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Genome-Wide Identification and Expression Analysis of the BTB Domain-Containing Protein Gene Family in Sugar Beet

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Abstract: Cercospora leaf spots (CLSs) is a fungal disease of sugar beet caused by *C. beticola*, which damages leaves and leads to yield cut on sugar beet worldwide. BTB protein genes are critical to plant defense against bacterial infection. Here, 49 members of the BTB protein gene family were identified from the big data of the sugar beet genome, and bioinformatics was used to analyze the BTB protein family. Through molecular techniques, *C. beticola* of CLS was identified. In addition, the transcriptome data of sugar beet resistant and susceptible materials after *C. beticola* infection were obtained. Three BTB genes most significantly related to *C. beticola* stress were screened from the transcriptome data. The three genes are *BvBTB1*, *BvBTB2*, and *BvBTB3*, their full-length cDNA sequences were acquired by RT-PCR. The phenotypes of sugar beet resistant and susceptible materials under different spore concentrations of *C. beticola* were analyzed. Further, under the stress of *C. beticola*, qRT-PCR results showed that the expression levels of *BvBTB1*, *BvBTB2*, and *BvBTB3* in roots and leaves were tissue-specific and expressed differently in various tissues. *BvBTB1*, *BvBTB2*, and *BvBTB3* were overexpressed in the resistant and susceptible materials within five days after *C. beticola* infection: the peak appeared on the fifth day, and the highest expression was 25 times that of the control group. However, the lowest was 1.1 times of the control group, moreover, the expression in the resistant material was higher than that in the susceptible material. Overall, these results showed that *BvBTB* genes were involved in the response in sugar beet to *C. beticola* infection. Therefore, the study provided a scientific theoretical basis for developing new resistant varieties in sugar beet.

Keywords: sugar beet; *C. beticola*; BTB genes; *C. beticola* stress; expression

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1. Introduction

The BTB protein family is a new protein family, and it has been found in plants in recent years. The BTB protein family contains a conserved BTB protein-protein interaction motif, members of the family are diverse, there are 150 and 80 members of the BTB gene family in Arabidopsis and rice, respectively [1].

The BTB protein family has performed an important role in plant growth and development, disease resistance, stress resistance, protein ubiquitination and degradation, cytoskeleton composition, ion channel and cell cycle regulation [2–4]. *NPR1* is an important gene of BTB protein genes under plant stress. *NPR1* was first cloned from Arabidopsis [5–8], and it has been shown that overexpression of *NPR1* can improve disease resistance. *NPR1* regulated systemic acquired resistance (SAR) in Arabidopsis, the interaction between nuclear localized *NPR1* and TGA transcription factors, led to the activation of defense genes. Overexpression of *NPR1* in Arabidopsis increased the resistance to *Pseudomonas syringae*, *Peronospora parasitica*, and *Erysiphe cichoracearum* [9]. The high expression of transgene Arabidopsis *AtNPR1* in rice enhanced the resistance of rice to bacterial blight and rice

blast [10–12], overexpression of transgene Arabidopsis *AtNPR1* in tomato showed resistance to broad spectrum to pathogens [11], overexpression of transgene Arabidopsis *NPR1* improved wheat sharp eyespot resistance in transgenic wheat [13,14]. Overexpression of *MpNPR1* in transgenic apples increased the resistance to pathogenic bacteria *Erwinia amylovora*, *Venturia inaequalis*, and *Gymnosporangium juniperi-virginianae. MnNPR1A* and *MnNPR1B*, the members of *NPR1* subfamily in banana, were transferred into Arabidopsis *NPR1* mutant, it was found that the two genes can reactivate the resistance of *NPR1* mutant to pathogens, and the two genes induced the normal expression of PR1 protein [15,16]. In cucumbers, *CsBTB* presented different expression patterns in cucumber tissues, and *CsBTB* expression levels were regulated by cold stress, salt stress and drought stress [17]. *CaBPM4* silencing significantly reduced the resistance to Phytophthora capsici and root activity by changing the transcriptional levels of *CaPR1*, *CaDEF1*, and *CaSAR82* which were related to immunity and defense [18,19]. Overexpression of *AtNPR1* in monocotyledonous plants improved the ability to resist pathogens, which suggested that monocotyledons and dicotyledons may have the same *NPR1* disease resistance regulation pathway. The highly expressed *AtNPR1* gene was introduced into common wheat, in order to obtain transgenic a transgenic wheat strain with significantly improved sharp eyespot resistance, a highly expressed *AtNPR1* gene was driven by the ubiquitin promoter, which further verified the important role of *AtNPR1* gene in plant disease resistance [11]. Specifically, *GmBTB* was a new nuclear protein of the BTB domain, which positively regulated the response of soybean to *Phytophthora sojae* infection. Overexpression of *GmBTB* can improve the resistance to *Phytophthora sojae* [5].

Sugar beet (*Beta vulgaris* L.), one of the main sugaring raw materials, is the second largest sugar crop in the world, 25% of sucrose is provided by sugar beet. CLS is a fungal disease caused by *Cercospora beticola.* CLS is a worldwide disease that affects sugar beet yield and CLS occurs in all sugar beet planting countries [20]. It is reported that sugar beet yield loss caused by CLS reached 50% [21]. *C. beticola* is a destructive pathogen in sugar beet, in the long-term evolution of plants, and complex disease resistance mechanisms against pathogen infection have been formed [22]. However, the pathogenesis and molecular mechanism of sugar beet defense response to CLS are not clear. Up to now, there are few studies on BTB genes in sugar beet, and identifying resistance to CLS has not been found.

2. Materials and Methods

2.1. Materials and Treating Methods

For many years, among our research materials, resistant material 'F85621', has been one of the most common materials with resistance to *C. beticola*, and susceptible material 'KWS9147', has been one of the most sensitive to *C. beticola*, both self-fertile materials were specially provided by the Sugar Beet Resistance Breeding Laboratory of Heilongjiang University, Harbin, China. The two materials were used for expression analysis and gene isolation in this study. 'F85621' and 'KWS9147' beet plants were grown at 25 °C in a 16-h/8-h light/dark photoperiod with 70% relative humidity for 15 days.

C. Beticola was isolated from infected beet plants in Heilongjiang, China (Yang et al., 2017) and cultivated at 25 °C for 15 days on PDA medium.

Pathogen isolation: tissue isolation method [23]. The surfaces of the collected diseased leaves were cleaned with tap water and wiped with absorbent paper, then a 0.5 × 0.5 cm^2 tissue block was taken from the diseased leaf with a sterile scalpel, the tissue blocks were placed on a PDA plate and cultured alternately at 28 °C for 12 h/12 h under light and dark for 15 days. Then, according to the morphological observation of *C. beticola*, *C. beticola* was continuously selected from the fungus in the culture medium until it was completely isolated.

