the G-box1-wt probe but not the other probes. This result demonstrated that SIBBX20
binds G-box1 in the \textit{SIDFR} promoter in vitro (Fig. 4c, d). Subsequently, a dual-luciferase system assay was performed to test whether \textit{SlBBX20} could activate the expression of \textit{SlDFR}. As shown in Fig. 4f, the ratio of LUC to REN in tobacco leaves co-injected with \textit{62SK-SlBBX20} and \textit{pAbAi-SlDFR} was increased by 2.6-fold relative to the negative control (\textit{62SK} and \textit{pAbAi-SlDFR}). These results provide evidence that \textit{SlBBX20} can activate the transcription of \textit{SlDFR} by binding the G-box1 \textit{cis}-element in its promoter.

\textbf{\textit{SlBBX20} interacts with \textit{SICSN5-2} in vivo}

To further analyze the molecular mechanism by which \textit{SIBBX20} regulates anthocyanin content, a yeast two-hybrid (Y2H) screen was performed using \textit{SIBBX20} as bait. We found that the protein encoded by Solyc06g073150 could interact with \textit{SIBBX20} in the Y2H screen. The full-length coding sequence of this gene is 1104 bp in length. The gene encodes a predicted protein consisting of 367 amino acid (aa) residues, which was referred to as \textit{SICSN5-2} in a previous study. We used three different methods to confirm the interaction between the \textit{SIBBX20} and \textit{SICSN5-2} proteins. First, the interaction was verified using the Y2H assay (Fig. 5a). The full-length \textit{SICSN5-2} protein was previously demonstrated to generate false-positive results in the Y2H assay. Therefore, a truncated \textit{SICSN5-2} construct (\textit{SICSN5-2}_{57-367}) was used to test the interaction. \textit{SIBBX20} was also divided into two fragments consisting of residues 56-203 or 101-203. The yeast cells cotransformed with the \textit{BD-SIBBX20}/\textit{BD-SIBBX20}_{56-203} and \textit{AD-SICSN5-2}_{57-367} plasmids grew on SD-Leu/Trp/His/Ade medium, but the negative control yeast cells and yeast cells cotransformed with \textit{BD-SIBBX20}_{101-203} and \textit{AD-SICSN5-2}_{57-367} did not grow on this medium, suggesting an interaction between \textit{SIBBX20}_{56-101} and \textit{SICSN5-2}_{57-367}.

Second, we employed a coimmunoprecipitation assay to confirm the interaction (Fig. 5b). \textit{SIBBX20-HA} and \textit{SICSN5-2-FLAG} plasmids were cotransformed into tobacco protoplasts and expressed for 8–10 h. The proteins were immunoprecipitated using an anti-FLAG antibody and immunoblotted using an anti-HA antibody. The \textit{SIBBX20-HA} protein was coimmunoprecipitated with \textit{SICSN5-2-FLAG}, but the negative control was not (Fig. 5b).

Finally, the interaction between \textit{SIBBX20} and \textit{SICSN5-2} was tested using the bimolecular fluorescence complementation (BiFC) assay. We observed YFP fluorescence...
in tobacco protoplast cells cotransformed with the SIBBX20-nYFP and SICSNS-5-2-cYFP plasmids but not in the negative control tobacco protoplast cells cotransformed with the empty vector and SIBBX20-nYFP or SICSNS-5-2-cYFP plasmids (Fig. 5c). These data provide in vivo evidence that SlBBX20 interacts with SlCSN5-2.

We also determined the subcellular localization of SlCSN5-2, and the results showed that SlCSN5-2 accumulates in both the nucleus and cytoplasm (Fig. 5d). This localization pattern was very similar to that of SlBBX20, suggesting that SlCSN5-2 and SlBBX20 work together.

