



# Article Identification of the *Brassica Campestris* ssp. *Chinensis BcHY5* Gene Important for Hypocotyl Length

Yiran Li<sup>1,2</sup>, Ying He<sup>1,2</sup>, Wenyuan Lin<sup>1,2</sup>, Cheng Jiang<sup>1,2</sup> and Xilin Hou<sup>1,2,\*</sup>

- State Key Laboratory of Crop Genetics & Germplasm Enhancement, Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (East China), Engineering Research Center of Germplasm Enhancement and Utilization of Horticultural Crops, Nanjing Agricultural University, Nanjing 210095, China
- <sup>2</sup> Nanjing Suman Plasma Engineering Research Institute, Nanjing 210095, China
- \* Correspondence: hxl@njau.edu.cn

**Abstract:** The primary domain/leucine zipper (bZIP) transcription factor, Elongated Hypocotyl (HY5), is crucial for the photomorphogenesis of seedlings. Here, we identified *BcHY5* as a regulator of hypocotyl length from the non-heading Chinese cabbage (NHCC) variety 'Suzhouqing', which encoded a protein comprised 164 amino acid residues. Ectopic expression of *BcHY5* in *Arabidopsis* shortens the length of the hypocotyl. Additionally, we discovered a protein called BcBBX24 containing the B-BOX (BBX) domain, which is the interacting partner of BcHY5. Yeast two-hybrid (Y2H), bimolecular fluorescence complementation (BiFC) and GST pull-down assays revealed that BcHY5 interacted with BcBBX24. Additionally, by physically binding to the promoter of *BcHY5*, BcBBX24 inhibited the transcriptional activity of BcHY5. Therefore, our findings reveal a transcriptional mechanism through which light response occurs in NHCC seedlings, where BcHY5 can interact with BcBBX24 can prevent *BcHY5* transcription.

Keywords: non-heading Chinese cabbage; BcHY5; photomorphogenesis; hypocotyl; BcBBX24



Light is one of the most important abiotic factors for plant growth and development. Plants have developed a sophisticated signaling network that includes photoreceptors, transcription factors, and downstream targets to take in light and produce their nutrition. Phytochromes (phyA-phyE) absorb red/far-red light, cryptochromes (cry1 and cry2) absorb blue/UV-A light, phototropin (phot1 and phot2) absorb blue light, and UVR8 photoreceptor absorbs UV-B light, dividing photoreceptors into four categories based on wavelength [1–4].

HY5, a bZIP protein, is essential for plant photomorphogenesis and takes part in various developmental processes, such as the development of hypocotyls, hormone regulation, and anthocyanin synthesis. In addition, it regulates nearly one-third of gene expression in *Arabidopsis thaliana* [5–8]. Constitutively photomorphogenic 1 (COP1), repressing photomorphogenesis in darkness, is a critical negative regulator in light signaling. It is an E3 ubiquitin ligase that aims to degrade the transcription factor HY5 in the dark. It then inhibits HY5 from interacting with other proteins or binding to downstream promoters, thereby regulating hypocotyl length [9,10]. *HY5* regulates hypocotyl by coordinating hormones, such as gibberellins (GAs), brassinosteroids (BRs), cytokinins (CTKs), abscisic acid (ABA), strigolactones (SLs), ethylene, and auxin (AUX), which promote photomorphogenesis and skotomorphogenesis [11–17]. Furthermore, *MYB12*, *MYB75/PAP1*, and *MYB111*, which influence the synthesis of anthocyanin, can all be expressed by direct binding of HY5 to the G-box or ACE-box of the promoter region of MYB family genes [7,18,19].

The Zn finger proteins in the *Arabidopsis* BBX family have one or two conserved B-box domains at the N-terminus [20]. There are 32 BBX family members, among which *BBX20*, *BBX21*, *BBX22*, and *BBX23* have been reported as positive regulators of photomorphogenesis, while *BBX24* and *BBX25* are negative regulators [20–26]. *BBX24* was initially identified



Citation: Li, Y.; He, Y.; Lin, W.; Jiang, C.; Hou, X. Identification of the *Brassica Campestris* ssp. *Chinensis BcHY5* Gene Important for Hypocotyl Length. *Agronomy* **2022**, *12*, 2573. https://doi.org/10.3390/ agronomy12102573

Academic Editor: Ali Raza

Received: 16 September 2022 Accepted: 12 October 2022 Published: 20 October 2022

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). from *Arabidopsis* as a protein conferring salt tolerance in yeast. Nevertheless, most of its functions are related to light signaling in *Arabidopsis*, interacting with HY5 and inhibiting the binding of *HY5* to downstream promoters [27].

NHCC (*Brassica campestris* ssp. *Chinensis*), native to China, is a popular cruciferous vegetable. It is uncertain whether BcHY5 employs comparable pathways in NHCC, despite the fact that research on plant photomorphogenesis, such as that of apple, tomato, and pear, has advanced significantly [28–31]. Our study identified the key gene *BcHY5* for photomorphogenesis in 'Suzhouqing' and constructed the *BcHY5* overexpression transgenic lines with shortened hypocotyls. In addition, we verified the physical interaction between BcHY5 and BcBBX24 through molecular biological approaches. Our findings provide new insights into the regulatory mechanisms of photomorphogenesis in NHCC and cruciferous crop breeding.

