

1 Evolution analysis and expression divergence of the chitinase gene family 2 against *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* infection in 3 *Brassica napus*

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8 **Abstract:** Blackleg and sclerotinia stem rot caused by *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* respectively are two
9 major diseases in rapeseed worldwide, which cause serious yield losses. Chitinases are pathogenesis-related proteins and play
10 important roles in host resistance to various pathogens and abiotic stress responses. However, a systematic investigation of the
11 chitinase gene family and its expression profile against *L. maculans* and *S. sclerotiorum* infection in rapeseed remains elusive. The
12 recent release of assembled genome sequence of rapeseed allowed us to perform a genome-wide identification of the chitinase gene
13 family. In this study, 68 chitinase genes were identified in *Brassica napus* genome. These genes were divided into five different
14 classes and distributed among 15 chromosomes. Evolutionary analysis indicated that the expansion of the chitinase gene family was
15 mainly attributed to segmental and tandem duplication. Moreover, the expression profiling of the chitinase gene family was
16 investigated using RNA sequencing (RNA-Seq) and the results revealed that some chitinase genes were both induced while the other
17 members exhibit distinct expression in response to *L. maculans* and *S. sclerotiorum* infection. This study presents a comprehensive
18 survey of the chitinase gene family in *B. napus* and provides valuable information for further understanding the functions of the
19 chitinase gene family.

20 **Keywords:** *Brassica napus*; chitinase gene family; expression pattern; *Leptosphaeria maculans*; *Sclerotinia sclerotiorum*

22 1. Introduction

23 Plant chitinases (EC 3.2.1.14) are enzymes that hydrolyze the N-acetyl glucosamine polymer chitin, a major component of fungal
24 cell walls and exoskeleton of insects (COLLINGE *et al.* 1993) and are considered as one group of pathogenesis-related (PR) proteins
25 (LEGRAND *et al.* 1987), which can be induced in response to the infection of various pathogenic micro-organisms. In the light of
26 classification of glycosyl hydrolases based on amino acid sequence similarities, plant chitinases have been put in glycoside hydrolase
27 family 18 (GH-18) and 19 (GH-19) (HENRISSAT 1991). According to the CAZy database ([http://www.cazy.org/Glycoside-
28 Hydrolases.html](http://www.cazy.org/Glycoside-Hydrolases.html)) (CANTAREL *et al.* 2009), plant chitinases have been grouped into five different classes ranging from I to V. Of these,
29 classes I, II and IV belong to the GH-19 family whereas the GH-18 family are composed of classes III and V chitinases (HENRISSAT
30 1991). The details of plant chitinase classification are described as follows. Class I chitinases have an N-terminal chitin-binding
31 domain and a GH-19 catalytic domain. Class II chitinases consist of only a catalytic domain with a high level of sequence and
32 structure similarity to class I chitinases but lack the chitin-binding domain and linker regions. Class IV chitinases show high homology
33 with class I chitinases but are smaller due to one deletion in the chitin-binding domain and three deletions in the catalytic domain (XU
34 *et al.* 2016). Both class III and V chitinases have a GH-18 catalytic domain and a consensus sequence DXDXE, but there is no

35 homology for other amino acids (UMEMOTO *et al.* 2015). GH-18 chitinases are widely distributed in plants, animals, fungi, bacteria
36 and viruses whereas GH-19 members almost exclusively exist in higher plants (PASSARINHO and DE VRIES 2002).