**SICSNS5 regulates anthocyanin accumulation**

To study the effects of SICSNS5-2 on anthocyanin biosynthesis, we used RNA interference to downregulate the expression of SICSNS5 (including SICSNS5-1 and SICSNS5-2) in stably transformed plants. It was impossible to independently silence SICSNS5-1 and SICSNS5-2 in tomato due to their high sequence similarity. While generating the transgenic plants, we observed many purple calli and shoots (Fig. 6a), similar to the effects of SIBBX20 overexpression. Strongly SICSNS5-silenced calli accumulated many anthocyanins but failed to grow into normal plants. The moderately silenced plants survived, but their growth was significantly restrained (Fig. 6b, c), indicating that the function of SICSNS5-2 in growth and development is indispensable. We selected a line in which SICSNS5 expression was moderately decreased to test the anthocyanin content and found that it was significantly increased compared with that in the WT plants (Fig. 6d). Correspondingly, when SICSNS5-2 was overexpressed in tomato, the anthocyanin content was significantly decreased compared with that in the WT plants (Fig. 6e), suggesting that SICSNS5 is a negative regulator of anthocyanin biosynthesis.

Furthermore, to confirm whether SICSNS5-2 regulates the accumulation of anthocyanin by DFR, we detected the expression level of SIDSFR in SICSNS5-RNAi plants. The expression level of SIDSFR was significantly upregulated compared with that of the WT plants, and the expression level of SICSNS5-2 was negatively correlated with that of SIDSFR (Fig. 6f). This result suggests that SICSNS5 negatively regulates the accumulation of anthocyanin in tomato by SIDSFR.

**SICSNS5-2 regulates accumulation of the SIBBX20 protein**

Previously, we revealed that SIBBX20 is modulated by the E3 ubiquitin ligase CRL4. SICSNS5-2 was reported to regulate CRL ubiquitin ligase and may regulate
ubiquitination of the SIBBX20 protein by modifying the E3 ubiquitin ligase CRL4. To explore whether SICSNS5-2 affects ubiquitination of the SIBBX20 protein, we conducted an immunoprecipitation assay to detect the ubiquitination of SIBBX20 with an anti-UBQ antibody. The results showed that upon SICSNS5-2 coexpression, ubiquitination of the SIBBX20 protein was enhanced, and expression of the SIBBX20 protein decreased with actin used as an input control (Fig. 7a).

*NbCSN5B* in tobacco is an ortholog of *SICSNS5-2*, and their gene sequences are highly similar (Supplementary Fig. S1). We used VIGS to silence *NbCSN5B* in tobacco. Relative *NbCSN5B* gene expression was confirmed to be downregulated in leaves (Fig. 7b). Then, we extracted protoplasts from tobacco and transiently expressed SIBBX20-HA in the protoplasts. After 10 h, the proteins were extracted and detected by western blotting (Fig. 7c). We found that the SIBBX20-HA protein accumulated to

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**Fig. 4 SIBBX20 activates the expression of DFR by binding its promoter.**

a) Schematic diagram of the G-box locations on the DFR promoter. P1 includes G-box1 and G-box2, and the black dots indicate the locations of the G-boxes. b) SIBBX20 was shown to bind the DFR promoter in a yeast one-hybrid assay. The yeast strain Y1HGold was transformed with the bait vector pAbAi-DFR and the prey vector AD-SIBBX20 and plated on SD-Leu-Ura medium with or without aureobasidin A (45 ng mL⁻¹). c) Wild-type and mutant probe sequences used for EMSAs. G-box1-wt and G-box2-wt were used as wild-type probes. The synthesized G-box1-mt and G-box2-mt sequences were mutation probes in which the cis-element sequences were replaced by GGGGGG or GGGGGGG, respectively. d) Affinity of SIBBX20 for two G-box motifs. BP indicates the binding probe; FP indicates the free probe. “+” and “-” indicate presence or absence in the EMSA, respectively. e) Schematic of the vector used for the dual-luciferase experiment. f) LUC/REN ratios. Luciferase activity was detected by dual-luciferase reporter assay.
higher levels in the NbCSN5B-silenced plants than in the control plants. In general, these results suggest that SICSNS5-2 interacts with SIBBX20 and promotes its ubiquitination and degradation to negatively regulate anthocyanin biosynthesis.