Purification of pathogen: the isolated fungus was eluted with sterile water and diluted to an average of only one spore per visual field, the spore was directly picked out on the PDA plate under the microscope and cultured alternately at 28 °C for 12 h/12 h under light and dark for 15 days for standby.

In this study, the ITS gene sequence was selected to amplify the isolated pathogenic strain. The ITS gene primers were designed by ourselves, and relevant articles were referred to while designing ITS gene primers [23,24]. The required primers are shown (Table 1), all primers were designed in this paper. The ITS sequence of the *C. beticola* strain was amplified by PCR, the PCR products were connected into λ DNA, and then the products of ligation were sent to the company for sequencing, *C. beticola* was obtained, 7×10^6 and 16×10^6 spores/mL spore suspensions were prepared.

Table 1. Primers for cloning and expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* and ITS primers.

Primer	Primer Sequences (5′–3′)	Tm
BvBTB1 Amplification primer	R: TTAAGAAATGGAGAACCTTCTTCTCCTTGG F: ATGATGAGTGCAACAGCGTTGAACCCT	62 °C
BvBTB2 Amplification primer	R: CTAATTTAGAGCATTAGGCTTGGTAAGTAACCT F: ATGGGGTCCAAGGAGTGGC	58 °C
BvBTB3 Amplification prime	R: GTTACCAAAGATGATCGAGTAGC F: TCTCCCAATGTTACACCACAAC	54 °C
BvBTB1 Fluorescent primer	R: TCGTTGGCTCCACTCCTTAGCTTTA F: GAGTACATGCCTCACAGAACAAGCG	60 °C
BvBTB 2 Fluorescent primer	R: AGTGGACTTGAGAAGCCCAAGAAGT F: AGTTGCTTTGGCTCTTGCTCATCTG	60 °C
BvBTB3 Fluorescent primer	R: TCCATCGATCGGAGAACAAAGCAGA F: ACTCCGATTCTCTCTCCTCCATTGC	60 °C
Gapdh Fluorescent primer	R: GTTGGAACACGGAA AGCC F: TGGAGAGGTGGAAGG	60 °C
ITS1 Amplification primer	TCCTCCGCTTATTGATATGC	60 °C
ITS4 Amplification primer	TCCGTAGGTGAACCTGCGG	60 °C

Three plants with the same growth and no mechanical damage were taken separately from the resistant and susceptible materials, the surfaces of plants were disinfected with 75% alcohol and then cleaned with sterile water, plants were recovered after one day. The spore concentrations of 7×10^6 spores/mL of *C. beticola* were used to infect the plants by spraying, after 12 h of infection, three leaves of the same part were taken separately from the two materials for RNA extraction and transcriptome analysis, according to the transcriptome data, three BTB genes with the most significant differences were selected.

The leaves of resistant and susceptible materials were sprayed with spore suspension with spore concentrations of 7×10^6 and 16×10^6 spores/mL of *C. beticola*, and the control group was sprayed with distilled water, the three plants with the same growth and no mechanical damage were taken separately in the same area of the small pot at each time period of 0, 120, 168, and 216 h for the test, at the same time, the samples were photographed to note their phenotypes.

'F85621' mRNA was used to amplify the full-length cDNA sequences of *BvBTB*. A high-fidelity enzyme from Takkara Bio, Dalian PrimeSTAR® Max DNA Polymerase, was used, and the reaction system was a total of 50 μL, including 25 μL Prime STAR Max Premix (2×), 10 μmol/L upstream and downstream primers 2 μL each, cDNA 2 μL (150~200 ng/μL), finally, making up to 50 μL with ddH_2O. The primers used are provided (Table 1). The PCR amplification conditions: 98 °C for 3 min; 98 °C for 10 s, 54, 58, and 62 °C for 15 s, 72 °C for 1 min, 30 cycles; 72 °C for 5 min.

2.2. Expression Analysis of BvBTB1, BvBTB2, and BvBTB3

The extraction of total RNA and reverse transcription were performed using TRIzol reagent (Invitrogen, Shanghai, China) and a ReverTra Ace Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The qRT-PCR was employed to measure the gene expression levels using a TB Green ® Premix EX Taq™ II collection kit (Takkara Bio, Dalian, China) with an ABI 7500 real-time fluorescence quantitative PCR instrument (Roche, Shanghai, China). The gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with

Gapdh as the internal control. The qPCR analyses were performed with three technical replicates. The primers used for expression analysis are shown (Table 1). The total reaction system was 20 μL, including 10 μL TB Green Premix Ex Taq II (2X), 10 μmol/L upstream and downstream primers 2 μL each, 2 μL ROX Reference Dye, 2 μL cDNA, making up to 50 μL with sterile H_2O. By using the Mx3000 p fluorescence quantitative PCR instrument for qRT-PCR, the reaction procedure is as follows: 95 °C for 30 s, 95 °C for 5 s, 60 °C for 34 s, 40 cycles; 95 °C 15 s, 60 °C 1 min, 95 °C 15 s. The significance was analyzed by the Spass software.

2.3. Bioinformatic Analysis of BTB Proteins and Genes

Sequences of all BTB protein genes are publicly available on NCBI, Bethesda, MD, USA. By using the SMART database (http://smart.embl-heidelberg.de, accessed on 21 June 2021), all proteins containing the BTB domain in sugar beet were obtained, verifying them through NCBI (https://www.ncbi.nlm.nih.gov, accessed on 23 June 2021) and Pfam database (http://pfam.sanger.ac.uk, accessed on 23 June 2021), finally, a total of 49 BTB protein genes and 49 proteins were obtained. The BTB protein and gene sequences were obtained. TBtools were used to predict the domain of the BTB protein family and to analyze cis elements of the BTB protein gene family.

The physical and chemical properties of the BTB protein family were analyzed by using the online software pI/MW (http://Web.Easy.Org/protparam, accessed on 29 June 2021).

2.4. Statistical Analysis

All experiments in this study were performed at least three times. Statistical significance between different measurements was examined by variance analysis. A difference was considered to be statistically significant when $p < 0.05$, different letters represent significant levels of $p < 0.05$.

