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Genome-Wide Identification and Analysis of CC-NBS-LRR family in response to Downy Mildew and Black Rot in Chinese Cabbage

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Abstract: The nucleotide-binding site-leucine-rich repeat (NBS-LRR) gene family is the largest group of plant disease resistance (R) genes widespread in response to viruses, bacteria, and fungi are usually involved in effectortriggered immunity (ETI). 4 members of the Chinese cabbage CCtype NBS-LRR family were investigated in this study. Gene and protein characteristics, such as distributed locations on chromosomes and gene structures were explored through comprehensive analysis. CC-NBS-LRR proteins were classified according to their conserved domains, and the phylogenetic relationships of CC-NBS-LRR proteins in *Brassica rapa*, *Arabidopsis thaliana*, and *Oryza sativa* were compared. Moreover, the roles of *BrCC-NBS-LRR* genes involved in pathogenesis-related defense were explored. First, the expression profiles of *BrCC-NBS-LRR* genes in response to downy mildew and black were determined. Second, sensitive and resistant Chinese cabbage inbred lines were screened by downy mildew and black rot. Finally, the differential expression levels of *BrCC-NBS-LRR* genes were monitored at different treatment times. Our study provides information on *BrCC-NBS-LRR* genes for the investigation of the functions and mechanisms of CC-NBS-LRR genes in Chinese cabbage.

Keywords: Chinese cabbage; CC-NBS-LRR; Downy Mildew; Black Rot

1. Introduction

Plants have developed specific mechanisms to protect themselves from abiotic or biotic stress[1,2]. Plant pathogen responses are rapidly activated when plants are attacked by pathogens, such as bacteria, fungi, oomycetes, viruses, nematodes, and insects, protecting plants from further harm[3]. Plant disease resistance (R) genes are involved in defense against pathogens, are triggered by pathogen signaling, and can target specific pathogens[4]. Most R genes encode a kind of a nucleotide-binding site-leucine-rich repeat (NBS-LRR) protein. The NBS domain belongs to the NB-ARC domain and contains three conserved motifs, namely, P-loop, kinase-2, which coordinates divalent metal ions, and kinase-3a-binding nucleotide, which is a critical region for nucleotide binding in ATP/GTPs and plays a role in defense signal transmission[5]. Most LRR domains with 20–30 amino acid residues consist of two segments: highly conserved segment (HCS) and variable segment (VS). However, the domains are usually 23–25 residues long, and a specific VS part in a plant LRR domain is involved in plant pathogen immune reaction and protein-to-protein interactions[6]. The NBS-LRR gene family is the largest class of R genes, which play multiple roles in the direct or indirect host-pathogen recognition and downstream signaling transduction [7,8].

NBS-LRR proteins are divided into two types according to their conservative functional domains. One contains a toll/interleukin 1 receptor (TIR) domain at the N terminal and the other is the non-TIR(CC-NBS-LRR type), which assembles a coiled-coil (CC) domain at the N terminal instead of a TIR domain. Other domains in the N-terminal of CC-NBS-LRR replace or coexist with CC domains, particularly zinc fingers or RPW8 domains[9,10]. CC-NBS-LRR genes are found in monocotyledons and dicotyledons, and *toll/interleukin-1 receptor-NBS-LRR* (TNL) genes are rare in monocotyledons(9), revealing that CC-NBS-LRR is widely present in plants. Furthermore, approximately 80% of reported R genes encode the NBS-LRR domain, and more than 50 NBS genes have been proved in response to disease resistance[11]. A NBS-LRR type R gene of Pi-ta played a defense role in *Magnaporthe grisea* of rice by interaction of the effector AVR-Pita[12]. And RRS1 protein of *Arabidopsis thaliana* interacted directly with PopP2, which was the pathogen protein of bacterial wilt[13]. RPS2 and RPM1 resistance genes from *Arabidopsis* were in response to *Pseudomonas syringae* by indirect interaction with AvrRpm1 and AvrB[14,15].The ectopic overexpression of *Arabidopsis* RPW8 enhanced the resistant ability to powdery mildew in grapevine[16].

Chinese cabbage is a popular vegetable worldwide, especially in Asia. The leaf heading, which forms at the late heading stage, is the mainly edible part and commodious organ and directly associated with the yield and quality of Chinese cabbage[17]. Downy mildew (DM) and black rot (BR) are the two common diseases affecting the heading leaves of Chinese cabbage in China and decreasing quality and yield[18]. Caused by *Hyaloperonospora parasitica*, DM is one of the three major diseases of Chinese cabbage and even the entire cruciferous family. BR is a bacterial disease caused by *Xanthomonas campestris* pv. *campestris* downson, which infects many *Brassica* species, such as cabbage (*Brassica oleracea* var), Chinese cabbage (*Brassica pekinensis*), and oil seed rape (*Brassica campestris*)[19,20]. Thus, screening the naturally resistant inbred lines of Chinese cabbage is necessary.

A total of 92 NBS-encoding resistance genes of *Brassica rapa* were identified without a reference genome[21], and the expression levels of 90 TIR-NBS-LRR genes against TuMV have been analyzed in *Brassica rapa* ssp. *Pekinensis*[22]. CC-NBS-LRR proteins in Chinese cabbage have not been explored, especially their roles in defense against bacterial and fungal infection. In our study, the CC-NBS-LRR genes of Chinese cabbage were identified and analyzed in a genome-wide range. A total of 40 genes encoding CC-NBS-LRR members were identified and further analyzed in terms of gene length and structure and chromosome location and distribution. The PI and conserved domains of CC-NBS-LRR proteins were also analyzed. A phylogenetic tree of CC-NBS-LRR genes in *Brassica rapa*, *Arabidopsis thaliana*, and *Oryza sativa* was built and used in exploring the homologous relationship between monocotyledons and dicotyledons. The expression profiles of *BrCC-NBS-LRR* genes were detected by infection with DM and BR. To further explore the roles of *BrCC-NBS-LRR* genes involved in pathogenesis-related defense, sensitive and insensitive inbred lines of the Chinese cabbage were infected by *Hyaloperonospora parasitica* and *Xanthomonas campestris*. *campestris* downson for the purpose of drawing the expression profiles and detecting the differential expression of *BrCC-NBS-LRR* genes in our study. Our study provides information on *BrCC-NBS-LRR* genes. This information is useful to the further investigation of the gene functions and mechanisms of CC-NBS-LRR genes in Chinese cabbage.

2. Results

2.1. Identification of the CC-NBS-LRR family in *Brassica rapa*

The CC-NBS-LRR family usually encodes two typical domains, including the CC domain at the C terminal and the NBS-LRR domain at the N terminal. All CC-NBS-LRR family genes were searched using the *Brassica rapa* genome on Brassica Database (BRAD). A total of 41 CC-NBS-LRR family candidates were found, and their protein sequences were verified using online tools for protein functional domains. The tools were PfamScan (<https://www.ebi.ac.uk/Tools/pfa/pfamscan/>), InterPro (a protein sequence analysis and classifications website, <http://www.ebi.ac.uk/interpro/>), and ScanProsite (<https://prosite.expasy.org/scanprosite/>). Finally, 40 genes were screened, all of which have intact CC, NBS, and LRR domains. The CC-NBS-LRR family genes of Chinese cabbage were named according to their genes that were ID-searched on BRAD. Blast searches for the homologous genes of *BrCC-NBS-LRR* in *Arabidopsis thaliana* were conducted using TAIR (<https://www.arabidopsis.org/>), and 20 genes were homologous to CC-NBS-LRR members shown in the Table S1. In our results, the main genetic characteristics of CC-NBS-LRR genes in *Brassica rapa* are summarized and shown in Table 1, including the gene ID chromosome location, the numbers of exon, protein length, molecular weights, and isoelectric point (PI). In all *BrCC-NBS-LRR* genes, exon length was between 1992 and 4842 bp, and amount varies from 1 to 12. As for the proteins, the length ranged from 666 aa to 1613 aa. The isoelectric points between 5.42 and 8.98 are shown in Table 1.