Discussion

Anthocyanins are natural plant pigments involved in regulating the coloration of specific plant organs, such as leaves, flowers, and fruits. Previous studies on the regulation of anthocyanin biosynthesis have mainly focused on the MBW complex. Some positive regulators of anthocyanin biosynthesis, such as SIAN2, SIAN1, and SIMYB75, and some negative regulators of anthocyanin biosynthesis, such as MdMYB16, FaMYB1, and PhMYB27, have been reported in different plants. Recently, some BBX proteins in apple (i.e., MdCOL4, MdBBX20, MdBBX22) were found to regulate anthocyanin biosynthesis. In pear, PpBBX16, PpBBX18, PpBBX21, and PpBBX24 were reported to be involved in anthocyanin synthesis. A recent study showed that MdBBX37 inhibits the transactivating activities of

Fig. 5 Interactions between SIBBX20 and SICSNS5-2. a Interaction between SIBBX20 and SICSNS5-2 in yeast two-hybrid experiments. The BD-SIBBX20 and AD-SICSNS5-2 (residues 57 to 367) plasmids were cotransformed into yeast strain AH109. b Interaction between SIBBX20 and SICSNS5-2 in communoprecipitation assays. SIBBX20-HA and SICSNS5-2-FLAG were simultaneously introduced into tobacco protoplasts. The proteins were immunoprecipitated using anti-FLAG matrix beads and analyzed by western blotting. c Interaction between SIBBX20 and SICSNS5-2 in BiFC assays. The pHBT-SIBBX20-nYFP and pHBT-SICSNS5-2-cYFP plasmids were cotransformed into Arabidopsis protoplasts. The pHBT-SIBBX20-nYFP and pHBT-SICSNS5-2-cYFP plasmids or pHBT-SICSNS5-2-cYFP and pHBT-SIBBX20-nYFP plasmids as a negative control were used. YFP fluorescence was observed using confocal laser scanning microscopy. d Subcellular localization of SICSNS5-2. SICSNS5-2 without a stop codon was cloned into pHBT-GFP. Tobacco protoplasts were extracted and cotransformed with plasmids encoding SICSNS5-2-GFP and a nuclear marker fused to RFP.
MdMYB1 and MdMYB9 and therefore downregulates anthocyanin biosynthesis.

In this study, we found that SlBBX20 interacts with SlCSN5-2 and promotes anthocyanin biosynthesis by binding the DFR promoter. Tomato plants overexpressing SlBBX20 accumulated high levels of anthocyanins. Anthocyanins are synthesized by the flavonoid pathway, which contains genes that contribute to anthocyanin biosynthesis at both the early and late stages. We found that most of these genes were upregulated in the SlBBX20-overexpressing lines, although the extent to which they were upregulated varied. BBX transcription factors were reported to regulate transcription by binding G-box cis-elements. Here, SlBBX20 was found to directly bind the first G-box in the SlDFR promoter and activate its expression, and the accumulation of anthocyanins was highly correlated with the expression level of SlBBX20. DFR is a key enzyme in the anthocyanin synthesis pathway, its mutation blocks the accumulation of anthocyanin in tobacco, and a white flower phenotype appeared. SlBBX20 was reported to promote anthocyanin biosynthesis by binding the promoters of DFR, ANS, and MYB1. PpBBX16 requires PpHY5 to increase the expression levels of genes related to anthocyanin biosynthesis. Thus, the mechanisms used by BBX20 to promote anthocyanin biosynthesis appear to be similar but vary among different plant species.

A large number of studies in Arabidopsis have shown that BBX family proteins are involved in photomorphogenesis. Our previous study showed that SlBBX20-overexpressing tomato plants exhibited enhanced photomorphogenesis. We identified the SlCSN5-2 protein as a binding partner of SlBBX20. The COP9 signalosome (CSN) plays an important role in plant photomorphogenesis and was originally discovered by cloning mutant alleles that disrupt photomorphogenesis in Arabidopsis. The accumulation of anthocyanins is an important phenomenon in photomorphogenesis. Recently, several BBX proteins were found to interact with the HY5 protein and regulate anthocyanin accumulation in Arabidopsis, apple, and pear. UV-B radiation induces the accumulation of anthocyanins using a signaling mechanism that depends on MdCOP1; This mechanism activates MdHY5 and promotes the binding of MdHY5 to the MdMYB gene-promoter region. In this study, we found that CSN5-2—a photomorphogenesis factor—could negatively regulate the accumulation of anthocyanins by promoting the accumulation of the SlBBX20 protein. Although the SlBBX20-OE plants accumulated many anthocyanins under light, anthocyanin...
accumulation in the SlBBX20-OE plants was reduced upon exposure to dark conditions for a period of time, and the expression of SlDFR was significantly downregulated compared with that under light (Supplementary Fig. S2a, b, d). suggested that the anthocyanin modulation by SlBBX20 is dependent on light. The transcription level of SlCSN5-2 was not significantly changed when the plants were exposed to dark conditions (Supplementary Fig. S2c). Previous studies have indicated that the protein level of COP9 is not affected by light, but light may regulate the activity of the SlCSN5-2 protein at the posttranslational level43. Therefore, we believe that light is required for anthocyanin regulation in the SlBBX20-SlCSN5-2 model. These data reveal a novel regulatory pathway involved in light-induced anthocyanin biosynthesis.