## 2. Materials and Methods

### 2.1. Plant Materials, Growth Conditions, and Treatments

The seeds of 'Suzhouqing' (*B. rapa* ssp. *chinensis* var. *communis* Tesnet Lee, cv., common type) were provided by the Cabbage Systems Biology Laboratory of the College of Horticulture, Nanjing Agricultural University (Nanjing, China), which were grown in illumination incubators under light 16 h/24 °C and dark 8 h/18 °C. The gene expression after treatments with salt (0.2 M NaCl), darkness, or ABA (0.1 M ABA) was studied in seedlings at the four-leaf stage. Leaves of the plants were collected after treatment for 0, 2, 4, 8, 12, and 24 h. The various treatment samples were sampled and frozen immediately in liquid nitrogen and stored at -70 °C. For each time point, three distinct leaves were independently replicated three times. In this study, the seeds of *hy5* were purchased from Arashare (https://www.arashare.cn/index/News/info/id/157.html, accessed on 10 August 2020). *Nicotiana benthamiana* and *Arabidopsis thaliana* wild-type (WT) were grown in illumination incubators under the same conditions.

# 2.2. Cloning and Analysis of BcHY5

The CDS (coding sequence) of the BcHY5 gene from 'Suzhouqing' was cloned using the primers BcHY5-F and BcHY5-R for *BcHY5* [32]. By using the online BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 5 June 2020), the orthologues of BcHY5 were searched. Using the Expasy website (https://web.expasy.org/protparam/, accessed on 10 June 2020), the physicochemical characteristics of BcHY5 were obtained. DNAman (6.0) was used to carry out the multiple sequence alignments of homologous proteins. The conserved motifs were analyzed using the MEME website (http://memesuite.org/tools/meme, accessed on 10 June 2020). The neighbor-joining phylogenetic tree was contrasted with MEGA7.0 (1000 bootstrap replicates) (Auckland, New Zealand). The primers are listed in Table S1.

#### 2.3. Subcellular Localization of BcHY5 in Tobacco

The *BcHY5* CDS without termination was integrated into the plant expression vector pRI101-GFP with primers BcHY5-GFP-F and BcHY5-GFP -R (Table S1). The recombinant plasmid *BcHY5-GFP* and empty vector plasmid 35S:GFP were transformed into *Agrobacterium tumefaciens* strain GV3101 cells. Histone H2B-RFP fusion protein was used as the nuclear marker [33]. When the OD<sub>600</sub> of bacteria liquid is 0.8–1.0, the transformed strains were injected into tobacco leaves. After 60 h, we collected the images using confocal laser scanning microscopy (Zeiss, LSM 500, Oberkochen, Germany).

# 2.4. Total RNA Extraction and Gene Expression Analysis by Quantitative Real-Time PCR

RNA from NHCC and *Arabidopsis* was extracted using an RNA simple Total RNA Kit (Tiangen, Beijing, China). The cDNA was obtained using the PrimeScript<sup>™</sup> RT reagent Kit with gDNA Eraser (Takara, China). For gene expression analysis, qRT-PCR was performed using Hieff<sup>®</sup> qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). The NHCC gene

*BcActin* (BraC09g068080.1) and *Arabidopsis* gene *AtActin* (AT1G80000) were used as reference genes. Relative gene expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method [34]. The primers are listed in Table S1.

# 2.5. Yeast Two-Hybrid Assay

The full-length CDS of *BcBBX24* was acquired from the 'Suzhouqing' cDNA using PrimerSTAR Max Premix (Takara, Dalian, China) and cloned into pGADT7 (AD) by specific primers BcBBX24-AD-F/R (Table S1). The CDS of *BcHY5* was cloned into pGBKT7 (BD) by specific primers BcHY5-BD-F/R (Table S1). To verify whether they were interact, we transformed recombinant plasmids (BcBBX24-AD, BcHY5-BD) and negative controls plasmid (AD, BD) into Y2H Gold yeast cells (Clontech, Mountain View, CA, USA) using the lithium acetate-mediated method. Meanwhile, the positive control used pGBKT7-53 and pGADT7-7. The transformed yeast strains were grown on SD/-Leu-Trp medium for 2–3 days and SD/-Leu-Trp-Ade-His (X- $\alpha$ -gal) medium for 3–4 days at 28 °C, respectively, and then observed and photographed.

# 2.6. BiFC Assay in Tobacco

Using specific primers BcHY5-nYFP-F/R and BcBBX24-cYFP-F/R, the CDSs of *BcHY5* and *BcBBX24* were constructed into pBiFC-VN173 and pBiFC-VC155, respectively (Table S1). The recombinant plasmids were subsequently transformed into *Agrobacterium tumefaciens* strain GV3101 cells. As a control, the pBiFC-VN173 and pBiFC-VC155 were employed. After being injected into tobacco leaves for 60 h, the fluorescence was observed by confocal laser scanning microscopy (Zeiss, LSM 500, Oberkochen, Germany).