37 In higher plants, the expression of chitinase genes is involved in defense against biotic and abiotic stress as well as in growth and
38 developmental processes (COLLINGE *et al.* 1993; PUNJA and ZHANG 1993). For instance, a class III chitinase gene, *Mtchitinase III-3*,
39 has been found to be induced upon the infection of fungi *Glomus mosseae* and *Glomus intraradices* in cortical root (BONANOMI *et al.*
40 2001). PSCHI4, a putative extracellular class II chitinase, is up-regulated in pine seedlings infected with the necrotrophic pathogen
41 *Fusarium subglutinans* f. sp. *Pini* (DAVIS *et al.* 2002). In *Arabidopsis thaliana*, a class IV chitinase gene *AtchitIV* accumulated very
42 rapidly in leaves after inoculation with *Xanthomonas campestris* and reached maximum mRNA accumulation after one hour infection
43 (GERHARDT *et al.* 1997). In addition, several transgenic studies showed that enhanced levels of chitinase genes in transgenic plants can
44 indeed improve resistance against pathogens and reduce the damage caused by fungi and some insect pests (LIN *et al.* 1995; DING *et al.*
45 1998; YAMAMOTO *et al.* 2000; WANG *et al.* 2005; PRASAD *et al.* 2013; CHEN *et al.* 2014). There are several reports of induced
46 expression of plant chitinases when plants were exposed to abiotic stresses such as heavy-metal stress (BEKESIOVA *et al.* 2008),
47 drought (HONG and HWANG 2002; LEE *et al.* 2008), salt (HONG and HWANG 2002), cold (YEH *et al.* 2000), heat (KWON *et al.* 2007),
48 UV light and wounding (BREDERODE *et al.* 1991). Furthermore, some chitinases are essential in physiological processes like somatic
49 embryo development (DEJONG *et al.* 1992) and formation of root nodules (OVTSYNA *et al.* 2000). In conclusion, chitinases play
50 important roles in plant defense and plant health.

51 Rapeseed (*Brassica napus*) is an important oilseed crop worldwide. This crop is affected by various fungal pathogens, especially
52 blackleg caused by *Leptosphaeria maculans* and sclerotinia stem rot by *Sclerotinia sclerotiorum*, which are the most destructive
53 rapeseed diseases in Canada, Australia, Europe and many other regions around the world (WEST *et al.* 2001). Recently, a few studies
54 have been conducted on chitinase genes responding to some pathogens infection in *B. napus* (RASMUSSEN *et al.* 1992a; RASMUSSEN *et al.*
55 1992b; GRISON *et al.* 1996; WANG *et al.* 2005; AHMED *et al.* 2012). For example, constitutive overexpression of a chimeric
56 chitinase gene in rapeseed had been shown to exhibit an increased resistance to three fungal pathogens compared with their
57 nontransgenic parental plants (GRISON *et al.* 1996). Co-expression of defensin gene *Rs-AFPI* from *R. sativus* and chimeric chitinase
58 gene *chit42* from *T. atroviride* in rapeseed via *Agrobacterium*-mediated transformation demonstrated enhanced resistance against
59 sclerotinia stem rot disease (ZARINPANJEH *et al.* 2016). In addition, global studies of transcriptome dynamics of defense responses to *L.*
60 *maculans* and *S. sclerotiorum* in *B. napus* presented that pathogen responsive genes including chitinases were rapidly induced during
61 early infection (LOWE *et al.* 2014; HADDADI *et al.* 2016; JOSHI *et al.* 2016; WU *et al.* 2016). However, to date, the chitinase genes in
62 rapeseed have not been systematically identified and thus the genetic resistance to *L. maculans* and *S. sclerotiorum* has been not yet
63 studied. Recently, the availability of the whole genome sequence and RNA-seq sequencing enable further investigations into chitinase
64 genes and their response to *L. maculans* and *S. sclerotiorum* infection on a genome-wide scale (CHALHOUB *et al.* 2014; WOODHOUSE
65 *et al.* 2014).

66 To further extend the understanding of the chitinase gene family, a global analysis, including identification, sequence features,
67 physical location, the evolutionary relationship and expression pattern of the chitinase gene family in response to *L. maculans* and *S.*
68 *sclerotiorum* infection in *B. napus* using the RNA-seq sequencing data collected in our lab and some transcriptome data from NCBI
69 database was performed. Expression analysis revealed that some chitinase genes were induced by both pathogens while others
70 displayed differential expression pattern in response to *L. maculans* and *S. sclerotiorum* infection, suggesting that they may have
71 distinct roles in different pathogens stress response. Together, our findings will be helpful for further understanding of the functions of
72 the chitinase gene family against different stress in rapeseed.