Table 1. CC-NB-LRR genes in Chinese cabbage.

Gene ID	Genome position	CDS length(bp)	Exon	Protein length(aa)	MW(Da)	PI
Bra011432	A01:2,191,374..2,196,276	2424	5	807	91259.06	5.96
Bra013947	A01:8,643,759..8,646,482	2724	1	907	103780.83	5.79
Bra026368	A01:9,720,079..9,723,012	2934	1	977	110513.54	6.56
Bra031482	A01:17,037,929..17,040,208	2145	2	714	81853.03	8.46
Bra035424	A01:16,404,803..16,407,820	2703	3	900	104139.92	6.56
Bra029405	A02:25352667..25357391	3489	4	1162	135161.42	6.22
Bra013134	A03:20,277,197..20,280,164	2736	3	911	104870.17	8.30
Bra013213	A03:19,840,894..19,844,167	2916	3	971	111041.47	7.81
Bra019063	A03:26,487,398..26,490,427	3030	1	1009	114155.02	6.30
Bra036995	A03:29,029,474..29,032,056	2583	1	860	97340.71	6.87
Bra018245	A05:6,909,119..6,911,701	2583	1	860	98194.11	6.50
Bra027332	A05:20,364,432..20,367,560	3129	1	1042	119330.79	8.42
Bra009882	A06:17,846,911..17,849,759	2466	5	821	93525.92	6.33
Bra018834	A06:1,698,860..1,701,415	2556	1	851	96871.93	5.85
Bra018835	A06:1,694,120..1,696,651	2532	1	843	95766.74	6.08
Bra018863	A06:1,554,378..1,556,936	2559	1	852	96608.49	5.99
Bra019752	A06:4,608,614..4,611,267	2583	2	860	98235.98	8.12
Bra019754	A06:4,599,425..4,602,097	2673	1	890	100984.19	6.63
Bra019755	A06:4,595,072..4,597,754	2586	2	861	98093.56	6.05
Bra026094	A06:6,170,240..6,176,552	4842	12	1613	182736.32	6.22
Bra016781	A08:19,944,556..19,947,218	2589	2	862	98259.93	5.64

Bra016782	A08:19,949,435..19,952,182	2748	1	915	104280.85	5.44
Bra016785	A08:19,969,925..19,972,603	2679	1	892	101874.73	6.10
Bra030778	A08:20,402,328..20,404,925	2598	1	865	98735.56	5.14
Bra030779	A08:20,398,567..20,401,178	2517	2	838	95047.27	5.82
Bra034631	A08:12,381,249..12,384,097	2655	3	884	99630.07	6.46
Bra017572	A09:16,599,373..16,602,112	2001	5	666	76247.74	5.48
Bra026682	A09:33,197,405..33,200,004	2514	2	837	95014.93	6.02
Bra026923	A09:34,338,460..34,341,119	2559	2	852	97168.91	6.09
Bra026924	A09:34,342,578..34,345,222	2544	2	847	96534.94	5.73
Bra026977	A09:34,580,053..34,582,713	2661	1	886	101121.24	5.72
Bra026978	A09:34,584,346..34,587,018	2673	1	890	101555.26	6.14
Bra026979	A09:34,589,539..34,592,283	2745	1	914	103823.01	6.74
Bra027097	A09:8,445,238..8,447,974	1992	3	663	75576.87	6.33
Bra027866	A09:9,368,958..9,371,944	2790	3	929	106985.17	6.51
Bra036845	A09:25,444,116..25,448,953	3876	5	1291	146912.49	6.22
Bra037123	A09:4,366,953..4,369,453	2415	2	804	93699.16	8.98
Bra037139	A09:4,297,244..4,300,267	2454	4	817	93032.88	5.42
Bra002495	A10:9,233,853..9,236,492	2640	1	879	99477.06	8.46
Bra015597	A10:747,361..750,005	2571	2	856	97894.04	7.43

2.2. Location and distribution of CC-NBS-LRR family genes on *Brassica rapa* chromosome

The physical chromosomal locations of *BrCC-NBS-LRR* family genes can be found on BRAD (<http://brassicadb.org/brad/index.php>), which were drawn and shown as Figure 1. Apart from A04 and A07, the *BrCC-NBS-LRR* family members showed asymmetric distribution on each chromosome of *Brassica rapa* from A1 to A10. Majority of *BrCC-NBS-LRR* genes were located on A09 with 12 members (30%), followed by A06 with 8 (15%) *BrCC-NBS-LRR* members. However, only one member was located on A02. Two members were located on A05 and A10, and four, five, and six members were located on A3, A01, and A08. A total of 24 *CC-NBS-LRR* family genes were found in *Arabidopsis thaliana*, 16 (67%) of which were located in Chr.1 and eight were located on Chr.5 (Table S2). However, 40 members of *BrCC-NBS-LRR* have uneven distribution on the eight chromosomes of Chinese cabbage because of chromosome expansion. Duplication analysis showed that only four pairs of genes had two copies, and two genes had three copies, which were distributed on different chromosomes. This result suggested that duplicate events in Chinese cabbage were caused by segment duplications without tandem.

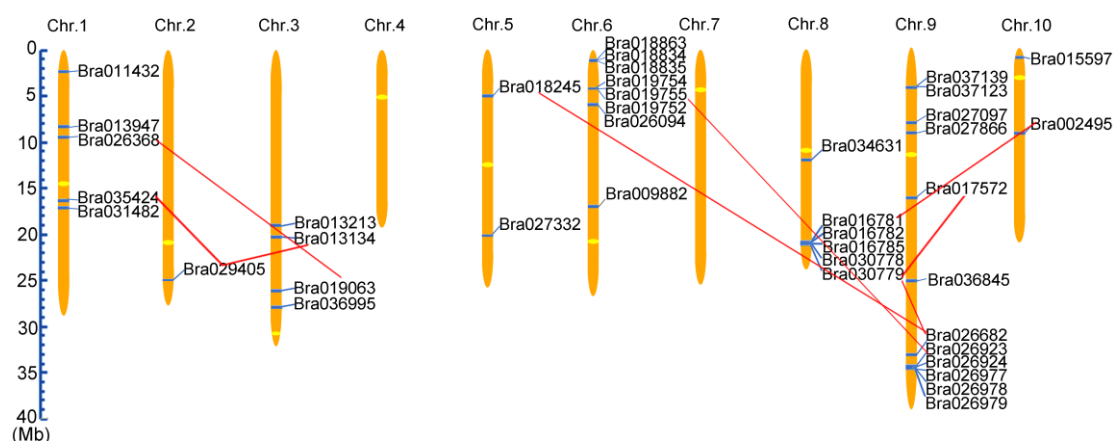


Figure 1. The chromosomal location of CC-NB-LRR genes from Chinese cabbage. The scale represents 40 Mb chromosomal distance. The chromosome numbers are labeled on the top of them. The duplicate gene are connected with red line.