# 2.7. GST Pull-Down Assay

The CDSs of *BcHY5* and *BcBBX24* were constructed into pET32a-His and pGEX4T1-GST by specific primers BcHY5-His-F/R and BcBBX24-GST-F/R, respectively (Table S1). Protein with a His-tagged or GST-tagged was expressed in *Escherichia coli BL21* strain in vitro and purified using a Ni-NTA Agarose Resin (Yeasen, Shanghai, China) and Glutathione Agarose Resin (Yeasen, Shanghai, China). The Glutathione Agarose Resin (Yeasen, Shanghai, China) was incubated with two purified proteins at 4 °C for 4 h. After 3 min of collection at 2500 rpm, the SDS loading buffer was added and brought to the boil. Afterward, samples were separated on 10% SDS-PAGE gel and were then transferred to a PVDF membrane. Anti-GST (Sangon, Shanghai, China) and Anti-His (Sangon, Shanghai, China) were used at a 1:1000 dilution, followed by the Anti-rabbit HRP secondary antibody 1:5000 (Sangon, Shanghai, China).

# 2.8. Overexpressing Transgenic Lines in Arabidopsis

We used the floral-dip method to transform *BcHY5-GFP/BcBBX24-GFP* into *Arabidopsis thaliana*. [35]. Resistance screening and gene relative expression detection were used to identify the transgenic lines, after which the phenotypes were evaluated.

#### 2.9. Hypocotyl Length Measurement

The sterilized seeds were sown on Murashige Skoog (MS) plates, kept in the dark at 4 °C for 3 days, and then moved to white light for 8 h to induce uniform germination. The seeds were then placed in a lighted incubator for cultivation. 6-day-old seedlings were scanned with a scanner, and the hypocotyl length was measured using ImageJ software v 1.8.0.

# 2.10. Yeast One-Hybrid Analysis

A 381 bp sequence upstream of the transcription start site of *BcHY5* was defined as the promoter region of *BcHY5*. BcBBX24 binding cis-acting elements on the *BcHY5* promoter were predicted by JASPAR (http://jaspar.genereg.net/, accessed on 10 October 2020).

Yeast one-hybrid assay was performed using the Matchmaker Gold Yeast One-Hybrid System Kit (TaKaRa, Dalian, China). Briefly, promoter fragments were ligated into the pAbAi vector by specific primers pAbAi-proBcHY5-F/R, and the BcBBX24 was cloned into the pGADT7 vector (AD). The pAbAi vector was linearized and transformed into the yeast strain Y1HGold. Transformants were selected on a medium containing SD/-Ura. The prey vectors were then transformed into Y1HGold cells containing pAbAi baits and tested on SD/-Leu/AbA plates. The primers used are listed in Table S1.

#### 2.11. Dual-Luciferase Assay

Dual-luciferase assay was performed with reference to Hellens [36]. The *BcBBX24* full-length CDS was cloned into the pRI101-GFP vector, while the promoter sequence was inserted into the pGreenII 0800-LUC vector. The recombinant plasmid was transformed into the *Agrobacterium* strain GV3101 (harboring the pSoup vector). The combined *Agrobacterium* solutions were injected into the tobacco leaves. Luciferase activity was detected after 60 h using a Dual-Luciferase Reporter Assay System (Princeton, PIXIS 1024B) and Dual-Luciferase Reporter Gene Assay Kit (Yeasen, China). The primers used are listed in Table S1.

## 2.12. Statistical Analysis

Experiments were carried out with three biological repeats and three technical repeats. Student's *t*-test was used to analyze the significant differences between each treatment.

#### 3. Results

# 3.1. The Characteristics of BcHY5

There has been a lot of reporting that HY5 plays a pivotal role in the growth and development of plants [6]. Therefore, according to the CDS of *AtHY5*, we obtained the CDS from the non-heading Chinese cabbage database after alignment, cloned from NHCC, and named *BcHY5*, which is the orthologue of *AtHY5*. The results showed that the length of *BcHY5* was 495 bp, encoding 164 amino acids. The molecular weight was 18.02 kDa, and its *pI* was 10.01. Multiple sequence alignments of HY5-like proteins from different plants, including BcHY5, showed that they had a conserved bZIP domain (Figure 1a).

The results of multiple sequence alignment showed that the homology of BcHY5 protein with MdHY5, PpHY5, NbHY5 and SlHY5 was 73.17%, 73.78%, 73.89% and 72.61%, respectively, while the homology with AtHY5, RsHY5, BrHY5, and BnHY5 was 93.25, 97.56, 97.56 and 99.38%, respectively, which further confirmed the conservation of cruciferous plants in the evolutionary process (Figure 1b, Table S2). In addition, the result of motif analysis showed that the identical motif existed in BcHY5, BrHY5, BnHY5, and RsHY5, which was similar to that in AtHY5. More specifically, motifs 2 and 4 located in the N-terminal region are highly conserved in all HY5 proteins, while NbHY5, SlHY5, PpHY5, and MdHY5 lack a motif 5 at the C-terminus (Figure 1c).

### 3.2. Subcellular Localization of BcHY5 Protein and Expression Patterns

To verify the subcellular localization of the BcHY5 protein, we constructed the 35S:BcHY5-GFP and expressed it in tobacco leaves alongside 35S: GFP as a control. The results showed that the BcHY5-GFP signal could be observed only in the nucleus of tobacco cells and overlapped with the Histone H2B-RFP nucleus marker protein (Figure 2a) [33].