3. Results

3.1. Analysis of Physicochemical Properties of the BTB Protein Family in Sugar Beet

According to the different domains in the BTB protein family in sugar beet, the BTB proteins were divided into nine subfamilies in sugar beet and played different roles, the nine subfamilies were: Ankyrin, Armadillo, MATH, NPH3, BACK, TAZ, TPR, BTB-only, and other. The physical and chemical properties of 49 members of the BTB protein family in sugar beet were analyzed by the online software pI/MW (http://Web.Easy.Org/protparam, accessed on 29 June 2021) (Table 2). Gene accession numbers of the BTB protein family are shown (Table 2). The number of amino acids encoded by 49 proteins ranged from 139 to 1120. The isoelectric points of 49 proteins ranged from 4.70 to 9.51, except for the proteins of the TAZ subfamily and NPH3 subfamily, the isoelectric points of other subfamilies were distributed between 5.5 and 7.63, which showed that most of the amino acids encoded by sugar beet BTB gene family are acidic amino acids. Except for BVRB—7 g 159950 and BVRB—05090, the average hydrophilicity and hydrophobicity of other proteins were negative, suggesting that most of the BTB protein family in sugar beet belonged to hydrophilic proteins. In addition, the number of phosphorylation sites of 49 BTB proteins ranged from 3 to 25, and the results of subcellular localization prediction showed that most proteins of the sugar beet BTB family were located in the nucleus, and the remaining proteins were located on the cell membrane and chloroplast respectively. The protein accession numbers of *BvBTB1*, *BvBTB2*, and *BvBTB3* were BVRB—5 g 109820, BVRB—000890, and BVRB—9 g 217510, respectively.

Table 2. Physical and chemical properties of the BTB protein family in sugar beet.

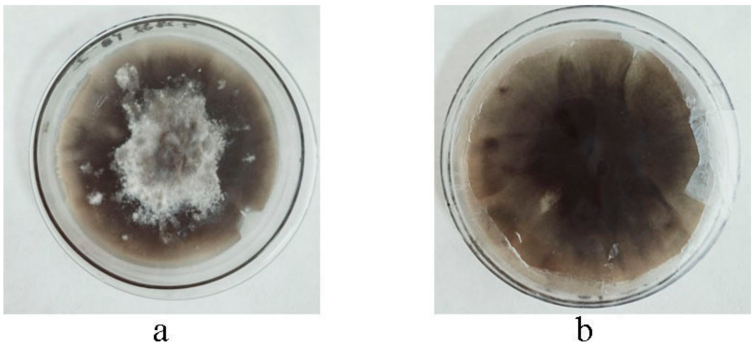
Gene Subfamily	Gene Accession Number	Protein Accession Number	Amino Acid Number	Isoelectric Point	Average Hydrophobicity	Number of Phosphorylation Sites	Subcellular Localization
NPH3	KMS95472.1	BVRB—007930	1120	5.91	−0.054	19	Nucleus
	KMT02116.1	BVRB—9 g 207600	539	6.68	−0.128	21	Nucleus
	KMT16927.1	BVRB—2 g 043970	615	7.50	−0.351	17	Nucleus
	KMT09829.1	BVRB—6 g 128050	625	7.54	−0.342	21	Nucleus
	KMT03832.1	BVRB—8 g 189750	621	6.73	−0.256	25	Cytoplasm
	KMT08787.1	BVRB—6 g 135110	1076	5.99	−0.722	24	Nucleus
	KMT13794.1	BVRB—4 g 081670	559	9.10	−0.258	13	Cytomembrane Nucleus
	KMT00896.1	BVRB—9 g 221680	695	6.88	−0.463	22	Cytomembrane
	KMT11299.1	BVRB—5 g 109820	581	8.18	−0.172	12	Cytomembrane Nucleus
	KMT09814.1	BVRB—6 g 127900	634	5.96	−0.185	15	Cytomembrane
	104899057	BVRB—7 g 164450	636	9.07	−0.292	15	Cytomembrane
	KMT05614.1	BVRB—7 g 167550	610	6.63	−0.327	23	Cytomembrane Nucleus
	KMT06491.1	BVRB—7 g 156670	617	6.00	−0.230	14	Cytomembrane
	KMT03943.1	BVRB—8 g 187520	633	5.74	−0.186	13	Cytomembrane
	KMT17316.1	BVRB—2 g 040100	688	5.64	−0.350	13	Cytomembrane Nucleus
	KMT09842.1	BVRB—6 g 128170	488	5.59	−0.301	11	Cytomembrane Nucleus
BACK	KMT11993.1	BVRB—5 g 099550	553	5.45	−0.265	9	Cytomembrane
	KMT20372.1	BVRB—1 g 003710	556	5.04	−0.369	10	Cytomembrane Chloroplast
	104898402	BVRB—7 g 159950	979	5.83	0.108	14	Nucleus
	104905652	BVRB—1 g 021120	805	5.95	−0.239	13	Cytomembrane
Arm	KMS95782.1	BVRB—005090	1012	6.88	0.068	15	Cytomembrane Nucleus
	KMT06143.1	BVRB—7 g 163130	709	6.14	−0.119	13	Nucleus
	104883420	BVRB—000890	698	6.13	−0.084	13	Nucleus
Ank	KMS99096.1	BVRB—3 g 066940	579	5.03	−0.260	11	Nucleus
	KMS96007.1	BVRB—003000	852	5.88	−0.453	15	Nucleus
	KMT15126.1	BVRB—3 g 062440	498	6.25	−0.273	12	Nucleus
	KMS96842.1	BVRB—8 g 199180	604	5.92	−0.271	13	Nucleus
MATH	KMT05007.1	BVRB—7 g 171670	398	5.72	−0.161	8	Nucleus
	KMS97744.1	BVRB—5 g 124160	407	7.16	−0.144	5	Nucleus
	KMS97745.1	BVRB—5 g 124170	405	5.98	−0.183	10	Nucleus
	KMT18392.1	BVRB—2 g 025440	420	5.98	−0.133	9	Nucleus

Table 2. *Cont.*

Gene Subfamily	Gene Accession Number	Protein Accession Number	Amino Acid Number	Isoelectric Point	Average Hydrophobicity	Number of Phosphorylation Sites	Subcellular Localization
TAZ	KMS97248.1	BVRB—7 g 176960	345	9.51	−0.323	5	Nucleus
	KMT09833.1	BVRB—6 g 128090	388	9.28	−0.321	8	Cytomembrane Nucleus
	KMT15541.1	BVRB—3 g 058930	139	9.18	−0.340	3	Nucleus
BTB-only	KMT11639.1	BVRB—5 g 109530	346	5.69	−0.330	8	Cytomembrane
	KMT12649.1	BVRB—4 g 091080	329	5.87	−0.141	8	Cytomembrane
	KMT19646.1	BVRB—1 g 010330	253	5.03	−0.313	8	Cytomembrane Nucleus
	KMT18872.1	BVRB—2 g 029750	279	5.43	−0.238	5	Cytomembrane Nucleus
TPR	KMT10233.1	BVRB—5 g 119930	886	5.72	−0.204	22	Nucleus
Other	KMS99416.1	BVRB—2 g 045380	484	5.35	−0.281	9	Chloroplast
	KMT12084.1	BVRB—5 g 100390	462	5.38	−0.152	17	Chloroplast
	KMT14083.1	BVRB—4 g 079080	558	6.56	−0.429	18	Chloroplast
	KMT08528.1	BVRB—6 g 138110	532	7.89	−0.434	16	Chloroplast Nucleus
	KMT12064.1	BVRB—5 g 100210	585	6.59	−0.394	20	Nucleus
	KMT05357.1	BVRB—7 g 174890	508	5.42	−0.359	16	Nucleus
	KMS93550.1	BVRB—030350	181	5.03	−0.160	4	Cytomembrane Nucleus
	KMT12599.1	BVRB—5 g 098380	433	4.70	−0.008	13	Nucleus
	KMT12434.1	BVRB—5 g 103650	436	4.93	−0.097	12	Nucleus
	KMT00450.1	BVRB—9 g 217510	481	6.48	−0.165	24	Nucleus