Interestingly, when we knocked down the expression of SlCSN5 in tomato using RNAi, anthocyanins accumulated in the calli and shoots, and strongly silenced plants accumulated abundant anthocyanins but failed to grow into normal plants. A moderate decrease in the level of SlCSN5 expression led to dwarfining, indicating the important function of SlCSN5 in tomato development. Tomato contains two CSN5 genes, namely, SlCSN5-1 and SlCSN5-2. Because their sequences are highly similar, it is difficult to individually interfere with the expression of these genes. Indeed, previous studies also reported that it was impossible to use different sequences to separately silence the two CSN5 genes in tomato37.

SICS5-VIGS plants were reported to be approximately 50% shorter in stature than controls37. The effects of SICS5 on growth and development may have been more severe in our stably transformed tomato lines. Among Arabidopsis thaliana, the csn5a mutant develops purple cotyledons44, but the reason is unknown. We found the sequence similarity between SICS5-2 and AtCSN5a to be 84%. We speculate that AtCSN5a utilizes a mechanism to regulate anthocyanin biosynthesis that is similar to that of SICS5-2.

CSN can regulate protein degradation through the ubiquitination pathway. The major activity of CSN is regulated by the fifth subunit (CSN5)45. CSN5 has been reported to be involved in deneddylation activity46. It can regulate the activity of CRLs (Cullin RING ligases) by covalently binding and removing RUB proteins47,48. Ubiquitinated proteins have been reported to accumulate in Arabidopsis csn mutants49. In the present study, we found that the ubiquitination of SlBBX20 was enhanced when it was coexpressed with SICS5. Furthermore, when we silenced the SICS5 homolog NbCSN5B in tobacco, accumulation of the SIBBX20 protein increased, indicating that CSN5 regulates accumulation of the SIBBX20 protein. Therefore, we infer that CSN5 is involved in ubiquitination and degradation of the SIBBX20 protein. Our previous study demonstrated that SIBBX20 can be ubiquitinated by the CUL4-DET1-DDB1 complex and eventually degraded by the 26 S proteasome34. CSN was
found to modify the activity of several CUL4-based E3 ubiquitin ligases to regulate plant photomorphogenesis. However, how CSN5 might participate in regulating the activity of CUL4-based E3 ubiquitin ligases has not been fully understood. Our work might provide insight into the modification of CSN5 to CRLs.

Materials and methods

Plant materials

The SIBBX20 gene was cloned into the overexpression vectors pHellsGate8 and pCAMBIA2300-HA. Using the “Alisa Craig” (LA2838A) tomato as the wild-type background, transgenic tomato plants were obtained by Agrobacterium tumefaciens-mediated transformation. The expression of SIBBX20 in the transgenic plants was quantified using qRT-PCR.

Gene expression analysis

TRizol reagent (Invitrogen, USA) was used to extract total RNA from leaves as previously described. cDNA was synthesized using a HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme, China). Gene-specific oligonucleotides were used to perform qRT-PCR in a Roche LightCycler 480 system. Relative gene expression was calculated by Microsoft Excel. Expression of the actin gene (SGN-U580609) was used as an internal control. The sequences of the gene-specific oligonucleotides used in the analysis are listed in Supplementary Table S2.