73 2. Results

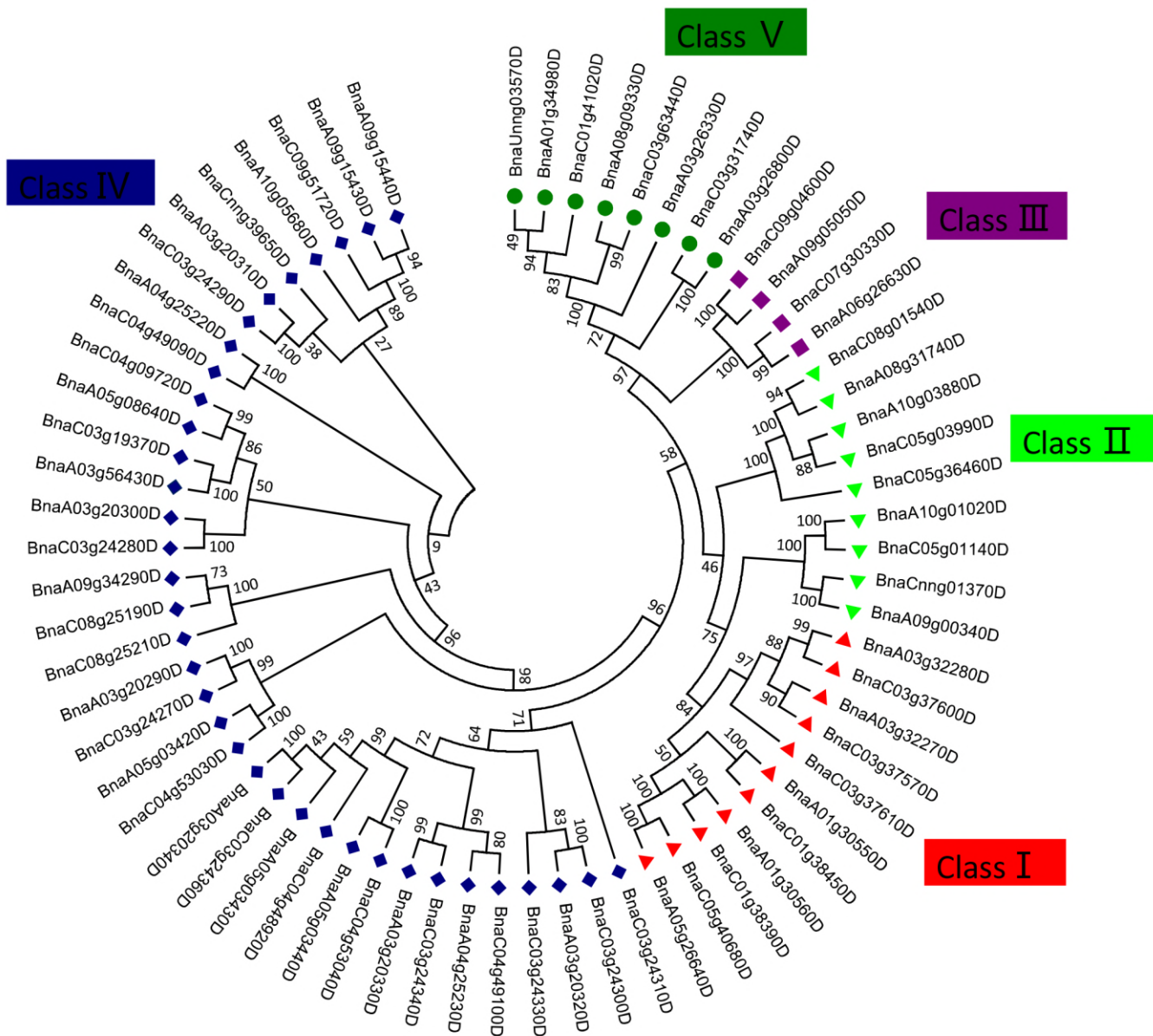
74 2.1 Identification and phylogenetic analysis of the chitinase gene family in *B. napus*

The complete genome sequence and gene annotation was used for the genome-wide identification of the chitinase gene family and a total of 68 putative chitinase genes were identified in the *B. napus* genome (Table 1). All these identified proteins have at least one typical “Glyco_hydro_19” or “Glyco_hydro_18” domain which is responsible for catalyzing the degradation of chitin. Of these, GH-18 family and GH-19 family include 12 and 56 putative chitinase genes, respectively. These chitinase genes in *B. napus* encode proteins ranging from 130 to 1005 amino acids in length with an average of 294. The average number of exons among these chitinase genes was 3.04, a value that is smaller than the average number of exons among all predicted *B. napus* genes (4.9). BLAST search of these 68 proteins against NCBI non-redundant database showed that the top matched hits were endochitinases, chitinases, chitinase-like proteins, which further confirm the reliability of the identified chitinase genes. Furthermore, the signal peptides in 54 predicted chitinase sequences were also identified. To examine the evolutionary relationships among the chitinase genes in *B. napus*, sequence alignment was performed with amino acid sequences (Supplementary Table 1) and an unrooted phylogenetic tree of the 68 chitinase genes using neighbor-joining method was constructed (Figure 1).