3.2. Isolation and Morphological Identification of C. beticola in Sugar Beet

Sugar beet diseased leaves with CLS cultured in PDA medium until *C. beticola* was isolated, and the morphology of *C. beticola* was observed and identified. The colony of *C. beticola* was round and gray white with a diameter of 0.5~6 μm, there was a dark brown and black matrix on the gray background of *C. beticola* (Figure 1a,b). The conidia of *C. beticola* were light brown, 2.5~4 μm wide, 50~200 μm long with few branches, and in the shape of a transparent needle, and had many diaphragms (Figure 1c,d).



a b

Figure 1. *Cont.*

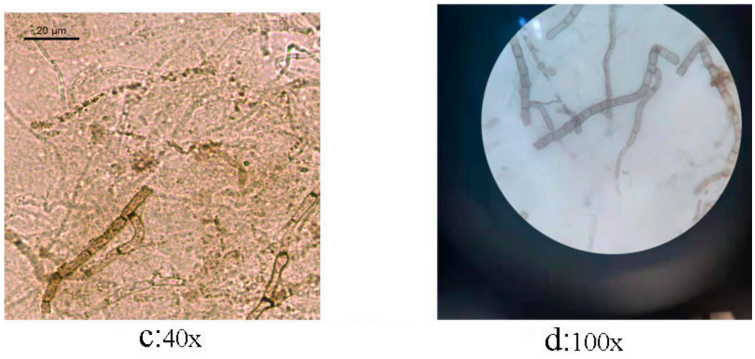


Figure 1. Colony and mycelium morphology of *C. beticola*. (**a**) The front morphology of the colony of *C. beticola*. (**b**) The back morphology of the colony of *C. beticola*. (**c**) Mycelial morphology of *C. beticola* under 40× microscope. (**d**) Mycelial morphology of *C. beticola* under 100× microscope.

3.3. Confirmation to C. beticola in Sugar Beet

The ITS sequence of the *C. beticola* strain was amplified by PCR (Figure 2), and the ITS sequence was obtained. The PCR products were connected to λ DNA, and then the products of ligation were sent to the company for sequencing. The results showed that the total length of CDS of the ITS gene was 700 bp, which was identified as *C. beticola* of CLS by molecular identification.

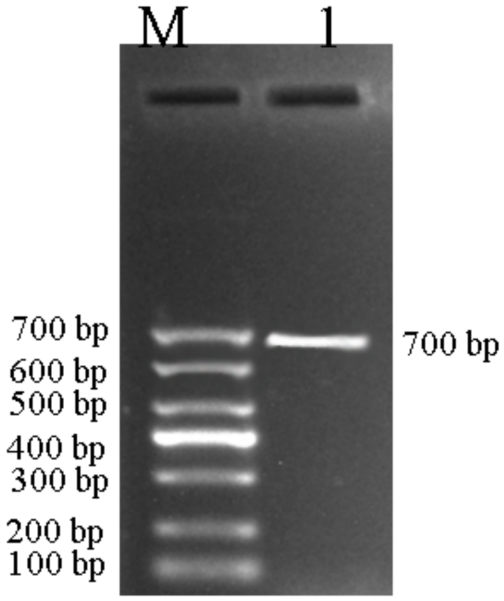


Figure 2. Agarose gel electrophoresis of PCR expansion of ITS sequence of *C. beticola*. M: DNA Marker I, the ladder was fabricated by Beijng Zoman Biotechnology Co., Ltd., Beijing, China; 1: ITS sequences of *C. beticola*, 700 bp.

3.4. Screening BvBTB Genes with Significant Differential Expression

The transcriptome data of the resistant material 'F85621' and susceptible material 'KWS9147' in sugar beet in response to the *C. beticola* infection were analyzed. The most significantly expressed *BvBTB1*, *BvBTB2*, and *BvBTB3* were found from the BTB protein gene family, expression of *BvBTB1* was the highest, it was about 252. In the resistant material, expression of *BvBTB1* was about 2 times that of *BvBTB2* and *BvBTB3*, while in susceptible materials, expression of *BvBTB1* was about 1.8 times that of *BvBTB2*, expression of *BvBTB1* was about 5.6 times that of *BvBTB3*. Further, expression of *BvBTB* genes in the resistant material was significantly higher than that in the susceptible material (Figure 3).

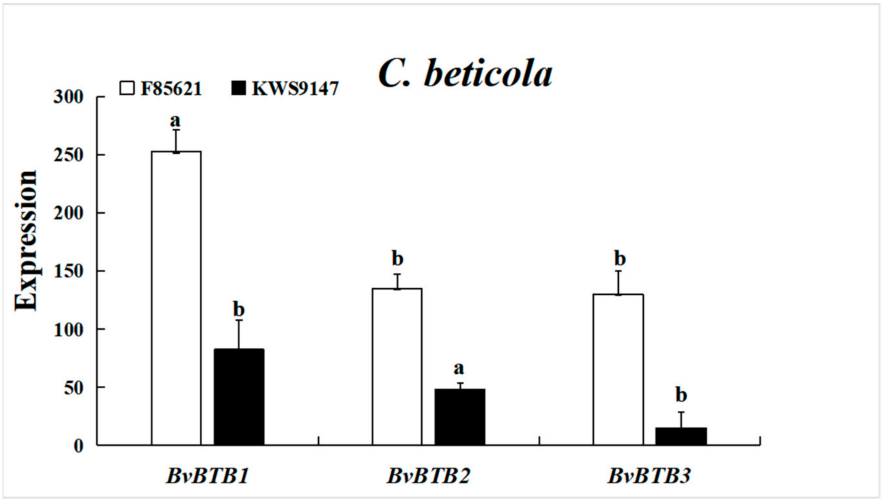


Figure 3. Expression of *BvBTB* genes in different transcriptomes in the materials. The transcriptomic data from sugar beet resistant material 'F85621'and susceptible material 'KWS9147' in response to *C. beticola* infection for 12 h. X-axial represents the gene names in the sugar beet transcriptomic data, while Y-axial represents *BvBTB* gene expression in the sugar beet transcriptomic data. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analyzed using variance analysis ($p < 0.05$), different letters represent significant levels of $p < 0.05$.