HY5 is a vital gene connecting light and hormones [6]. It has been reported that HY5 is involved in the ABA signal pathway, light signal, and salt stress [37–39]. To explore the biological function of BcHY5, qRT-PCR was performed to examine the expression patterns of *BcHY5* in NHCC (Figure 2b). Influenced by light and circadian rhythm, the expression of *BcHY5* without any treatment increased first and then decreased, reaching the maximum at 8 h, which was 2.5 times that at 0 h (Figure S1). The expression of *BcHY5* was reduced within the first 2 h. The expression level of *BcHY5* started to increase dramatically with the lengthening of the treatment after 4 h. After spraying ABA for 12 h, the expression increased 9.2 times that before treatment. During the first two hours in the dark, there

was little change in the expression of *BcHY5*. The expression of *BcHY5* started to rise after 4 h of treatment and peaked at a level 13.7 times that before treatment. In the case of salt treatment, expression of *BcHY5* decreased first, then increased, and then reduced, reaching its peak 4 h after treatment.



**Figure 1.** The characteristics of BcHY5 protein. (a) Amino acid sequence alignment of HY5-like proteins in *Brassica oleracea* (BoHY5, XP\_013609113.1), *Raphanus sativus* (RsHY5, NP\_001330553.1), *Brassica rapa* (BrHY5, XP\_009121971.1), *Arabidopsis thaliana* (AtHY5, NP\_568246.1), *Solanum lycopersicum* (SIHY5, NP\_001234820.1), *Malus domestica* (MdHY5, NP\_001280752.1), *Nicotiana tabacum* (NtHY5, OIT35567.1), and *Pyrus ssp* (PpHY5, QRR19189.1). (b) HY5 phylogenetic analysis in different species. (c) The motif analysis of HY5. The relevant sequence information is shown at the bottom of the figure, and the *p*-value represents the significance of each motif.



**Figure 2.** Subcellular localization of BcHY5 and expression patterns of BcHY5 in NHCC. (**a**) Subcellular localization of BcHY5. Histone H2B-RFP fusion protein was used as the nuclear marker. Scale bars =  $50 \ \mu\text{m}$ . (**b**) Expression patterns of *BcHY5* gene in NHCC. Error bars represent the standard deviation among independent replicates. Asterisks (\*\* p < 0.01, Student's *t*-test) indicate statistical significance.

# 3.3. Overexpression of BcHY5 Results in Shorter Hypocotyl Length in Arabidopsis

Two lines of *Arabidopsis* with *BcHY5* overexpression were obtained in order to investigate the role of *BcHY5* (Figure 3a). The result showed that the hypocotyl length of the transgenic lines was significantly shorter than that of the wild-type and *hy5*, and the hypocotyl length of the complement line *hy5* returned to normal (Figure 3b,c). *AtHY2* (AT3G09150) and *AtYUC8* (AT4G28720), two genes that have been identified as important regulators of hypocotyl length, were selected to investigate the expression differences between WT and transgenic lines [40–42]. As an auxin synthesis gene, *AtYUC8* is involved in the regulation of hypocotyl length, which can restore hypocotyl shortening caused by the *ztl* mutant, and is a positive regulator of hypocotyl length; *hy2* plants have significantly longer hypocotyl length. Therefore, *AtHY2* expression was increased in *BcHY5-OX* lines. The relative expression of *AtYUC8* was down 50% in two transgenic lines relative to WT, and the mRNA level of *AtHY2* was generally higher relative to WT (Figure 3d).



**Figure 3.** The *BcHY5*-overexpression in *Arabidopsis*. (**a**) The expression level of *BcHY5* in WT and transgenic lines. (**b**,**c**) Hypocotyl phenotypes and length of WT, *hy5*, and transgenic lines *BcHY5* of 6-day-old seedlings. (**d**) The analysis of genes' relative expression level related to hypocotyl length of 6-day-old seedlings. The unit of hypocotyl length is millimeters. Error bars represent the standard deviation among independent replicates. Asterisks (\*\* *p* < 0.01, Student's *t*-test) indicate statistical significance.

# 3.4. BcHY5 Interacts with BcBBX24

In *Arabidopsis thaliana*, BBX proteins can interact with HY5 [43]. We screened a BBX24 orthologous protein interacting with BcHY5 in NHCC using a yeast two-hybrid assay and named it BcBBX24 (Figure 4a). To further demonstrate the interaction between BcHY5 and BcBBX24, we fused BcHY5 to the N-terminus of the yellow fluorescent protein and BcBBX24 to the C-terminus of the yellow fluorescent protein and then co-expressed them in tobacco. Consequently, we observed a strong yellow fluorescent signal in the nucleus. Nevertheless, when BcHY5-nYFP and cYFP, or BcBBX24-cYFP and nYFP were co-expressed, tobacco cells showed no fluorescence (Figure 4b). Moreover, GST-tagged BcBBX24 was able to pull-down His-tagged BcHY5 in the pull-down assay (Figure 4c). These results proved that BcHY5 interacted with BcBBX24 in vitro.

#### 3.5. The Hypocotyl Length of BcBBX24-OX\*BcHY5-OX Transgenic Lines Returned to Normal

We generated the *BcBBX24-OX\*BcHY5-OX* transgenic lines to explore the relationship between BcBBX24 and BcHY5 further. The results showed that the hypocotyl length of *BcBBX24-OX* was longer than WT, but the hypocotyl length of double overexpressing transgenic lines had been reduced and was near WT (Figure 5a,b). Two genes associated with hypocotyl length had significant changes in their expression (Figure 5c). These results identified that BcHY5 could repress the expression of BcBBX24.