Measurement of the total anthocyanin content

The methanol-HCl method was used to extract anthocyanins from tomato leaves. Approximately 2 g of tomato leaves ground with liquid nitrogen was soaked in 5 ml of 1% (v/v) methanol-HCl and extracted overnight in the dark at 24 °C. A spectrophotometer (UV-1600, Shimadzu, Japan) was used to measure the absorbance of each sample at 530, 620, and 650 nm. The following formula was used to calculate the relative anthocyanin content: optical density (OD) = (OD530 - OD620) - 0.1 (OD650 - OD620). One unit of anthocyanin represented a change in the OD of 0.1.

RNA sequencing

Three biological replicates from 4-week-old tomato seedlings that overexpressed SIBBX20 and WT tomato seedlings were selected for the extraction of total RNA and RNA sequencing. We used the average RPKM (reads per kilobase per million reads) value as a measure of gene expression. Genes showing at least a twofold change in expression with an FDR-adjusted p-value of less than 0.05 were defined as differentially expressed genes (DEGs). The heat map was plotted with log2RPKM values to visually show differences in expression levels.

Yeast one-hybrid assay

The full-length SIBBX20 gene was amplified with tomato cDNA as the template and inserted into pGADT7 to obtain the prey vector (AD-SIBBX20). Fragments of the DFR, CHS1, CHS2, F3H, F3’5’H, and FLS promoters were amplified with tomato genomic DNA as the template and cloned into pAbAi to obtain a bait vector (pAbAi-DFR, CHS1, CHS2, F3H, F3’5’H, and FLS). The yeast strain Y1HGold transformed with bait vector was cultured on SD-Ura medium and in a 30 °C incubator for three days. Subsequently, the prey vector was transformed into the yeast strain Y1HGold previously transformed with the bait vector, and the resulting strain was plated on SD-Leu-Ura medium. The positive clones were diluted with 0.9% NaCl to an OD600 of 0.1, after which 2 μL of each suspension was spotted on SD-Leu medium with or without aureobasidin A (45 ng mL−1).

Transient dual-luciferase assay

The full-length SIBBX20 ORF was amplified by using tomato cDNA as a template and inserted into the pGreen II 62-2K vector. Promoter fragments from DFR (bp 1 to 1490) were cloned into the reporter vector, pGreen II 0800-LUC. The constructed vectors were individually introduced into Agrobacterium strain GV2260. The Agrobacterium liquid introduced into the reporter vector and effector vector were mixed and injected into tobacco leaves. Transient expression was evaluated three days after infiltration. The firefly luciferase activity was detected by a dual-luciferase reporter assay system (Promega, USA).

Electrophoretic mobility shift assays (EMSAs)

The SIBBX20 gene was cloned into pET28a to express the His-tagged SIBBX20 protein. Based on the DFR promoter sequence, distinct 30-bp single-stranded fragments containing the cis-acting elements AACGTG or CACATGG were synthesized (TsingKe, China) and labeled using the Biotin 3’ End DNA Labeling Kit (Thermo Scientific, USA). The cis-acting element was replaced with a series of guanosines to obtain the mutated fragment. The labeled DNA fragment and purified His-SIBBX20 protein were incubated in the reaction mixture for 30 min as described previously. The protein-DNA complexes were separated in 6.5% native PAGE gels. The gels were transferred to a nylon membrane (Beyotime Biotechnology, China). After UV cross-linking, chemiluminescence was used to observe the migration of the biotin-labeled probe on the membranes.

Yeast two-hybrid assay

The SIBBX20 coding sequence was inserted into the prey vector pGBKT7 (BD) to yield BD-SIBBX20, which was used as bait to screen a tomato yeast two-hybrid library, which showed that SICSN5-2 and SIBBX20
interact. Furthermore, the coding sequence for a truncated SICSN5-2 protein construct lacking 56 amino acids at its N-terminus (residues 57 to 367, SICSN5-257-367) was amplified and cloned into the bait vector pGADT7 (AD) to yield AD-SICSN5-2. The BD-SIWBX20 and AD-SICSN5-2 plasmids were cotransformed into yeast strain AH109. After transformation, yeast AH109 cells were grown on SD-Trypt-Leu medium for 3 days. A single clone was spotted in SD -Trypt-Leu-His-Ade medium, and the growth of yeast cells was observed. The pGBKTK7 and AD-SICSN5-2 plasmids or BD-SIWBX20 and pGADT7 plasmids were used as negative controls.