Table 1 Chitinase genes in the *B.napus* genome and and their sequence characteristics

Gene	Family	Class	Chr	Start	End	Strand	Protein Length(aa)	Number of Exons	Signal Peptide
BnaA01g30550D	Glyco_hydro_19	I	A01	20941303	20942028	+	130	1	YES
BnaA01g30560D	Glyco_hydro_19	I	A01	20943578	20945861	+	145	4	YES
BnaA03g32270D	Glyco_hydro_19	I	A03	15569491	15570916	-	168	3	NO
BnaA03g32280D	Glyco_hydro_19	I	A03	15575380	15578982	-	190	6	YES
BnaA05g26640D	Glyco_hydro_19	I	A05	19447511	19449216	+	196	2	YES
BnaC01g38390D	Glyco_hydro_19	I	C01	37477744	37480353	-	216	3	YES
BnaC01g38450D	Glyco_hydro_19	I	C01	37494610	37495630	-	227	2	NO
BnaC03g37570D	Glyco_hydro_19	I	C03	23003071	23005099	-	231	4	YES
BnaC03g37600D	Glyco_hydro_19	I	C03	23016407	23018656	-	239	3	YES
BnaC03g37610D	Glyco_hydro_19	I	C03	23021408	23025003	-	242	2	YES
BnaC05g40680D	Glyco_hydro_19	I	C05	38780461	38782151	+	245	2	YES
BnaA08g31740D	Glyco_hydro_19	II	A08	2107446	2110073	+	245	3	YES
BnaA09g00340D	Glyco_hydro_19	II	A09	146643	148245	-	245	2	YES
BnaA10g01020D	Glyco_hydro_19	II	A10	536566	537828	-	255	2	YES
BnaA10g03880D	Glyco_hydro_19	II	A10	2063411	2066519	-	255	3	YES
BnaC05g01140D	Glyco_hydro_19	II	C05	600283	601194	-	256	2	YES
BnaC05g03990D	Glyco_hydro_19	II	C05	1957654	1959846	-	257	3	YES
BnaC05g36460D	Glyco_hydro_19	II	C05	35739352	35741554	+	261	9	YES
BnaC08g01540D	Glyco_hydro_19	II	C08	1206975	1209667	-	261	3	YES
BnaCnng01370D	Glyco_hydro_19	II	Cnn	1527030	1528502	+	263	2	YES
BnaC07g30330D	Glyco_hydro_18	III	C07	34864788	34866603	+	263	3	YES
BnaC09g04600D	Glyco_hydro_18	III	C09	2638283	2639657	+	263	3	YES
BnaA09g05050D	Glyco_hydro_18	III	A09	2477135	2478513	+	264	3	NO
BnaA06g26630D	Glyco_hydro_18	III	A06	18298306	18300242	-	307	3	YES
BnaA03g20290D	Glyco_hydro_19	IV	A03	9648039	9649318	-	269	3	YES
BnaA03g20300D	Glyco_hydro_19	IV	A03	9652001	9653298	-	269	3	YES
BnaA03g20310D	Glyco_hydro_19	IV	A03	9663327	9664876	-	272	2	YES
BnaA03g20320D	Glyco_hydro_19	IV	A03	9678956	9680269	-	272	2	YES
BnaA03g20330D	Glyco_hydro_19	IV	A03	9684457	9685621	-	274	2	YES
BnaA03g20340D	Glyco_hydro_19	IV	A03	9698939	9700499	-	275	2	YES