**Figure 4.** BcHY5 interacts with BcBBX24. (**a**) Interaction between BcHY5 and BcBBX24 in the yeast two-hybrid assay. The full-length *BcHY5* was fused with the GAL4 DNA-binding domain (BD) and GAL4 activation domain (AD) fusions of BcBBX24. The yeast cells were grown on SD-TL and SD-TLHA with added  $\alpha$ -Gal. (**b**) Bimolecular fluorescence complementation (BiFC) assay. BcHY5 was fused with the N-terminal (nYFP) portion of YFP, while BcBBX24 was fused with the C-terminal (cYFP) portion of YFP. Different constructs were co-transformed into tobacco leaves, and fluorescence was observed under a confocal microscope. YFP, yellow fluorescent protein. Bars, 100 µm and 50 µm. (**c**) GST pull-down assay. GST-tagged BcBBX24 was co-incubated with His-tagged BcHY5, immunoprecipitated with GST resin, and detected using anti-His antibody and anti-GST antibody.



**Figure 5.** The *BcHY5* \* *BcBBX24*-overexpression in *Arabidopsis.* (**a**,**b**) Hypocotyl phenotypes and length of Col-0, *BcBBX24-OX*, and *BcBBX24-OX* \* *BcHY5-OX* transgenic lines of 6-day-old seedlings. (**c**) The analysis of genes' relative expression level related to hypocotyl length of 6-day-old seedlings. The unit of hypocotyl length is millimeters. Asterisks (\*\* p < 0.01, Student's *t*-test) indicate statistical significance.

а

С

#### 3.6. BcBBX24 Binds to the Promoter of BcHY5

BBX proteins can directly bind to the G-box elements of target genes [44,45]. We analyzed the promoter of *BcHY5* and discovered that it contained G-box sites in the *BcHY5* promoter, and we split the promoter of *BcHY5* into two segments based on the position of the G-box sites (Figure 6a). To verify the interaction between BcBBX24 and the *BcHY5* promoter, we performed a Y1H assay to confirm the interaction between BcBBX24 and the *BcHY5* promoter. The results showed that BcBBX24 could bind the promoter of *BcHY5*, and two G-box sites both could interact with BcBBX24 (Figure 6b). Also, dual-luciferase activity assaywas used to determine the BcBBX24 on the expression of BcHY5. We generated the *35S:BcBBX24-GFP* and pro*BcHY5-*pGreen II 0800-LUC. The luciferase activity of pro*BcHY5* was significantly lower in the co-expression combination of BcBBX24 and pro*BcHY5* compared with the empty load (Figure 6c,d). These results suggested that BcBBX24 was bound to the *BcHY5* promoter and negatively regulated its expression.



**Figure 6.** BcBBX24 binds to the *BcHY5* promoter and represses its expression. (a) Distribution of light-responsive cis-elements in the *BcHY5* promoter region. Blue triangles indicate G-boxes (G1-box: -220 bp to -226 bp; G2-box: -92 bp to -98 bp), and green triangles indicate the MYB motif. (b) Yeast one-hybrid assays of BcBBX24 and the promoter of *BcHY5*. (c) Representative images of transient expression in *Nicotiana benthamiana*. (d) Activation effects of the BcBBX24 on the *BcHY5* promoter. LUC/Ren, ratio to firefly to Renilla luciferase activity. Asterisks (\*\* p < 0.01, Student's *t*-test) indicate the statistical significance of the difference between the two groups.

# 4. Discussion

*HY5*, a major transcription factor for plant growth and development, regulates the expression of several genes via various pathways [6]. Previous studies have shown that HY5 regulates photomorphogenesis, temperature, and anthocyanin synthesis pathways [6].

In our study, *BcHY5*, an orthologue of *HY5*, was cloned from the non-heading Chinese cabbage 'Suzhouqing'. Sequence analysis revealed that *BcHY5* has a typical bZIP domain, participating in DNA-binding and transcription factor dimerization. The transgenic lines that overexpressed *BcHY5* had considerably shorter hypocotyl lengths than WT. This study is the first to show that the *BcHY5* gene regulates hypocotyl length in NHCC, enhancing our understanding of the seedlings' hypocotyl lengths. Different trends of ABA, dark, and

salt treatment generated *BcHY5* (Figure 2b). The functions of *BcHY5* upon exposure to abiotic stresses and ABA signaling also need further study.

In order to maintain its activity for light-mediated seedling growth, HY5 can interact with other proteins, the most typical of which are the BBX family proteins [27]. In Arabidopsis, the BBX family has 32 members. BBX24 has been identified as a negative regulator of photomorphogenesis and has been shown to respond to salt stress [25,46,47]. Our results suggested that BcHY5 interacted with BcBBX24 in vitro (Figure 4a). Interestingly, compared with *BcHY5-OX* lines and *BcBBX24-OX* lines, the hypocotyl length of *BcHY5-OX\*BcBBX24-*OX lines, the hypocotyl length of double overexpression lines, recovered to a normal level close to that of the wild-type. The related genes also changed, indicating that the two proteins inhibited each other's function in hypocotyl regulation. Studies have confirmed that BBX family proteins can bind to T/G-box elements in the promoters of downstream target genes [20]. We observed that the *BcHY5* promoter contains multiple G-box elements and speculated that BcBBX24 might bind to the *BcHY5* promoter. We demonstrated that BcBBX24 can bind to the promoter of BcHY5 and inhibit BcHY5 expression by yeast onehybrid assay, and the dual-luciferase assay. As expected, BcBBX24 was able to bind the promoter of *BcHY5*, and the luciferase activity assay showed that BcBBX24 could inhibit the expression of *BcHY5* (Figure 5b,c).