2.3. Phylogenetic analysis of the CC-NBS-LRR family in *Brassica rapa*, *Arabidopsis thaliana*, and *Oryza sativa*

Arabidopsis thaliana is a dicotyledonous model plant, whereas *Oryza sativa* is a typical monocotyledonous plant[23]. To further analyze the phylogenetic relationship of CC-NBS-LRR family genes among Chinese cabbage, *Arabidopsis thaliana*, and rice, we aligned a series of multiple protein sequences and built a phylogenetic tree with the Neighbor-joining method. MEGA7.0 was used, and 40 CC-NBS-LRR members of *Brassica rapa*, 24 AtCC-NBS-LRR genes, and eight candidates belonging to rice were included (Figure 2, Table S3). The CC-NBS-LRR proteins were divided into nine subgroups according to their protein sequence features, namely, Groups A to I. The largest subgroup was Group A with 14 members including 12 BrCC-NBS-LRR and two AtCC-NBS-LRR proteins. Groups E and H had four proteins each and thus had the smallest subgroups in terms of number. All the proteins in Group E were Chinese cabbage CC-NBS-LRR proteins, and two proteins in Group H were Chinese cabbage proteins and two were *Arabidopsis thaliana*, showing that Bra013134 shared high homology with AT1G58400 and Bra035424 shared high homology with At1G59620. Two types of CC-NBS-LRR proteins were found in Group B, including four AtCC-NBS-LRR members and seven BrCC-NBS-LRR members with a total of 11 members. Similar to Group B, Group C contained four AtCC-NBS-LRR and 5 BrCC-NBS-LRR proteins. Seven CC-NBS-LRR proteins, including two *Brassica rapa* and five *Oryza sativa* members, were found. Groups F and G had three types of CC-NBS-LRR proteins, such as *Arabidopsis thaliana*, *Oryza sativa*, and *Brassica rapa* proteins. In Group F, two AtCC-NBS-LRR, two OsCC-NBS-LRRs, and four BrCC-NBS-LRR proteins were found, whereas Group G had one *Oryza sativa*, two *Arabidopsis thaliana*, and the three *Brassica rapa* members. These results showed that the phylogenetic tree of all the CC-NBS-LRR members are inclined to species differences because of the loose functional domain structures of CC domain, NBS, and LRRs.

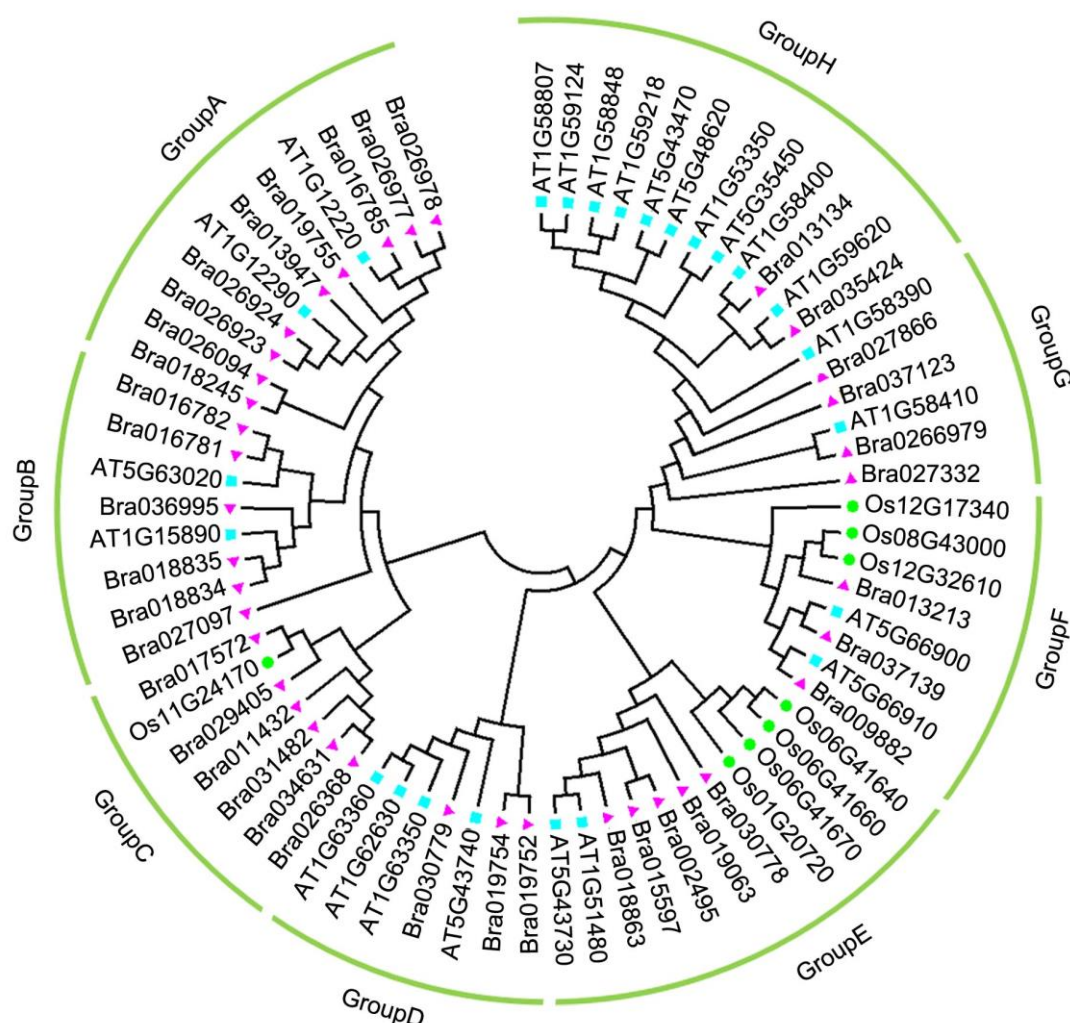


Figure 2. Phylogenetic relationships of CC-NB-LRR protein among *Brassica rapa*, *Arabidopsis thaliana* and *Oryza sativa*. The unroot phylogenetic tree was constructed by MEGA 6.0 by g Neighbor-Joining method with 1000 bootstrap replicates. CC-NB-LRRs of different plants are indicated with different colors and shapes.

2.4. Gene structures and protein function domains of CC-NBS-LRR family in Chinese cabbage

The gene structures and protein function domains of the BrCC-NBS-LRR family were analyzed using a phylogenetic tree built by aligning all the protein sequences of BrCC-NBS-LRR proteins through the neighbor-joining method, and the phylogenetic relationships among them were explored. This family was divided into five subgroups, namely, Groups I, II, III, IV, and V, as shown in Figure 3A. The IV Group had 11 members, which was the largest group. Group III was the smallest, with only four BrCC-NBS-LRR proteins. Groups I, II, and V had eight, eight, and six members, respectively (Figure 3A).

The BrCC-NBS-LRR gene structures were analyzed on the basis of the alignments between whole cDNA and genomic DNA sequences, which were downloaded from BRAD and blasted in National Center for Biotechnology Information (NCBI). Then, the structures were drawn with Gene Structure Display Server, as shown in Figure 3B. Nearly half of all BrCC-NBS-LRR genes had only one exon (17 of 39), and nine genes had two exons and one intron. However, Bra026094 had most exons up to 12. The rest of the CC-NBS-LRR genes contained more than three exons. Specifically, six members had three exons, two had four, and four had five (Figure 3B). In terms of structure performance,

most *BrCC-NBS-LRR* genes have similar exons and introns in the same phylogenetic subfamily.

The conserved protein motifs of *BrCC-NBS-LRR* were searched on the online tool MEME, which was set to monitor 10 putative protein domains (shown in Figure 3) with the protein sequences listed in Tables S3 and sequence logos of motifs in Figure S1. All the *CC-NBS-LRR* proteins of Chinese cabbage existed at motif 6, which encodes LRR domains. However, the encoding proteins of motifs 2, 3, 4, 7, and 10 belong to NB-ARC function domains, which were identified as central nucleotide-binding domains of resistance (R) proteins in plants. Moreover, the motif 3 in each *BrC-NBS-LRR* protein may be an essential motif of NBS. And 33 (85%) members contained motif 2, and 32 (82%) *BrCC-NBS-LRR* proteins had motif 4, except Br011432, Br031482, Br017572, Br037139, Br009882, and Br037123. All 40 *BrCC-NBS-LRR* proteins contained one or more NB-ARC domain motifs. In our searched results, the functions of motifs 1, 5, 8, and 9 were undefined, although motifs 1 and 5 were highly conserved in the *CC-NBS-LRR* family of Chinese cabbage. Similar motifs were found in the same *BrCC-NBS-LRR* superfamily, and Groups I, II, and III showed higher degrees of conservation than Groups IV and V.