3.5. Agarose Gel Electrophoresis of PCR Amplification of BvBTB1, BvBTB2, and BvBTB3

Gene accession numbers of *BvBTB1*, *BvBTB2*, and *BvBTB3* were found in the NCBI, Bethesda, MD, USA database, which were KMT11299.1, 104883420, and KMT00450.1, respectively, the three full-length cDNA sequences were acquired, and their lengths were 1746, 2097, and 1518 bp, respectively, primers were designed according to the cDNA sequences for RT-PCR amplifying, the amplified bands were about 1000 (Figure 4a), 2097 (Figure 4b), and 1518 bp (Figure 4c), respectively.

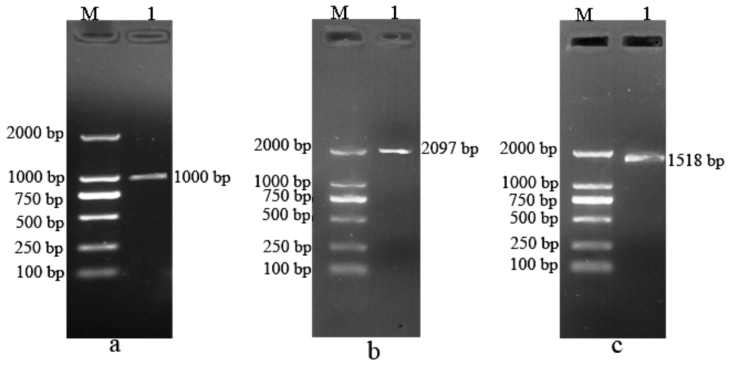


Figure 4. Agarose gel electrophoresis of PCR amplification of *BvBTB1*, *BvBTB2*, and *BvBTB3*. M: D2000 DNA marker, the ladder was fabricated by Tiangen Biochemical Technology (Beijing) Co., Ltd. (Beijing, China); (**a**) *BvBTB1*, (**b**) *BvBTB2*, (**c**) *BvBTB3*; 1: Target gene.

3.6. Conserved Domains of BvBTB1, BvBTB2, and BvBTB3 Proteins

Conserved domains of the *BvBTB* proteins were analyzed: *BvBTB1*, *BvBTB2*, and *BvBTB3* proteins all had a unique conserved domain BTB belonging to the BTB protein family. In addition to the BTB domain, *BvBTB1* protein also had an NPH3 domain, and *BvBTB2* protein also had eight ARM domains with unknown roles, however, *BvBTB3* protein had only the BTB domain (Figure 5).

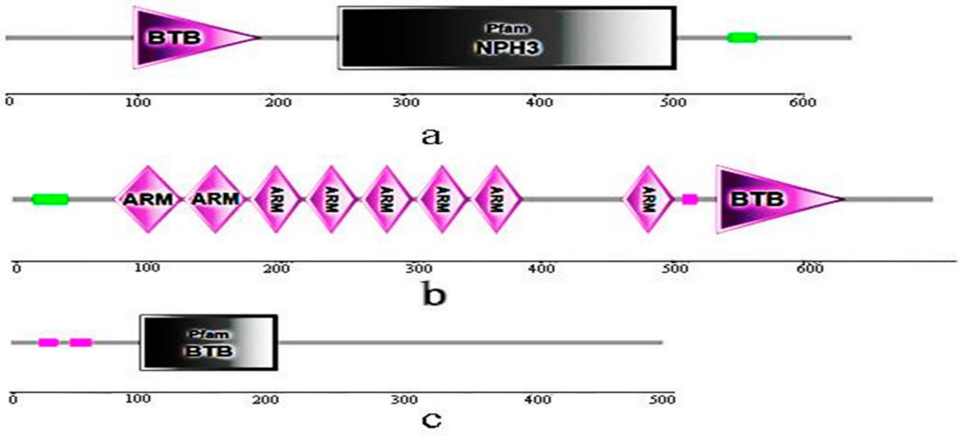


Figure 5. Conserved domains of *BvBTB1*, *BvBTB2*, and *BvBTB3* protein. (**a**) Conserved domain of *BvBTB1* protein. (**b**) Conserved domain of *BvBTB2* protein. (**c**) Conserved domain of *BvBTB3* protein.

3.7. Analysis of Promoter Elements of BvBTB1, BvBTB2, and BvBTB3

The promoter elements of *BvBTB* genes with 2000 bp upstream of the transcription beginning site were analyzed, and their regulatory roles at the transcription level were also analyzed. Most cis regulatory elements of *BvBTB* genes were screened, which were related to light, defense, stress, MeJA response, endosperm, meristem expression, and circadian rhythm control, such as G-boxlight element related to light response, TCA-element related to hormone and to salicylic acid response, and TC-rich repeats element related to stress response including defense and stress response. Among them, *BvBTB* genes contained the most important core TGA element related to bacterial defense (Figure 6).

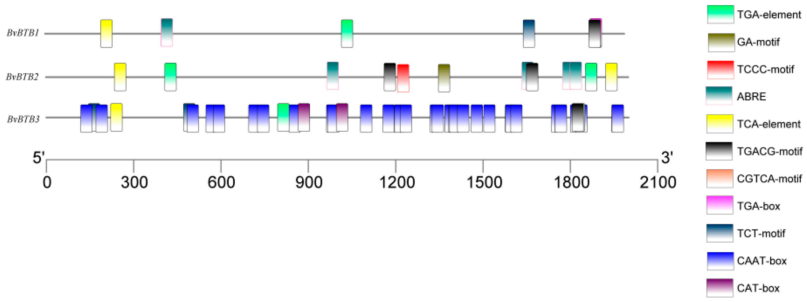


Figure 6. Analysis of promoter elements of *BvBTB*, *BvBTB2*, and *BvBTB3.*

3.8. Phenotypic Analysis of Sugar Beet Resistant and Susceptible Materials at Different Time under Different Spore Concentrations of C. beticola

The resistant material was 'F84621', the sugar beet susceptible material was 'KWS9147'. The leaves of resistant and susceptible materials were sprayed with spore suspension with spore concentrations of 7×10^6 and 16×10^6 spores/mL, the leaves were taken and photographed at 0, 5, 7, and 9 d to observe the phenotype. Phenotype: irregular brown spots first appeared on the leaves, and the disease spots gradually expanded from the outer leaves to the middle and inner leaves. The analysis showed that the disease degree of leaves treated with spore suspension with high concentrations was significantly higher than that treated with spore suspension with low concentrations, the disease severity of susceptible materials was higher than that of resistant materials, in addition, as treating time increases, the spread of CLS also hastened and the damage was also more serious (Figure 7).