Previous studies have only explored the relationship between the two from the perspective of their interaction [25,27]. Our results confirmed that BcBBX24 not only interferes with the expression of BcHY5 via interacting with BcHY5 but also inhibits the transcription of *BcHY5* by binding to the promoter of *BcHY5*. Therefore, we proposed the possibility of regulatory pathways in non-heading Chinese cabbage between BcHY5 and BcBB24. First, BcHY5 interacts with BcBBX24 in a manner similar to that of *Arabidopsis*, which inhibits BcHY5 binding to the promoter of downstream genes to regulate the expression of downstream genes [48]. The second is that BcBBX24 binds to the promoter of *BcHY5* and prevents the transcription of *BcHY5*, which further affects the regulation of downstream genes. It is important to continue researching how the BcHY5-BcBBX24 module accurately controls downstream genes.

We have revealed that BcHY5 interacts with BcBBX24, a negative regulator of photomorphogenesis, to positively regulate hypocotyl length. These findings provide more possibilities for elucidating the mechanism of BcHY5 and BcBBX24 co-regulating the photomorphogenesis of NHCC (Figure 7). We will keep focusing on whether the components of the BcHY5-BcBBX24 module complete each other in the light signaling pathway.



Figure 7. The pattern of BcBBX24 and BcHY5 co-regulating the photomorphogenesis of NHCC.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102573/s1; Table S1: Primers were used in this study. Table S2: Proteins sequences of HY5. Figure S1: The expression of *BcHY5* without any treatment.

**Author Contributions:** Y.L. and X.H. planned and designed the research; Y.L., Y.H. and W.L. performed the experiments and analyzed the data; Y.L. wrote the manuscript; X.H. contributed to the project, the manuscript writing and revision; C.J. revised the manuscript; All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by The China Agriculture Research System (CARS-23-A-16), the Key Projects of the National Key Research and Development Plan (2017YFD0101803), Nanjing Science and Technology project (202109022).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank everyone who helped us in this study.

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

## References

- 1. Mawphlang, O.I.L.; Kharshiing, E.V. Photoreceptor Mediated Plant Growth Responses: Implications for Photoreceptor Engineering toward Improved Performance in Crops. *Front. Plant Sci.* **2017**, *8*, 1181. [CrossRef] [PubMed]
- Furuya, M.; Schäfer, E. Photoperception and signalling of induction reactions by different phytochromes. *Trends Plant Sci.* 1996, 1, 301–307. [CrossRef]
- 3. Cashmore, A.R.; Jarillo, J.A.; Wu, Y.J.; Liu, D. Cryptochromes: Blue light receptors for plants and animals. *Science* **1999**, *284*, 760–765. [CrossRef] [PubMed]
- 4. Chen, M.; Chory, J. Phytochrome signaling mechanisms and the control of plant development. *Trends Cell Biol.* **2011**, *21*, 664–671. [CrossRef] [PubMed]
- Oyama, T.; Shimura, Y.; Okada, K. The Arabidopsis HY5 gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. *Genes Dev.* 1997, 11, 2983–2995. [CrossRef]
- 6. Gangappa, S.N.; Botto, J.F. The Multifaceted Roles of HY5 in Plant Growth and Development. *Mol. Plant* **2016**, *9*, 1353–1365. [CrossRef]
- Shin, D.H.; Choi, M.; Kim, K.; Bang, G.; Cho, M.; Choi, S.B.; Choi, G.; Park, Y.I. HY5 regulates anthocyanin biosynthesis by inducing the transcriptional activation of the MYB75/PAP1 transcription factor in Arabidopsis. *FEBS Lett.* 2013, 587, 1543–1547. [CrossRef]
- Zhang, H.; He, H.; Wang, X.; Wang, X.; Yang, X.; Li, L.; Deng, X.W. Genome-wide mapping of the HY5-mediated gene networks in Arabidopsis that involve both transcriptional and post-transcriptional regulation. *Plant J.* 2011, 65, 346–358. [CrossRef]
- 9. Hardtke, C.S.; Gohda, K.; Osterlund, M.T.; Oyama, T.; Okada, K.; Deng, X.W. HY5 stability and activity in arabidopsis is regulated by phosphorylation in its COP1 binding domain. *EMBO J.* 2000, *19*, 4997–5006. [CrossRef]
- Chen, Z.; Huang, Y.; Yang, W.; Chang, G.; Li, P.; Wei, J.; Yuan, X.; Huang, J.; Hu, X. The hydrogen sulfide signal enhances seed germination tolerance to high temperatures by retaining nuclear COP1 for HY5 degradation. *Plant Sci.* 2019, 285, 34–43. [CrossRef]
- 11. Chen, H.; Zhang, J.; Neff, M.M.; Hong, S.W.; Zhang, H.; Deng, X.W.; Xiong, L. Integration of light and abscisic acid signaling during seed germination and early seedling development. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 4495–4500. [CrossRef] [PubMed]
- De Lucas, M.; Daviere, J.M.; Rodriguez-Falcon, M.; Pontin, M.; Iglesias-Pedraz, J.M.; Lorrain, S.; Fankhauser, C.; Blazquez, M.A.; Titarenko, E.; Prat, S. A molecular framework for light and gibberellin control of cell elongation. *Nature* 2008, 451, 480–484. [CrossRef] [PubMed]
- Feng, S.; Martinez, C.; Gusmaroli, G.; Wang, Y.; Zhou, J.; Wang, F.; Chen, L.; Yu, L.; Iglesias-Pedraz, J.M.; Kircher, S.; et al. Coordinated regulation of Arabidopsis thaliana development by light and gibberellins. *Nature* 2008, 451, 475–479. [CrossRef] [PubMed]
- 14. Shi, Q.M.; Yang, X.; Song, L.; Xue, H.W. Arabidopsis MSBP1 is activated by HY5 and HYH and is involved in photomorphogenesis and brassinosteroid sensitivity regulation. *Mol. Plant* **2011**, *4*, 1092–1104. [CrossRef]
- 15. Rasmussen, A.; Mason, M.G.; De Cuyper, C.; Brewer, P.B.; Herold, S.; Agusti, J.; Geelen, D.; Greb, T.; Goormachtig, S.; Beeckman, T.; et al. Strigolactones suppress adventitious rooting in Arabidopsis and pea. *Plant Physiol.* **2012**, *158*, 1976–1987. [CrossRef]
- 16. Yu, Y.; Wang, J.; Zhang, Z.; Quan, R.; Zhang, H.; Deng, X.W.; Ma, L.; Huang, R. Ethylene promotes hypocotyl growth and HY5 degradation by enhancing the movement of COP1 to the nucleus in the light. *PLoS Genet.* **2013**, *9*, e1004025. [CrossRef]