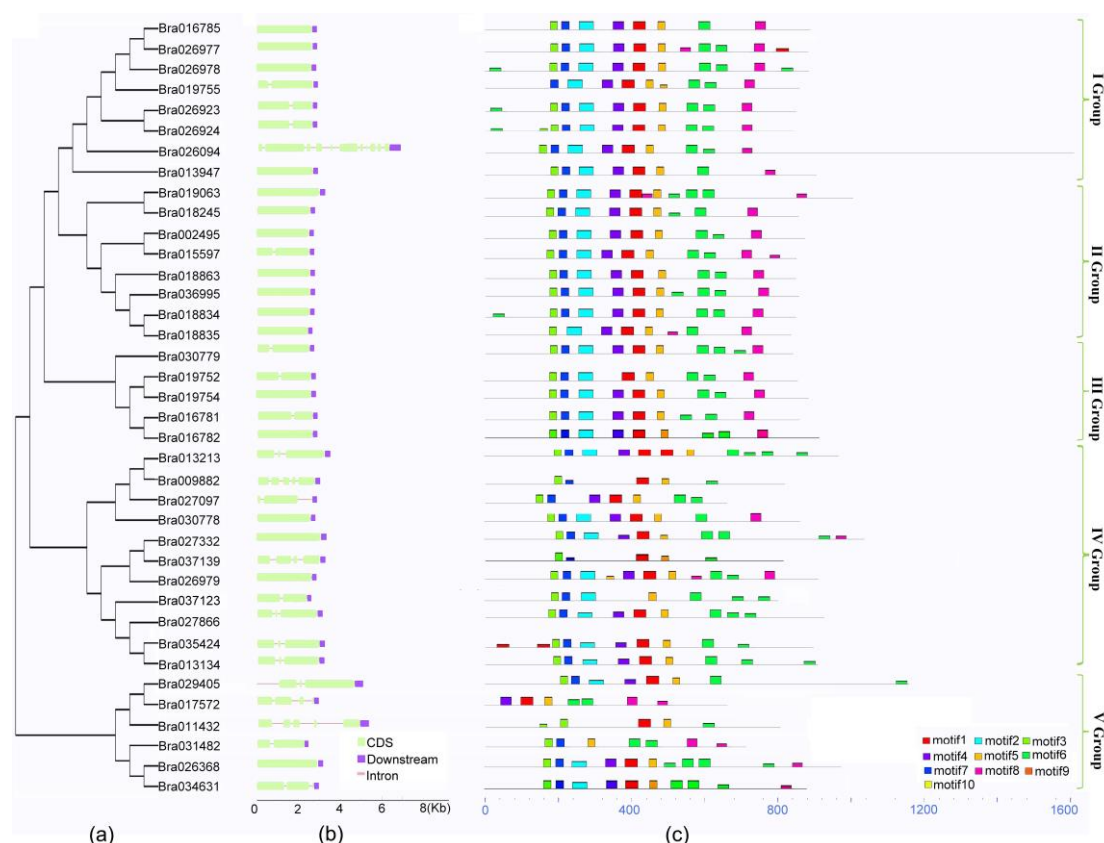


Figure 3. The Phylogenetic tree, gene structure and multiple motifs of *CC-NBS-LRR* family in Chinese cabbage. (a) Phylogenetic tree was constructed based on the protein sequence of Chinese cabbage by MAGE6.0;(b) The gene structure of Chinese cabbage *CC-NBS-LRRs*. Exon and intron were represented by green boxes and lines;(c)The multiple conserved motifs of Chinese cabbage *CC-NBS-LRR* proteins. Different colored boxes represent function motifs , identified by an online tool MEME . Motifs 2, 3, 4, 7, 10 were NB-ARC function domains.

2.5. Cis-acting elements analysis of the *CC-NBS-LRR* family promoter in *Brassica rapa*

The promoter sequences of *BrCC-NBS-LRR* genes obtained from BRAD were approximately 265–1500 bp upstream of transcriptional start sites (Table S5). They were scanned with the online tool New PLACE for the prediction of putative cis-acting regulatory DNA elements. The disease-resistant related cis-elements of *BrCC-NB-LRR* pro-

motors were further analyzed and drawn on Figure 4. A total of 12 types of disease-resistant related cis-element were found, namely, AGCBOXNPGLB, BIHD1OS, ELRECOREPCR1, GCCCORE, GT1CONSENSUS, GT1GMSCAM4, MYB1LEPR, SEBFCONSSTPR10A, TL1ATSAR, WBOXATNPR1, WBOXPCWRKY1, and WRKY71OS (Figure 4). They were distributed randomly in their promoters with different items (Figures S2 and S3). The cis-elements of GT1CONSENSU was detected in each *BrCC-NBS-LRR* promoter. Especially, in GT1CONSENSU, the GT-1-like factors bind the PR-1a promoter influences the expression level of SA-inducible gene [24]. GT1CONSENSU had the largest number of cis-elements among the CC-NBS-LRR members of Chinese cabbage (up to 411). *BrCC-NBS-LRR* proteins contained different GT1CONSENSU elements with numbers ranging from 3 to 24. WRKY71OS was the second largest number of cis-elements in *BrCC-NBS-LRR* with a total of 206, which are related to the WRKY proteins that bind specifically to TGAC-containing W box elements within the pathogenesis-related class 10 (PR-10) genes[25]. Most CC-NBS-LRR members contained WRKY71OS elements, except *Bra019754*. A total of 38 *BrCC-NBS-LRR* members contained GT1GMSCAM4 in their promoters, the expression of which is induced by pathogens and mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. WBOXATNPR1 and WBOXPCWRKY1, a salicylic acid (SA)-induced WRKY DNA binding protein and a "W box" WRKY protein that binds specifically to DNA sequence motifs[26], appeared in 31 and 25 *BrCC-NBS-LRR* gene promoters with a total of 96 and 20 items (Figure 4B). A total of 68 BIHD1OS elements were present in 32 *BrCC-NBS-LRR* genes; these elements are the binding sites of OsBIHD1 in disease resistance responses[27]. SEBFCONSSTPR10A, a binding site of the potato silencing element binding factor (SEBF) gene found in the promoter of the pathogenesis-related gene PR-10a, was present in 18 *BrCC-NBS-LRR* genes promoters with totally 29. Nearly 10 elements were found in other *BrCC-NBS-LRR* gene promoters, such as ELRECOREPCR1, which contained the consensus sequence of the elements W1 and W2 of PR1-1/PR1-2 promoters, and the WRKY1 protein binding site had 10 items in the 10 promoters of *BrCC-NBS-LRRs*. Five MYB1LEPR cis-elements were found in five *BrCC-NBS-LRR* genes and were related to Pti4(ERF), which regulates defense-related gene expression assisted by GCC box and non-GCC box cis elements[28]. Two GCC-box cores were present in many pathogen-responsive genes on the *Bra026682* promoter, and only one TL1ATSAR was detected in the promoter regions of 13 NPR1-responsive ER-resident genes on the *Bra019754* promoter. Only one AGCBOXNPGLB was conserved in most PR-protein genes on the *Bra037139* promoter. Furthermore, 40 cis-elements were present on *Bra011432* and *Bra034631*, and only five cis-elements were present on *Bra026924* gene promoters listed in Supplemental Figures 2 and 3. These results suggested that the expression of homology *BrCC-NBS-LRR* genes might be regulated by different mechanisms, and these genes play different roles in disease resistance in Chinese cabbage.