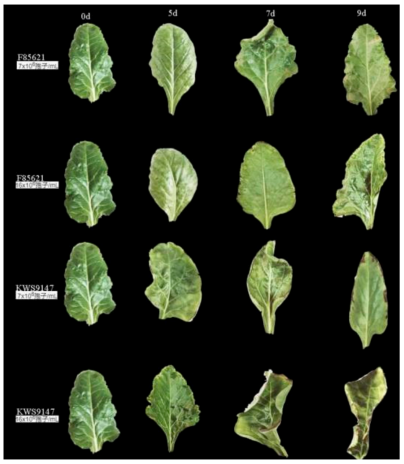


Figure 7. Phenotypic analysis of sugar beet resistant and susceptible material at different times under different spore concentrations of *C. beticola*.

3.9. Tissue Expression Patterns of BvBTB1, BvBTB2, and BvBTB3 in Sugar Beet Resistant Material 'F85621' and Susceptible Material 'KWS9147'

The relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* in roots and leaves in sugar beet resistant and susceptible materials were analyzed, the relative expression of *BvBTB1* in leaves in the resistant material 'F85621' was about 17 times that of roots, in the susceptible material 'KWS9147', the relative expression of *BvBTB1* in leaves was about 6 times that of roots, the relative expression of *BvBTB2* in leaves in the resistant and susceptible materials was about 6.5 times and 2.2 times that of roots, respectively; however, the relative expression of *BvBTB3* in leaves in the resistant and susceptible materials was about 5.8 times and 2.5 times that of roots, respectively. In conclusion, the results showed that the expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* in leaves was significantly higher than that in roots, and the relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* had significant tissue-specificity (Figure 8).

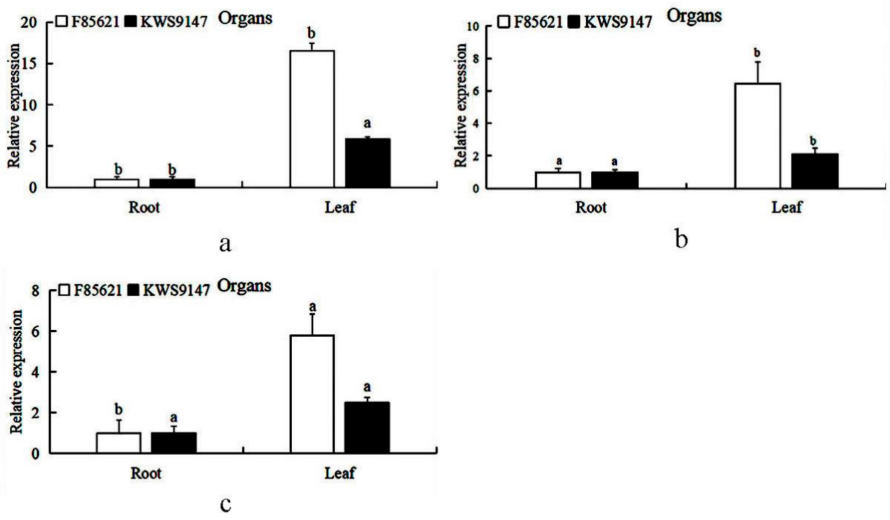


Figure 8. Tissue expression pattern of *BvBTB*, *BvBTB2*, and *BvBTB3* in sugar beet resistant material 'F85621' and susceptible material 'KWS9147'. (**a**) Tissue expression pattern of *BvBTB1* in sugar beet resistant material 'F85621' and susceptible material 'KWS9147'. (**b**) Tissue expression pattern of *BvBTB2* in sugar beet resistant material 'F85621' and susceptible material 'KWS9147'. (**c**) Tissue expression pattern of *BvBTB3* in sugar beet resistant material 'F85621' and susceptible material 'KWS9147'. The housekeeping gene of beet *Gapdh* was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analyzed using variance analysis ($p < 0.05$), different letters represent significant levels of $p < 0.05$.

3.10. The Expression Levels of BvBTB1, BvBTB2, and BvBTB3 after High and Low Spore Concentrations of C. beticola Infection at Different Times in Leaves in Sugar Beet Resistant Material 'F85621' and Susceptible Material 'KWS9147'

The sugar beet resistant material was 'F84621', while the sugar beet susceptible material was 'KWS9147'. The leaves of the resistant and susceptible materials were sprayed with spore suspension with spore concentrations of 7×10^6 and 16×10^6 spores/mL. Then, the leaves were taken at 0, 120, 168, and 216 h. The expression patterns of *BvBTB1*, *BvBTB2*, and *BvBTB3* after high and low spore concentrations of *C. beticola* infection at different times in leaves in the resistant material 'F85621' and susceptible material 'KWS9147' in sugar beet were analyzed by qRT-PCR. Under the stress of high and low spore concentrations of *C. beticola* within 216 h, the relative expression trend of *BvBTB1*, *BvBTB2*, and *BvBTB3* in leaves first increased rapidly, then quickly decreased, and finally remained the same as the initial amount of the control group. The three genes increased quickly at 120 h, which was significantly higher than the initial amount of the control group, reached the peak at 120 h, then decreased rapidly, finally stabilized, and finally decreased to the same level as the initial amount of the control group (Figure 9). Under the stress of high spore concentrations of *C. beticola*, the relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* in leaves in the resistant material reached the peak at 120 h, which was about 24, 10, and 5 times that of the control group. However, the relative expression in leaves in the susceptible material was about 10, 6, and 2 times that of the control group (Figure 9b,d,f). By comparison, the comprehensive analysis showed that the relative expression of *BvBTB1* was higher than that of *BvBTB2* and the expression of *BvBTB2* was higher than that of *BvBTB3*. Moreover, the relative expression of three genes after high spore concentrations of *C. beticola* infection was significantly higher than that under the stress of low spore concentrations of *C. beticola*. Similarly, the relative expression of the three genes in the resistant material was significantly higher than that in the susceptible material (Figure 9).