- 17. Xu, D.; Li, J.; Gangappa, S.N.; Hettiarachchi, C.; Lin, F.; Andersson, M.X.; Jiang, Y.; Deng, X.W.; Holm, M. Convergence of Light and ABA signaling on the ABI5 promoter. *PLoS Genet.* **2014**, *10*, e1004197. [CrossRef]
- 18. Chattopadhyay, S.; Ang, L.H.; Puente, P.; Deng, X.W.; Wei, N. Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *Plant Cell* **1998**, *10*, *673–683*. [CrossRef]
- 19. Stracke, R.; Favory, J.J.; Gruber, H.; Bartelniewoehner, L.; Bartels, S.; Binkert, M.; Funk, M.; Weisshaar, B.; Ulm, R. The Arabidopsis bZIP transcription factor HY5 regulates expression of the PFG1/MYB12 gene in response to light and ultraviolet-B radiation. *Plant Cell Environ.* **2010**, *33*, 88–103. [CrossRef]
- 20. Gangappa, S.N.; Botto, J.F. The BBX family of plant transcription factors. Trends Plant Sci. 2014, 19, 460–470. [CrossRef]
- 21. Wei, C.Q.; Chien, C.W.; Ai, L.F.; Zhao, J.; Zhang, Z.; Li, K.H.; Burlingame, A.L.; Sun, Y.; Wang, Z.Y. The Arabidopsis B-box protein BZS1/BBX20 interacts with HY5 and mediates strigolactone regulation of photomorphogenesis. *J. Genet. Genom.* **2016**, *43*, 555–563. [CrossRef]
- 22. Xu, D.; Jiang, Y.; Li, J.; Lin, F.; Holm, M.; Deng, X.W. BBX21, an Arabidopsis B-box protein, directly activates HY5 and is targeted by COP1 for 26S proteasome-mediated degradation. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 7655–7660. [CrossRef] [PubMed]
- Chang, C.S.; Maloof, J.N.; Wu, S.H. COP1-mediated degradation of BBX22/LZF1 optimizes seedling development in Arabidopsis. *Plant Physiol.* 2011, 156, 228–239. [CrossRef] [PubMed]
- Zhang, X.; Huai, J.; Shang, F.; Xu, G.; Tang, W.; Jing, Y.; Lin, R. A PIF1/PIF3-HY5-BBX23 Transcription Factor Cascade Affects Photomorphogenesis. *Plant Physiol.* 2017, 174, 2487–2500. [CrossRef] [PubMed]
- Jiang, L.; Wang, Y.; Li, Q.F.; Bjorn, L.O.; He, J.X.; Li, S.S. Arabidopsis STO/BBX24 negatively regulates UV-B signaling by interacting with COP1 and repressing HY5 transcriptional activity. *Cell Res.* 2012, 22, 1046–1057. [CrossRef]
- 26. Gangappa, S.N.; Holm, M.; Botto, J.F. Molecular interactions of BBX24 and BBX25 with HYH, HY5 HOMOLOG, to modulate Arabidopsis seedling development. *Plant Signal. Behav.* **2013**, *8*, e25208. [CrossRef]
- Job, N.; Yadukrishnan, P.; Bursch, K.; Datta, S.; Johansson, H. Two B-Box Proteins Regulate Photomorphogenesis by Oppositely Modulating HY5 through their Diverse C-Terminal Domains. *Plant Physiol.* 2018, 176, 2963–2976. [CrossRef]
- Yang, C.; Xie, F.; Li, L. Phenotypic Study of Photomorphogenesis in Arabidopsis Seedlings. *Methods Mol. Biol.* 2021, 2297, 41–47. [CrossRef]
- 29. Humplik, J.F.; Tureckova, V.; Fellner, M.; Bergougnoux, V. Spatio-temporal changes in endogenous abscisic acid contents during etiolated growth and photomorphogenesis in tomato seedlings. *Plant Signal. Behav.* **2015**, *10*, e1039213. [CrossRef]
- Shalmani, A.; Fan, S.; Jia, P.; Li, G.; Muhammad, I.; Li, Y.; Sharif, R.; Dong, F.; Zuo, X.; Li, K.; et al. Genome Identification of B-BOX Gene Family Members in Seven Rosaceae Species and Their Expression Analysis in Response to Flower Induction in Malus domestica. *Molecules* 2018, 23, 1763. [CrossRef]
- 31. An, J.P.; Qu, F.J.; Yao, J.F.; Wang, X.N.; You, C.X.; Wang, X.F.; Hao, Y.J. The bZIP transcription factor MdHY5 regulates anthocyanin accumulation and nitrate assimilation in apple. *Hortic. Res.* 2017, *4*, 17023. [CrossRef] [PubMed]
- Li, Y.; Liu, G.F.; Ma, L.M.; Liu, T.K.; Zhang, C.W.; Xiao, D.; Zheng, H.K.; Chen, F.; Hou, X.L. A chromosome-level reference genome of non-heading Chinese cabbage [Brassica campestris (syn. Brassica rapa) ssp. chinensis]. *Hortic. Res.* 2020, 7, 212. [CrossRef] [PubMed]
- 33. Goodin, M.M.; Dietzgen, R.G.; Schichnes, D.; Ruzin, S.; Jackson, A.O. pGD vectors: Versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. *Plant J.* **2002**, *31*, 375–383. [CrossRef] [PubMed]
- 34. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001, 29, e45. [CrossRef] [PubMed]
- 35. Clough, S.J.; Bent, A.F. Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* **1998**, *16*, 735–743. [CrossRef]
- Hellens, R.P.; Allan, A.C.; Friel, E.N.; Bolitho, K.; Grafton, K.; Templeton, M.D.; Karunairetnam, S.; Gleave, A.P.; Laing, W.A. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods* 2005, 1, 13. [CrossRef]
- Yu, Y.; Wang, J.; Shi, H.; Gu, J.; Dong, J.; Deng, X.W.; Huang, R. Salt Stress and Ethylene Antagonistically Regulate Nucleocytoplasmic Partitioning of COP1 to Control Seed Germination. *Plant Physiol.* 2016, 170, 2340–2350. [CrossRef]
- Bhagat, P.K.; Verma, D.; Sharma, D.; Sinha, A.K. HY5 and ABI5 transcription factors physically interact to fine tune light and ABA signaling in Arabidopsis. *Plant. Mol. Biol.* 2021, 107, 117–127. [CrossRef]
- Osterlund, M.T.; Hardtke, C.S.; Wei, N.; Deng, X.W. Targeted destabilization of HY5 during light-regulated development of Arabidopsis. *Nature* 2000, 405, 462–466. [CrossRef]
- Saitoh, A.; Takase, T.; Abe, H.; Watahiki, M.; Hirakawa, Y.; Kiyosue, T. ZEITLUPE enhances expression of PIF4 and YUC8 in the upper aerial parts of Arabidopsis seedlings to positively regulate hypocotyl elongation. *Plant Cell Rep.* 2021, 40, 479–489. [CrossRef]
- 41. Kim, B.C.; Soh, M.C.; Kang, B.J.; Furuya, M.; Nam, H.G. Two dominant photomorphogenic mutations of Arabidopsis thaliana identified as suppressor mutations of hy2. *Plant J.* **1996**, *9*, 441–456. [CrossRef] [PubMed]
- 42. Parks, B.M.; Quail, P.H. Phytochrome-Deficient hy1 and hy2 Long Hypocotyl Mutants of Arabidopsis Are Defective in Phytochrome Chromophore Biosynthesis. *Plant Cell* **1991**, *3*, 1177–1186. [CrossRef] [PubMed]
- Bursch, K.; Toledo-Ortiz, G.; Pireyre, M.; Lohr, M.; Braatz, C.; Johansson, H. Identification of BBX proteins as rate-limiting cofactors of HY5. *Nat. Plants* 2020, *6*, 921–928. [CrossRef] [PubMed]