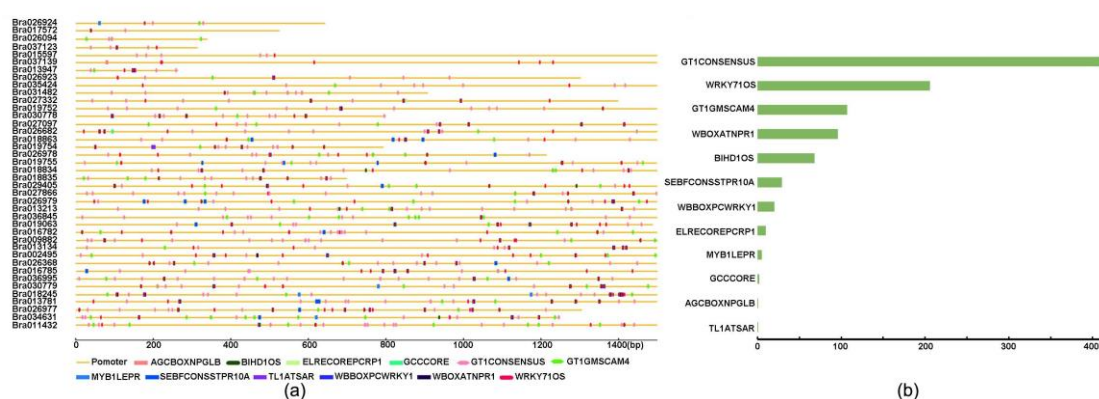


Figure 4. The disease resistant related cis-elements on CC-NB-LRR promoters of Chinese cabbage. (a), The distribution of disease resistant related cis-elements on *BrCC-NB-LRR* promoters; (b) The total number of each disease resistant related cis-element on Chinese cabbage CC-NB-LRR promoters.

2.6. Tissue expression patterns of CC-NBS-LRR genes in Chinese cabbage

To explore the expression patterns of *BrCC-NBS-LRR* genes in the roots, stems, leaves, flowers, and siliques, the total mRNAs of the different tissues of the Chinese cabbage cultivar A160 were isolated and reversed-transcribed into cDNAs, which were amplified with specific primers (Table S4) and normalized with *BrActin*. Expression levels were detected through real-time PCR, and a **hot map** was drawn, as shown in Figure S4. The leaves had the highest number of *BrCC-NBS-LRR* genes that exhibited significantly high expression levels. The expression levels of *Bra027097*, *Bra027866*, and *Bra036845* in the leaves were 10-fold those in the other tissues. However, *Bra026094* showed high expression levels in all tissues, especially in the roots, stems, flowers, and siliques. In the siliques, only three *BrCC-NBS-LRR* genes showed prominently enhanced expression levels. These results showed that the *BrCC-NBS-LRR* expression levels in the four tissues varied, indicating that they play different roles in the development of Chinese cabbage.

2.7. Expression profiles of *BrCC-NBS-LRR* genes in response to phytopathogens

To further analyze the potential functions of *BrCC-NBS-LRR* genes in response to fungal diseases, the inbred A160 lines of Chinese cabbage in the five-leaf stage were inoculated with *Hyaloperonospora parasitica* or distilled water as a control. They were harvested after 1, 3, 6, 12, and 24 hours for short treatments and 3, 5, 7, 10, and 14 days for long treatments for the detection of the expression levels of *BrCC-NBS-LRR* genes through real-time PCR. **Hot maps were** drawn, as shown in Figures 5A and 5B. After the short treatment, we found that most (63%) of the CC-NBS-LRR family genes were up-regulated after 1 h of inoculation. This result indicated that they quickly respond to DM. For example, *Bra034631* expression was induced and increased 11-fold within 1 h. As for *Bra031482*, the expression was up-regulated 10-fold. The expression level of *Bra026978* increased with treatment time, showing that *Bra026978* was continuously induced by DM. The expression levels of numerous *BrCC-NBS-LRR* dramatically changed after 12 h of treatment. However, the expression levels of *BrCC-NBS-LRRs* were relatively evenly up-regulated 3, 5, 7, and 10 days after inoculation (Figure 5B). In addition, the expression levels of *Bra016782* and *Bra016782* were consistently high at all treatment periods. Although most *BrCC-NBS-LRR* genes (88%) showed decreased expression 14 days after the inoculation of DM relative to the expression levels detected at 0 day of inoculation (Figure 5B). Prominent diseased plaques were observed in Chinese cabbage leaves after 14 days of treatment. The expression of *Bra019755* was up-regulated 18-fold in 3, 5, 7, and 10 days after inoculation and then declined rapidly to 1.

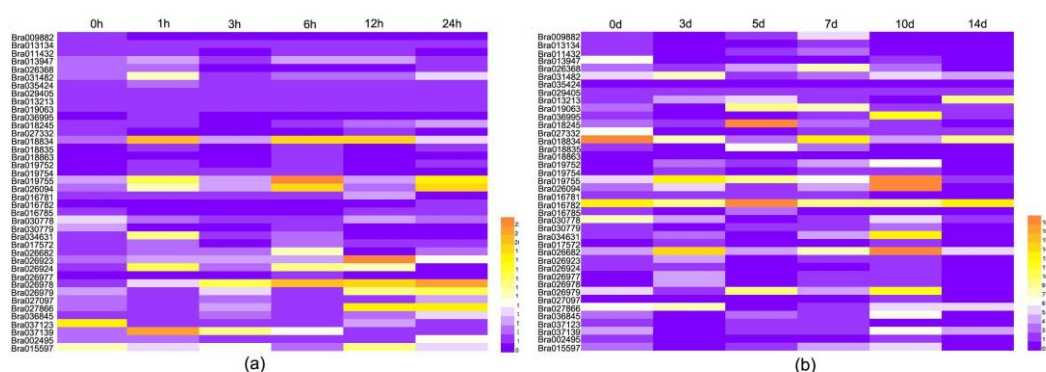


Figure 5. Expression patterns of Chinese cabbage CC-NBS-LRR genes in response to DM infected represented by heat map. (a) The expression levels of CC-NBS-LRR genes in response to DM infected for 0, 1, 3, 6, 12 and 24 hours; (b) The expression levels of CC-NBS-LRR genes in response to DM infected for 0, 3, 5, 7, 10 and 14 days.

Expression levels after exposure to *Xanthomonas campestris*, a kind of bacterial BR, were further detected through qPCR. The results are shown in Figures 6A and B. Different *BrCC-NBS-LRR* members were induced at different treatment times (0, 1, 3, 6, 12, and 24 h [short treatments] and 3, 5, 7, 10, and 14 days [long treatments]). Seven of the 40 *BrCC-NBS-LRR* genes (18%) were up-regulated more than twofold after 1 h of inoculation relative to the expression at 0 h, and the expression levels of *Br018245*, *Bra030778*, and *Bra017572* increased 3.5-, 7.3-, and 2.2-fold after 3 h of BR treatments. Six hours after inoculation, only *Bra016785* showed an increase in expression level. The expression of the others showed no change or were down-regulated. However, more *BrCC-NBS-LRRs* (15 of 40) were up-regulated after 12 h of treatment. The expression levels of CC-NBS-LRRs were smooth and steady compared with those after the short treatments. *Bra030778* was up-regulated in the long treatments (3, 5, 7, 10, and 14 days), and the expression levels of *Bra029405*, *Bra019063*, *Bra017572*, and *Bra015597* increased remarkably 3 days after inoculation. The expression levels of *Bra030779* and *Bra015597* remarkably increased 5 days after inoculation. After 7 days of BR treatment, 17 CC-NBS-LRR genes showed up-regulated expression, but 93% of the *BrCC-NBS-LRR* genes showed down-regulated expression 14 days after inoculation. Most of the Chinese cabbages showed numerous disease spots. Compared to long time treatment, these dramatically differential expression of *BrCC-NBS-LRR* genes in the 24 h short treatment suggested that they respond quickly to BR.

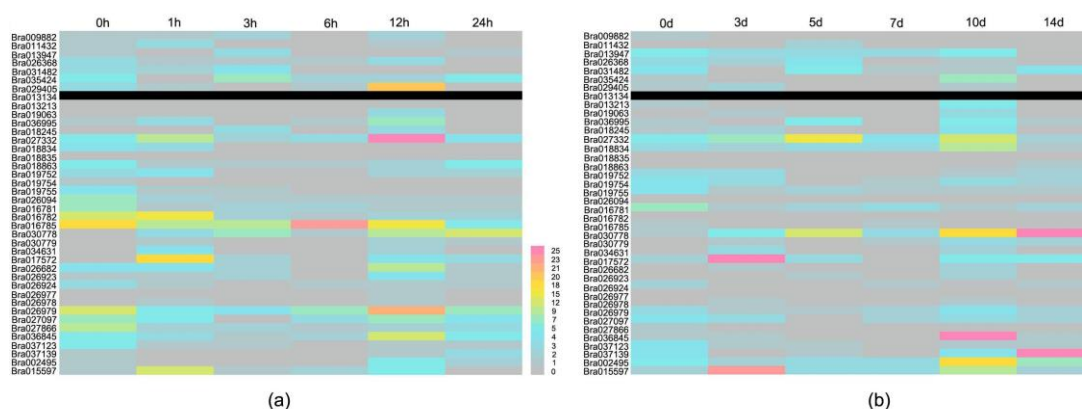


Figure 6. Expression patterns of Chinese cabbage CC-NBS-LRR genes in response to BR infected represented by heat map. (a) The expression profile of CC-NBS-LRR genes in response to BR infected for 0, 1, 3, 6, 12 and 24 hours; (b) The expression profile of CC-NBS-LRR genes in response to BR infected for 0, 3, 5, 7, 10 and 14 days.