BnaA03g56430D	Glyco_hydro_19	IV	A03	711494	712848	+	275	2	YES
BnaA04g25220D	Glyco_hydro_19	IV	A04	18233599	18234851	-	275	2	NO
BnaA04g25230D	Glyco_hydro_19	IV	A04	18235520	18236868	-	279	2	NO
BnaA05g03420D	Glyco_hydro_19	IV	A05	1888936	1890632	-	279	3	YES
BnaA05g03430D	Glyco_hydro_19	IV	A05	1893397	1894609	-	280	2	YES
BnaA05g03440D	Glyco_hydro_19	IV	A05	1904615	1905892	-	280	2	YES
BnaA05g08640D	Glyco_hydro_19	IV	A05	4802795	4804226	+	281	2	YES
BnaA09g15430D	Glyco_hydro_19	IV	A09	8977793	8978880	+	281	2	YES
BnaA09g15440D	Glyco_hydro_19	IV	A09	8979421	8980691	+	281	2	NO
BnaA09g34290D	Glyco_hydro_19	IV	A09	25164422	25167344	+	281	6	YES
BnaA10g05680D	Glyco_hydro_19	IV	A10	3646850	3650151	-	282	2	YES
BnaC03g19370D	Glyco_hydro_19	IV	C03	10063312	10065182	-	282	2	YES
BnaC03g24270D	Glyco_hydro_19	IV	C03	13607893	13609216	-	282	2	YES
BnaC03g24280D	Glyco_hydro_19	IV	C03	13614270	13615575	-	282	3	YES
BnaC03g24290D	Glyco_hydro_19	IV	C03	13628566	13630189	-	283	2	YES
BnaC03g24300D	Glyco_hydro_19	IV	C03	13641790	13643090	-	284	2	YES
BnaC03g24310D	Glyco_hydro_19	IV	C03	13644193	13644910	-	302	2	YES
BnaC03g24330D	Glyco_hydro_19	IV	C03	13646581	13649085	-	302	2	YES
BnaC03g24340D	Glyco_hydro_19	IV	C03	13654943	13656106	-	302	2	NO
BnaC03g24360D	Glyco_hydro_19	IV	C03	13695822	13697335	-	318	2	YES
BnaC04g09720D	Glyco_hydro_19	IV	C04	7384173	7385277	+	319	5	YES
BnaC04g48920D	Glyco_hydro_19	IV	C04	47325431	47326573	+	320	2	YES
BnaC04g49090D	Glyco_hydro_19	IV	C04	47408585	47409809	-	322	2	NO
BnaC04g49100D	Glyco_hydro_19	IV	C04	47411832	47413177	-	322	2	YES
BnaC04g53030D	Glyco_hydro_19	IV	C04	680771	682319	-	322	3	YES
BnaC04g53040D	Glyco_hydro_19	IV	C04	688006	689125	-	322	2	NO
BnaC08g25190D	Glyco_hydro_19	IV	C08	27018000	27020278	+	323	6	NO
BnaC08g25210D	Glyco_hydro_19	IV	C08	27024727	27025633	+	329	2	NO
BnaC09g51720D	Glyco_hydro_19	IV	C09	948730	950079	-	334	3	YES
BnaCnng39650D	Glyco_hydro_19	IV	Cnn	38235388	38236682	+	342	3	YES
BnaC03g63440D	Glyco_hydro_18	V	C03	52860028	52861720	+	346	3	NO
BnaA01g34980D	Glyco_hydro_18	V	A01	304675	306686	+	363	3	YES
BnaUnng03570D	Glyco_hydro_18	V	Unn	5067935	5068651	-	371	1	NO
BnaA03g26800D	Glyco_hydro_18	V	A03	13187584	13189629	+	381	8	YES
BnaA03g26330D	Glyco_hydro_18	V	A03	12888937	12890809	+	383	3	YES
BnaC03g31740D	Glyco_hydro_18	V	C03	19525415	19530521	+	424	18	YES
BnaA08g09330D	Glyco_hydro_18	V	A08	8944530	8946552	-	513	3	YES
BnaC01g41020D	Glyco_hydro_18	V	C01	240723	242688	+	1005	5	YES



89
 90 **Figure 1 Phylogenetic tree of the chitinase gene family in *Brassica napus*.** The chitinase protein sequences were used to construct
 91 multiple sequence alignments using MUSCLE program within MEGA 7.0 software. Phylogenetic analysis was performed using
 92 MEGA 7.0 with the neighbor-joining method with 1,000 bootstrap replications.

93 2.2 Conserved motifs and gene structures of the chitinase gene family in *B. napus*

94 The above phylogenetic tree highlighted that the 68 chitinase genes could be divided into five well-supported subfamilies, which
 95 were consistent with Class I, II, III, IV, and V. As expected, the chitinase genes of Glyco_hydro_19 and Glyco_hydro_18 families
 96 were clustered into two relatively distinct branches. Chitinase genes from subfamilies Class III and Class V are in the Glyco_hydro_18
 97 clade, whereas subfamilies Class I, II, and IV that belong to Glyco_hydro_19 clade were clustered together and showed close
 98 relationships (Figure 2). According to the phylogenetic tree, chitinases in different subfamilies had various characteristics. Among the
 99 five subfamilies, Class IV was found to be the biggest group with 36 members, accounting for almost a half of the chitinase gene
 100 family, whereas there were 11, 9, 4, 8 chitinase genes in Class I, II, III, V subfamilies, respectively.