Bimolecular fluorescence complementation (BiFC)

The SIWBX20 coding sequence was inserted into pHBT-nYFP to yield pHBT-SIWBX20-nYFP. The full-length SICSN5-2 coding sequence was cloned into pHBT-cYFP to yield pHBT-SICSN5-2-cYFP. Two plasmids, pHBT-SIWBX20-nYFP and pHBT-SICSN5-2-cYFP, were cotransformed into Arabidopsis protoplasts. After the protoplasts had been cultured for 12 h, YFP fluorescence was observed by confocal microscopy.

Coimmunoprecipitation

For coimmunoprecipitation assays, tobacco protoplasts coexpressing SIWBX20-HA and SICSN5-2-FLAG or expressing Mer as a control were collected, resuspended in extraction buffer and centrifuged at 12000 × g at 4 °C for 10 min. Five microliters of anti-FLAG matrix beads (Sigma, USA) were added to the supernatant and incubated at 4 °C for 2 h to capture the epitope-tagged protein. Finally, anti-HA (MBL, Japan) or anti-FLAG (Sigma, USA) antibodies were used for western blot analysis. Anti-UBQ (Millipore, USA) was used to detect ubiquitination of the SIWBX20 protein, and anti-actin was used as a control.

Subcellular localization

The SICSN5-2 coding sequence without a stop codon was amplified and cloned into pHBT-GFP using genespecific primers (Supplementary Tables S1). Tobacco protoplasts were extracted and cotransformed with the SICSN5-2-GFP plasmid and a nuclear marker fused to RFP. After expression in protoplasts for 10 h, fluorescence was observed under a laser scanning confocal microscope.

Downregulation of CSN5 expression by virus-induced gene silencing (VIGS)

Because the two CSN5 sequences in tomato (CSN5-1 and CSN5-2) and two CSN5B sequences in tobacco (CSN5B-1 and CSN5B-2) are highly homologous, it is difficult to individually interfere with the two CSN5 sequences in a single species. Therefore, we elected to interfere with the expression of both. We employed virus-induced gene silencing (VIGS) and stable RNA interference (RNAi) to downregulate the expression of CSN5 in tobacco and tomato, respectively. For VIGS, we selected a unique sequence from NbCSN5B and ligated the sequence into pTRV2. The recombinant plasmid was transferred into Agrobacterium GV3101. Agrobacterium strains transformed with NbCSN5B-TRV2 and TRV1 or TRV2 and TRV1 were mixed and injected into tobacco leaves. After 10 days, the protoplasts were extracted from the tobacco, and SIWBX20-HA was transiently expressed. After SIWBX20-HA had been expressed in the protoplasts for 10 h, SIWBX20-HA was extracted and detected by western blotting. A unique sequence was selected from SICSN5 and used to construct an RNAi vector for stable transformation. The sequences of the gene-specific oligonucleotides used in the analysis are listed in Supplementary Table S1.

Statistical analysis

Statistical analyses were performed using Prism 6 and SPSS 26.0. All of the experiments were repeated at least three times. Statistically significant differences were determined by subjecting the data to one-way ANOVA. The data are reported as the mean value ± SE. * indicates \( P < 0.05 \), and ** indicates \( P < 0.01 \).

Acknowledgements

This work was supported by grants from the Fundamental Research Funds for the Central Universities (2662019PY048) and the National Natural Science Foundation of China (31772313, 31972421, and 31991182). We thank Professor Robert M. Larkin and Xiangzong Meng for critical reading of our manuscript.

Author contributions

T.W., D.L., H.P. and Z.Y. conceived and designed the research. D.L., A.L., C.Z. and W.S. performed the experiments and carried out the fieldwork. D.L. and C.K. analyzed the data and wrote the manuscript. J.Z., C.Y., Y.L., H.L., H.P. and T.W. provided advice related to the research. All the authors have confirmed the final version of the manuscript.

Conflict of interest

The authors declare no competing interests.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1038/s41438-021-00595-y.

Received: 27 October 2020 Revised: 24 March 2021 Accepted: 19 April 2021

Published online: 01 July 2021

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