2.8. Associated the expression of *BrCC-NBS-LRR* in different inbred lines of Chinese cabbage in response to phytopathogen

To further analyse the expression characteristics of *BrCC-NBS-LRR* in response to fungal and bacterial disease, 24 inbred lines of Chinese cabbage were applied to screen the sensitive and resistant lines to DM and BR. On basis of statistics, inbred lines A95 and A167 showed the most sensitive compared to other 23 lines to DM and BR, respectively, to the opposite A24 and A96 were resistant.

And then the expression level of inbred lines A95 and A24 were detected when they were exposed to DM for 1day and 14 days, shown as Figure 7. The expression levels of most *BrCC-NBS-LRR* genes in the sensitive inbred line A95 and resistant A24 were up-regulated in response to DM. A total of 11 *BrCC-NBS-LRR* genes showed similar expression characteristics. The expression levels in the two lines were induced by DM to a

higher degree than by the control. Moreover, the expression levels in A24 were even remarkably higher than those in A95 not only 1 day of DM treatment but also after the control treatment, particularly those of *Bra011432*, *Bra026368*, *Bra030778*, *Bra026682*, *Bra034631*, *Bra017572*, *Bra027332*, *Bra018245*, *Bra018863*, *Bra019755*, and *Bra037139*. Meanwhile, 13 CC-NBS-LRR family members of Chinese cabbage had the same trend of expression as above in the long treatments (14 days), particularly *Bra013947*, *Bra026368*, *Bra029405*, *Bra030778*, *Bra030779*, *Bra036995*, *Bra027097*, *Bra018863*, *Bra019752*, *Bra019755*, *Bra036845*, *Bra037139*, and *Bra015597*. The trends of *Bra026368*, *Bra030778*, *Bra018863*, *Bra019755*, and *Bra015597* in the 1 day short treatment were consistent with those in the 14 day long treatment. By contrast, *Bra009882* and *Bra026978* expression in A24 and A95 decreased obviously after 1 day of DM treatment.

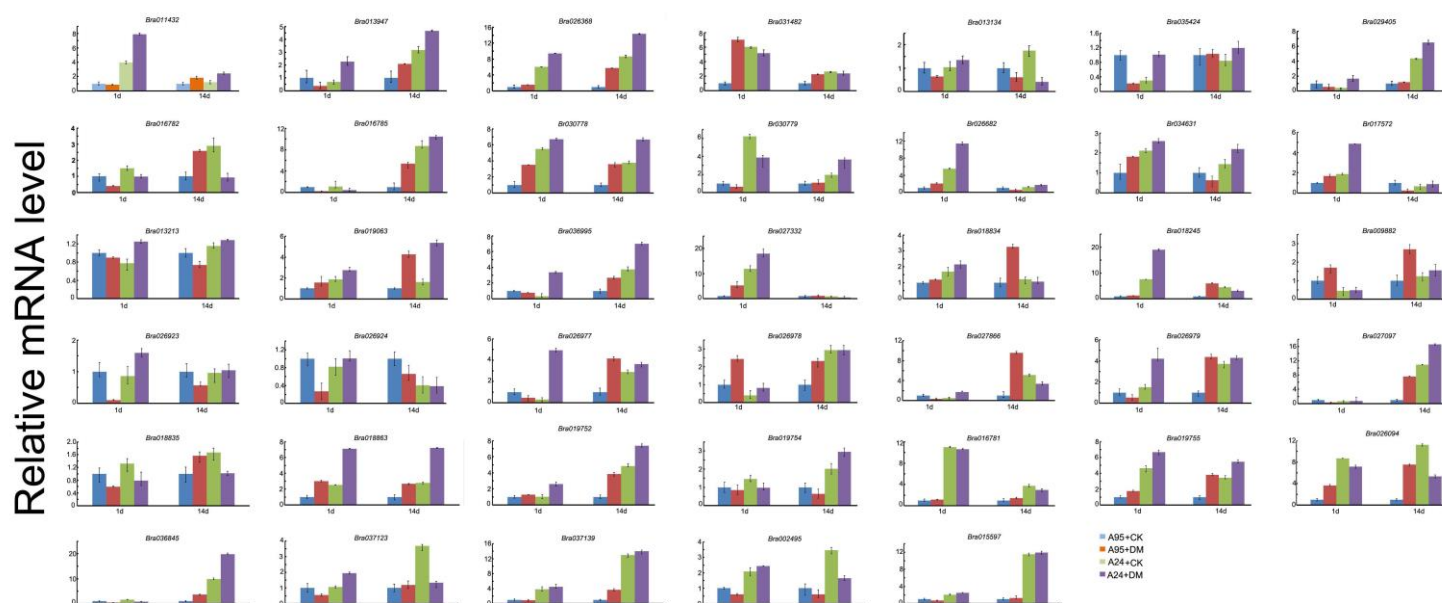


Figure 7. The expression levels of CC-NBS-LRR genes in Chinese cabbage inbred line A95 and A24 after DM infection. The CC-NBS-LRR genes expression level of A95 were detected by quantitative real-time PCR after DM infection (A95+DM and A24+DM) for 24h (short) and 14 days (long), and water spraying as control marked A95+CK and A24+CK. The represent results performed in triplicate. Error bars represent $\pm$ SE.

As for disease stress due to bacterial BR, the sensitive line A167 and resistant line A96 were also inoculated with *Xanthomonas campestris*, and the expression levels of the BrCC-NBS-LRR family were detected through qPCR 1 and 14 days after inoculation, as shown in Figure 8. A total of 14 BrCC-NBS-LRR genes were induced by BR after 1 day of treatment in A167 and A96 and six BrCC-NBS-LRR genes were induced after 14 days of treatment. In addition, the expression levels in A96 were even higher than those in A167 after inoculation. Especially, the expression levels of *Bra035424* and *Bra030778* in A167 were up-regulated more than 11-fold. Only one member showed the same expression trend as above (*Bra027866*). However, the expression of *Bra030779* were down-regulated after 1 day of BR treatment. These results revealed that the CC-NBS-LRR gene family of Chinese cabbage may play different roles in response to a disease and the type of role depends on treatment time and disease types.