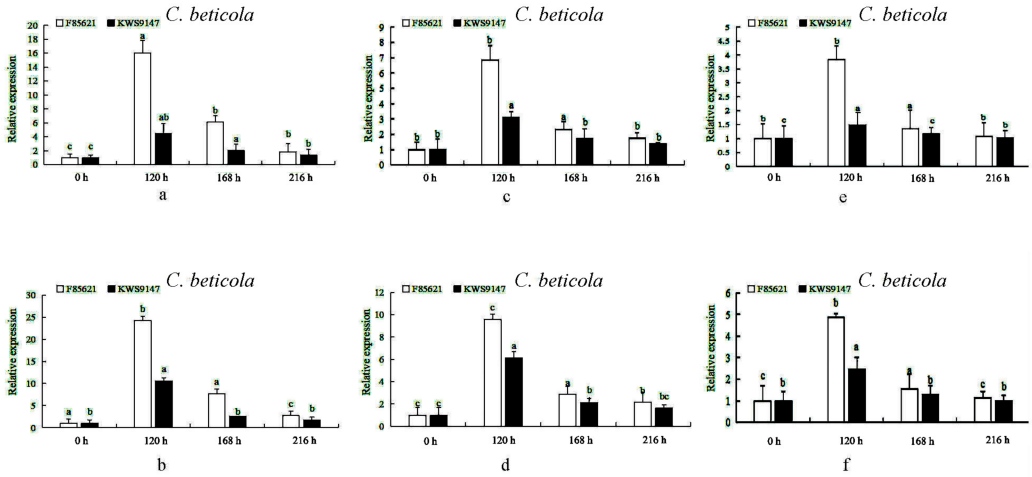


Figure 9. The relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* after high and low spore concentrations of *C. beticola* infection at different times in leaves in the resistant material 'F85621' and susceptible material 'KWS9147'. X-axial represents the times of *C. beticola* infection in sugar beet, while Y-axial represents the relative expression of *BvBTB* in sugar beet materials 'F85621' and 'KWS9147' on *C. beticola* infection. (**a**) The relative expression of *BvBTB1* in leaves at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after low spore concentrations of *C. beticola* with 7×10^6 spores/mL infection. (**b**) The relative expression of *BvBTB1* in leaves at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after high spore concentrations of *C. beticola* with 16×10^6 spores/mL infection. (**c**) The relative expression of *BvBTB2* in leaves at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after low spore concentrations of *C. beticola* with 7×10^6 spores/mL infection. (**d**) The relative

expression of *BvBTB2* in leaves at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after high spore concentrations of *C. beticola* with 16 × 10^6 spores/mL infection. (**e**) The relative expression of *BvBTB3* in leaves at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after low spores concentration of *C. beticola* with 7 × 10^6 spores/mL infection. (**f**) The relative expression of *BvBTB3* in leaves at 0, 120, 168, 216 h of sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after high spore concentrations of *C. beticola* with 16 × 10^6 spores /mL infection. The housekeeping gene of beet *Gapdh* was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analyzed using variance analysis ($p < 0.05$), different letters represent significant levels of $p < 0.05$.

3.11. The Expression Levels of BvBTB1, BvBTB2, and BvBTB3 after High and Low Spore Concentration of C. beticola Infection at Different Times in Roots in Sugar Beet Resistant Material 'F85621'and Susceptible Material 'KWS9147'

The sugar beet resistant material was 'F84621', while the sugar beet susceptible material was 'KWS9147'. The leaves of resistant and susceptible materials were sprayed with spore suspensions with spore concentrations of 7 × 10^6 and 16 × 10^6 spores/mL. Then, the roots were taken at 0, 120, 168 and 216 h. The expression pattern of *BvBTB1*, *BvBTB2*, and *BvBTB3* after high and low spore concentrations of *C. beticola* infection at different times in roots in the resistant material 'F85621' and susceptible material 'KWS9147' were analyzed by qRT-PCR. The sugar beet resistant material 'F85621' and susceptible material 'KWS9147' under the stress of high and low concentration of *C. beticola* within 216 h, the relative expression trend of *BvBTB1*, *BvBTB2*, and *BvBTB3* in roots first increased rapidly, then quickly decreased rapidly, and finally remained the same as the initial amount of the control group. The three genes increased quickly at 120 h, which was significantly higher than the initial amount of the control group, reached the peak at 120 h, then decreased rapidly, and finally stabilized and decreased to the same level as the initial amount of the control group (Figure 10). Under the stress of high spore concentration of *C. beticola*, the expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* in the roots in the resistant material reached the peak at 120 h, which was about 9.5, 4.3 and 3.3 times that of the control group. However, the relative expression in the roots of the susceptible material was about 7, 3, and 1.9 times that of the control group (Figure 10b,d,f). By comparison, the comprehensive analysis showed that the relative expression of *BvBTB1* was higher than that of *BvBTB2* and the expression of *BvBTB2* was higher than that of *BvBTB3*. Moreover, the expression of three genes after high spore concentration of *C. beticola* infection was significantly higher than that under the stress of low spore concentration of *C. beticola*. Similarly, the expression of the three genes in the resistant material was significantly higher than that in the susceptible material (Figure 10). The relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* after high and low spore concentrations of *C. beticola* infection at different times in leaves and roots in the sugar beet resistant material 'F85621' and susceptible material 'KWS9147' had similarities.

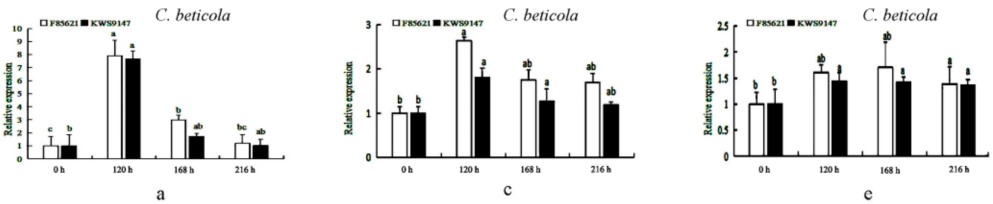


Figure 10. *Cont.*

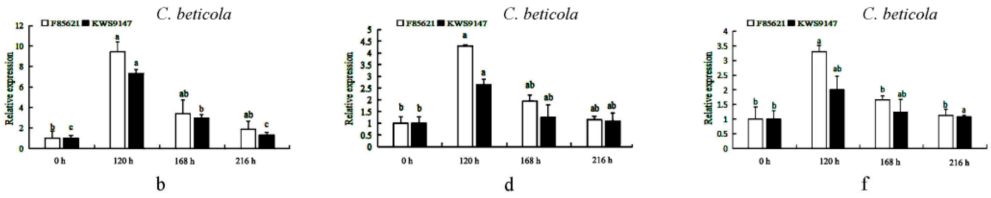


Figure 10. The relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* after high and low spore concentrations of *C. beticola* infection at different times in roots in the resistant material 'F85621' and susceptible material 'KWS9147'. X-axial represents the times of *C. beticola* infection in sugar beet, while Y-axial represents the relative expression of *BvBTB* in sugar beet materials 'F85621' and 'KWS9147' on *C. beticola* infection. (**a**) The relative expression of *BvBTB1* in roots at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after low spore concentrations of *C. beticola* with 7×10^6 spores/mL infection. (**b**) The relative expression of *BvBTB1* in roots at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after high spore concentrations of *C. beticola* with 16×10^6 spores/mL infection. (**c**) The relative expression of *BvBTB2* in roots at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after low spore concentrations of *C. beticola* with 7×10^6 spores/mL infection. (**d**) The relative expression of *BvBTB2* in roots at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after high spore concentrations of *C. beticola* with 16×10^6 spores/mL infection. (**e**) The relative expression of *BvBTB3* in roots at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after low spore concentrations of *C. beticola* with 7×10^6 spores/mL infection. (**f**) The relative expression of *BvBTB3* in roots at 0, 120, 168, 216 h in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' after high spore concentrations of *C. beticola* with 16×10^6 spores/mL infection. The housekeeping gene of beet *Gapdh* was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and was statistically analyzed using the variance analysis ($p < 0.05$), different letters represent significant levels of $p < 0.05$.