- 44. Crocco, C.D.; Botto, J.F. BBX proteins in green plants: Insights into their evolution, structure, feature and functional diversification. *Gene* **2013**, *531*, 44–52. [CrossRef]
- Sarmiento, F. The BBX subfamily IV: Additional cogs and sprockets to fine-tune light-dependent development. *Plant Signal. Behav.* 2013, *8*, e23831. [CrossRef]
- 46. Nagaoka, S.; Takano, T. Salt tolerance-related protein STO binds to a Myb transcription factor homologue and confers salt tolerance in Arabidopsis. *J. Exp. Bot.* **2003**, *54*, 2231–2237. [CrossRef]
- 47. Yan, H.; Marquardt, K.; Indorf, M.; Jutt, D.; Kircher, S.; Neuhaus, G.; Rodriguez-Franco, M. Nuclear localization and interaction with COP1 are required for STO/BBX24 function during photomorphogenesis. *Plant Physiol.* **2011**, *156*, 1772–1782. [CrossRef]
- Gangappa, S.N.; Crocco, C.D.; Johansson, H.; Datta, S.; Hettiarachchi, C.; Holm, M.; Botto, J.F. The Arabidopsis B-BOX Protein BBX25 Interacts with HY5, Negatively Regulating BBX22 Expression to Suppress Seedling Photomorphogenesis. *Plant Cell* 2013, 25, 1243–1257. [CrossRef]