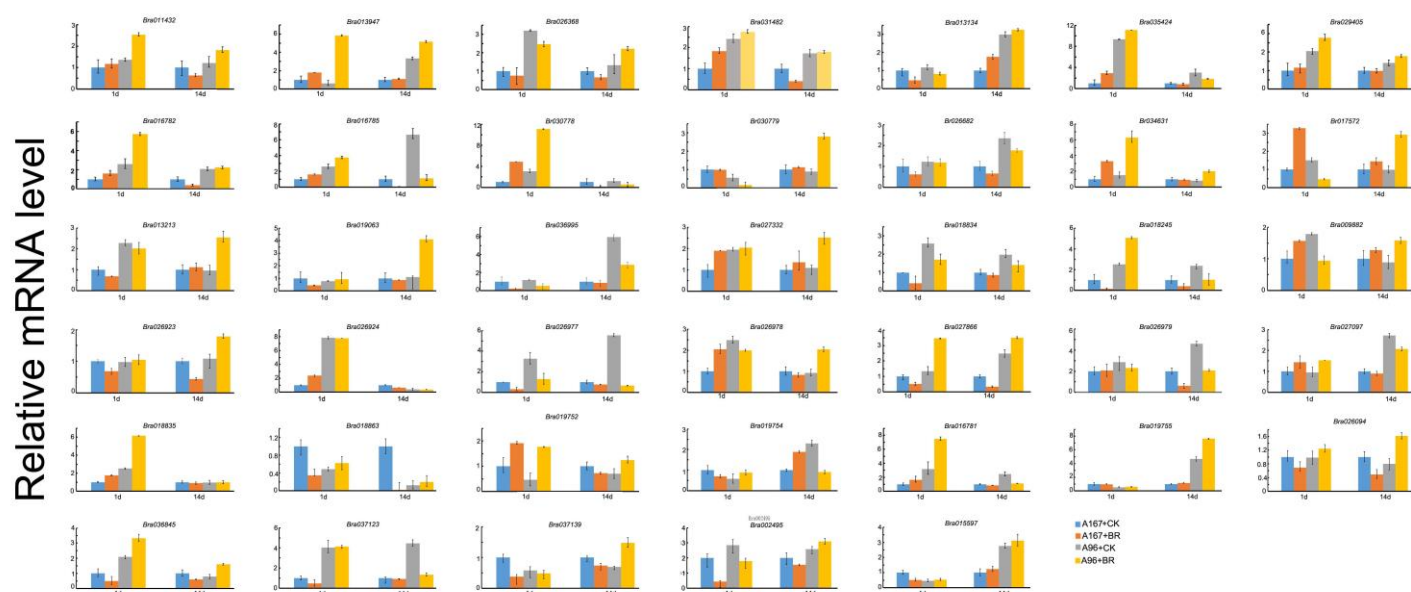


Figure 8. The expression levels of CC-NBS-LRR genes in Chinese cabbage selfing line A167 and A96 after BR infection. The CC-NBS-LRR genes expression level of A95 were detected by quantitative real-time PCR after SM infection (A167+BR and A96+BR) for 24h (short) and 14 days (long), and water spraying as control marked A167+CK and A96+CK. The represent results performed in triplicate. Error bars represent $\pm$ SE.

3. Discussion

Plant disease resistance genes (R genes) have mechanisms for recognizing and providing direct or indirect protection against pathogens in plants and play a critical role in ETI [29]. Of all the five classes of R genes, the NBS-LRR class encodes the largest protein family with one or more NBSs and an LRR domain at the C terminal [30]. The NBS-LRR protein family usually contains two major subfamily *TNL* genes and *non-toll/interleukin-1 receptor-NBS-LRR (nTNL)* genes, which is composed CC-NBS-LRR [31]. In our study, 40 CC-NBS-LRR members in Chinese cabbage were searched and identified on the basis of the entire genome of Chinese cabbage. Although 47.1% NBS encodings of *Brassica rapa* appeared to have undergone tandem duplication and to be distributed in tandem arrays [32], all *BrCC-NBS-LRRs* underwent segmental duplication without tandem and were unevenly distributed on A1–10, with five pairs of two copies (Figure 1) and two pairs of three copies. No *BrCC-NBS-LRR* members were found on Chr.A4 and Chr.A7, but 12 members (30%) located on A09 with the rest genes unevenly distributed on each chromosome. *Brassica rapa* and *Arabidopsis thaliana* belong to *Brassicaceae* plants and shared high homology with each other. A total of 21 *AtCC-NBS-LRR* genes were found on the whole genome, 17 members were found on Chr.1, and only one was found in Chr.5 (Table S2). Compared with *AtCC-NBS-LRRs*, *BrCC-NBS-LRRs* expanded 1.9-fold and were distributed symmetrically.

With the use of the phylogenetic tree of the encoded protein sequence, *BrCC-NBS-LRRs* were divided into five subfamilies according to their protein and gene structures. The protein structures of *BrCC-NBS-LRRs* were usually composed of three parts: one or more NBSs, an LRR domain at the C terminal, and a CC domain at the N terminal. NBS-encoding domains are necessary to the recognition of diverse pathogens in plants [33]. Five kinds of NBS-encoding motifs were found, such as motifs 2, 3, 4, 7, and 10 (Figure 3), and motif 3 was the most conserved in the *BrCC-NBS-LRRs*. Motif 6, which lies at the N-terminal, was found in each member. It encodes an LRR domain. LRRs are irregular and variable and responsible for interactions between resistance proteins on account of a hydrophobic backbone for three-dimensional integrity and many free solvent-exposed residues of LRR domain [34,35]. The CC domains were detected in all the CC-NBS-LRR members of *Brassica rapa* on InterPro. The CC motif was analogous to the

LRR motif in the critical core of the three-dimensional structure composed of hydrophobic chains and free solvent-exposed residues. The CC domain of a CC-NBS-LRR gene of LR10 in wheat directly recognizes pathogens; this result indicated that the CC domain function of CC-NBS-LRRs need to be further explored and excavated [36].

The cis-acting elements of gene promoters were essential for transcriptional expression, and the types of cis-acting elements indicated the potential functions of genes, particularly in response to pathogens[37]. All the BrCC-NBS-LRR promoters were downloaded from BRAD and analyzed with an online tool New PLACE for the searching of cis-acting regulatory elements. A total of 12 disease-resistance-related cis-acting elements were found in the promoters of BrCC-NBS-LRR. The GT1CONSENSU (consensus GT-1) elements dominated as investors and appeared on the promoters in tandem. It first reported as the binding sites of many light-regulated genes in many species, for example, *ribulose biphosphate carboxylase small chain*, *phytase* gene[38,39]. The binding of GT-1-like factors to the *PR-1a* promoter can reduce TMV infection and SA treatment and influence the expression level of SA-inducible genes[24]. The function of GT1GMSCAM4 were analogous to that of GT1CONSENSU. WRKY71OS, WBOXATNPR1, and WBBOXPCWRKY1 are typical disease-related elements found in BrCC-NBS-LRRs. WRKY71OS not only is involved in plant defense signaling by binding specifically to W box elements of the PR-10 but also plays a role in GA and ABA signaling pathways[25,40]. "W-box" sequences were found in the promoters of WBOXATNPR1 and WBBOXPCWRKY1 to bind with NPR1 and PR1 participating in plant defense response[41,42]. A total of 68 BIHD1OS elements were found in BrCC-NBS-LRR genes, that is the binding site of BELL-type transcription factor of OsBIHD1 associated with resistance and response in rice[27]. Some minor cis-acting elements are associated with disease resistance, such as SEBFCONSSTPR10A, ELRECOREPCR1, MYB1LEPR, TL1ATSAR, GCCCORE, and AGCBOXNPGLB.

In recent years, a large number of plant genomes have been sequenced, and an increasing number of NBS-encoding families of different species have been analyzed and surveyed[36]. In addition five *Brassicaceae* NBS-LRR genes were comprehensively analyzed and compared on the basis of established cross-species phylogenetic and syntenic relationships of NBS genes for the study of the R gene in *Brassicaceae*, particularly in *Arabidopsis lyrata*, *Arabidopsis thaliana*, *Brassica rapa*, *Capsella rubella* (127), and *Thellungiella salsuginea*[43]. NBS-LRR genes exert critical effects on response to multiple pathogens, including viruses, bacteria, and fungi[44]. CC-NBS-LRR as a main subfamily of NBS-LRR is involved in disease resistance in plants, such as *Arabidopsis thaliana*, rice, and wheat. However, they are rarely reported in Chinese cabbage in terms of response to DM and BR. In our study, the A160 inbred lines of Chinese cabbage were inoculated with *Hyaloperonospora parasitica* and *Xanthomonas campestris*, and short and long treatments were performed for exploring the expression profile characteristics. In the short treatments, hot maps were drawn after 1, 3, 6, 12, and 24 h of treatment (Figures 5 and 6). No inoculation was performed in the control treatment. We found that most BrCCNBSLRR genes were induced drastically 1 h after inoculation, and a peak in expression level occurred 12 h after inoculation with *Hyaloperonospora parasitica* and *Xanthomonas campestris*. Bra026978 was continuously induced by *Hyaloperonospora parasitica*. The expression levels of Bra018834, Bra019755, and Bra026094 were up-regulated by *Hyaloperonospora parasitica* at each treatment time in the short treatments, but no obvious change in response to BR was observed. Bra016785 and Bra027332 had high expression levels during BR inoculation. Meanwhile, 3, 5, 7, 10, and 14 day treatments were considered long treatments. The expressed BrCC-NBSL-RRs after inoculation with DM were more abundant than those observed after BR inoculation in the short and long treatments. Moreover, the differentially expressed genes responding to each pathogen slightly varied. Bra030778 was induced markedly at each treatment time in the short and long treatments using BR, but no change in response to DM was observed.