4. Discussion

In China, CLS is such a common leaf disease of sugar beet that in the main sugar beet producing areas, sugar beet yield was reduced or even lost, which seriously affected the stability of sugar beet yield. Here, cultivating varieties resistant to CLS is the main objective in sugar beet breeding [20,25]. Studies have shown that BTB genes are involved in the establishment of plant SAR and ISR, which are important for plants to produce resistance to broad-spectrum pathogens [17]. Based on the important role of BTB protein genes in plant, it was of great significance to analyze the structural composition of *BvBTB* genes and the expression induced by *C. beticola*.

Studies have shown that after being induced by exogenous signals, Arabidopsis NPRl protein interacted selectively with other proteins in the nucleus and activated genes related to SAR after being induced by exogenous signals [11], *NPR1* is an important gene of BTB protein genes under plant stress. Here, *BvBTB* genes were obtained from sugar beet resistant material, and bioinformatics analysis was carried out. BvBTB proteins have high similarity with BTB proteins in other plants reported, BvBTB proteins may interact with other downstream proteins through conserved domains in the nucleus to induce various disease resistance reactions. Furthermore, BvBTB proteins contained other domains besides the BTB domains, which suggested that besides retaining the basic functions of BTB genes, *BvBTB* genes might also have a new mechanism for further studies. The results of the yeast two-hybrid experiment and pull-down experiment showed that TGA1 and TGA4 belonging to class I, TGA2, TGA5, and TGA6 belonging to class II, and TGA3 and TGA7 belonging to class III can interact with *NPR1* [5], all *BvBTB* genes had TGA promoter elements, it was speculated that TGA elements interacted with *BvBTB* genes to improve plant disease resistance by combining TGA and PR promoter, which further explained the conservation of their interaction, moreover, most cis regulatory elements of *BvBTB* genes were screened.

BvBTB genes contained the most important core TGA element related to bacterial defense, when the plant is infected by pathogens, it is speculated that the *NPR1* monomer may enter the nucleus through acting nuclear localization domain, or *NPR1* may interact directly with some TGA transcription factors, which effectively induces the downstream resistance gene *PR* expression and established systemic acquired resistance.

Overexpression of *NPR1* in rice significantly improved its resistance to rice bacterial blight, after inoculation with Gibberella [14], the expression of wheat *TaNPR1* was up-regulated in response to the induction of Gibberella, which showed *NPRl* genes played an important role in plant resistance to many diseases. After rice black-streaked dwarf virus infected maize, the expression of *ZmNPR1* was rapidly up-regulated, and the constitutive expression of *ZmNPR1* was also detected in different parts in the later stage [26]. After *Phytophthora sojae* infection, the expression of *GmBTB* in the resistant and susceptible varieties first increased significantly to the peak, and then decreased sharply, and the expression levels of *GmBTB* in the tissues of soybean resistant varieties were much higher than that in the tissues of soybean susceptible varieties [5], which was similar to the expression trend of *BvBTB* genes after *C. beticola* infection, the relative expression of *BvBTB1* was higher than that of *BvBTB2*, and the relative expression of *BvBTB2* was higher than that of *BvBTB3*, the relative expression of three genes after high spore concentrations of *C. beticola* infection was significantly higher than that under the stress of low spore concentrations of *C. beticola*, and the relative expression of three genes in the resistant material was significantly higher than that in the susceptible material. The relative expression of *BvBTB1*, *BvBTB2*, and *BvBTB3* after high and low spore concentrations of *C. beticola* infection at different times in leaves and roots in sugar beet resistant material 'F85621' and susceptible material 'KWS9147' had similarities, it was speculated that *BvBTB* genes played a key role in the resistance to CLS and the growth and development in sugar beet. The relative expression of *BvBTB* genes reached the peak at 120 h, which was much greater than the initial amount of the control group, there was a significant difference between roots and leaves, it was speculated that the immune stress response caused by *C. Beticola* as a pathogen appeared in the early stage of sugar beet infection. The expression of *BvBTB* genes at high spore concentrations of *C. beticola* was significantly higher than that at low spore concentrations of *C. beticola*, it was speculated that the change in the relative expression of *BvBTB* genes was related to the number and growth distribution of *C. beticola.* QTL mapping showed that CLS was regulated by multiple quantitative trait genes, and the pathogenesis was complex [5]. At present, no target resistance gene has been cloned in sugar beet. In this study, the amplified bands of *BvBTB* genes were obtained, and the expression pattern induced by *C. beticola* was obtained, which laid a foundation for further study on the disease resistance mechanism of CLS.

5. Conclusions

In this study, the BTB Domain-containing protein gene family in sugar beet was identified, and bioinformatics was used to analyze the BTB protein family. Through molecular techniques, *C. beticola* of CLS was identified. In addition, transcriptome data of sugar beet resistant and susceptible materials after *C. beticola* infection was obtained, by contrast, the expression levels of *BvBTB1*, *BvBTB2*, and *BvBTB3* in roots and leaves in the resistant and susceptible materials after high and low spore concentrations of *C. beticola* infection were analyzed by qRT-PCR, which provided a theoretical basis for the in-depth study on BTB genes in sugar beet, and laid a foundation for developing new resistant varieties and transgenic resistant varieties of sugar beet.

Author Contributions: Conceptualization, Q.Y.; methodology, Q.Y. and Y.L. (Yu Liu); software, Q.Y. and Y.L. (Yu Liu); validation, C.Z., X.W. and Y.L. (Yanli Li); formal analysis, X.W. and Y.L. (Yanli Li); investigation, C.Z.; writing-original draft preparation, Q.Y.; writing-review and editing, Q.Y. and G.D.; supervision, G.D. and L.C.; funding acquisition, G.D. and L.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that none of the authors has competing interests.

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