An increasing number of CC-NBS-LRRs have been reported, such as Lr10, which is involved in (CC-NBS-LRR)-mediated resistance[45]; Yr10 (CC-NBS-LRR), which plays a key role in resistance to *Pst*[46]; *Lr34* (CC-NBS-LRR), which enhances resistance during adult stages; TaRPM1, which is involved in resistance to *Pst*[47], Pm21 from the wild species shows high resistance to Bgt [48]. And in rice Pb1 show resistance to rice blast only during adult stages[49]. To further identify the function of *BrCC-NBS-LRRs*, the expression characteristics were carried out on the sensitive inbred lines A95, A167 and resistant lines to A24 and A96 to DM and BR for 1 day and 14 day treatment, as shown in Figures 7 and 8. There were 11 *BrCC-NBS-LRR* genes show similar expression characteristics induced by DM than control, what is more A24 were even higher than A95 DM for 1 day treatment and for 13 for the long treatment(14 days). However 5 of the *BrCC-NBS-LRR* genes showed the same expression trends for both short and long *Hyaloperonospora parasitica* inoculation. As to bacterial BR, 14 *BrCC-NBS-LRR* genes were induced by BR treatment in A167 and A96 1 day after inoculation and six genes after the 14 day treatment. The expression levels of *BrCC-NBS-LRR* genes after 1 day of treatment with DM and BR were compared. *Bra030778* and *Bra034631* were induced and had the same expression level. In the long treatment of DM and BR, three *BrCC-NBS-LRRs* (*Bra013947*, *Bra019755*, and *Bra015597*) showed higher expression levels. These results revealed that *BrCC-NBS-LRR* family genes play various roles in response to phytopathogens, and the roles depend on disease type and treatment time.

4. Materials and Methods

4.1. Plant Materials

A total of 24 inbred lines of Chinese cabbage from Northeast Agricultural University were cultivated in a greenhouse subjected to day (28 °C)/night (18 °C) and 16 h light/8 h dark cycles. For the expression profiles of DM and BR, the *Brassica rapa* inbred line A160 of the fifth leaf was sprayed with pathogens and harvested 3, 5, 7, 10, and 14 days after infection, and non-infected Chinese cabbage was used as the control. And for screening the sensitive and insensitive plant, the fourth leaves of 24 inbred lines were infected by both *Hyaloperonospora parasitica* and *Xanthomonas campestris* for 14 days until invasion of the two diseases. The whole leaf and lesion areas of Chinese cabbage were surveyed, and three replications of biological duplication were carried out. The lesion area of leaves were measured, and according to statistics the inbred lines with lesion area taking up greater than 75% as sensitive line, at the same time less than 20% was used as the resistant line. Water was used as the control. Inbred lines A95 and A167 showed were the most sensitive to DM and BR, respectively, whereas A24 and A96 were resistant. The leaves of the sensitive and resistant plants were harvested and frozen in liquid nitrogen and then stored at -80 °C for RNA isolation.

4.2. Identification and analysis of CC-NBS-LRR in Chinese cabbage

All the CC-NBS-LRR members of Chinese cabbage were obtained from BRAD (<http://brassicadb.org/brad/>). They were identified with the NB-ARC domain according to the Hidden Markov Model of pfam00931 and the LRR domain of PLN03210 at the C terminal with the BLAST program of the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The CC domain of *BrCC-NBS-LRRs* were confirmed using MPI Bioinformatics Toolkit (<https://toolkit.tuebingen.mpg.de/#/tools/quick2d>)[50]. The nucleotide and protein sequences of *BrCC-NBS-LRRs* were downloaded from BRAD. The other CC-NBS-LRR sequence of *Arabidopsis thaliana* and *Oryza sativa* were downloaded from TAIR (<http://www.arabidopsis.org/>) and Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>), which were confirmed using the Basic Local Alignment Search Tool of NCBI. InterPro (<http://www.ebi.ac.uk/interpro/scan.html>) and Pfam (<http://pfam.xfam.org/search#tabview=tab1>) were used for further confirmation[51].

4.3. The cis-acting elements in promoters, chromosomal locations, and gene structures

The promoter sequences of *BrCC-NBS-LRR* genes were searched on BRAD (<http://brassicadb.org/brad/>), and all of them were analyzed using New PLACE (<https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace>), which is a database containing plants used in the analysis of cis-acting regulatory elements[52]. Information on chromosomal location was obtained from BRAD and assessed by MapInspect. The genome and CDS sequences of CC-NBS-LRR were downloaded from BRAD, which were placed in the Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>) for the reconstruction of the gene structure[53].

4.4. Phylogenetics and conserved motif analysis of CC-NBS-LRR protein

The CC-NBS-LRR protein sequences of *Brassica rapa* and *Arabidopsis thaliana* and *Oryza sativa* were downloaded from BRAD, TAIR, and Rice Genome Annotation Project. First, multiple sequences were aligned with MEGA6, then the phylogenetic trees were calculated and constructed using the neighbor joining method within 1000 replicates[54].

The protein sequences were analyzed using the online tool MEME Suite with the following settings: site distribution, any number of repetitions; number of motifs, 10.

4.5. Total RNA extraction and real-time PCR Analysis

The plant described in the Plant Materials section were harvested for RNA extraction with TRIzol™ reagent (Invitroge, Thermo Fisher, US), and 2 mg of RNA was reversed-transcribed to cDNA with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kits (TransGen, Beijing, China). Approximately 15 µL of total volume were used for real-time PCR, containing 1 µL of specific primers, 3 µL of cDNA samples diluted 30–50-fold and 7.5 µL of SYBR Green real-time PCR master mix (TOYOBO, Japan). The reactions were amplified using a qTower3G (Analytik Jena, Germany) real-time PCR detection system and tubulin as an internal reference. The primers are listed in Supplementary Table 1.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Sequence logos of motifs in *BrCC-NBS-LRR*, Figure S1: Sequence logos of motifs in *BrCC-NBS-LRR*, Figure S2: The number of different type cis-elements on each *BrCC-NBS-LRR* promoter, Figure S3: The total disease resistant related cis-element number on each Chinese cabbage *CC-NB-LRR* promoter, Figure S4: Hot map of *BrCC-NBS-LRRs* in root, stem, leaf, flower and silique, Table S1: The homologous gene of *BrCC-NBS-LRRs* in *Arabidopsis thaliana* and their positive function, Table S2: The Chromosome locations of CC-NBS-LRR in *Arabidopsis thaliana* and Chinese cabbage, Table S3: CC-NBS-LRR genes in *Arabidopsis thaliana*, rice and Chinese cabbage, Table S4: Motif sequences detected by MEME, Table S5: The promoters Cis-elements of CC-NB-LRR genes in Chinese cabbage, Table S6: Primers of qPCR.

Author Contributions: Yan Liu, Aoxue Wang and Yaowei Zhang planned and designed the study and wrote the manuscript. Yan Liu and Na Yang performed the experiments. Xiaolong Zhu and Kexin Han implemented the software. Dalong Li and Ran Gu provided supplementary experimental results. Junyu Bai collected all of the plant materials in this work. All authors read and approved the final manuscript.

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

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