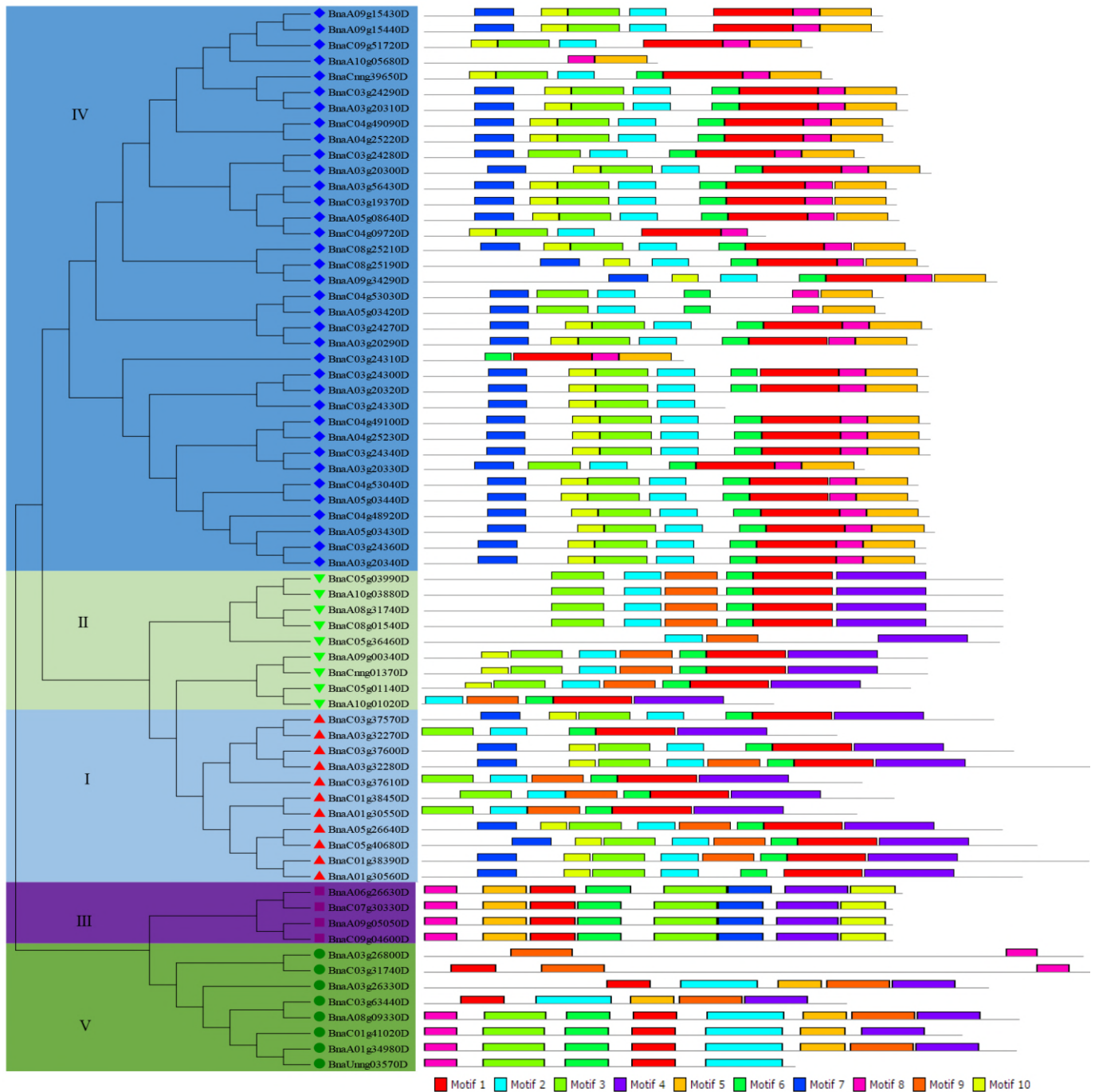
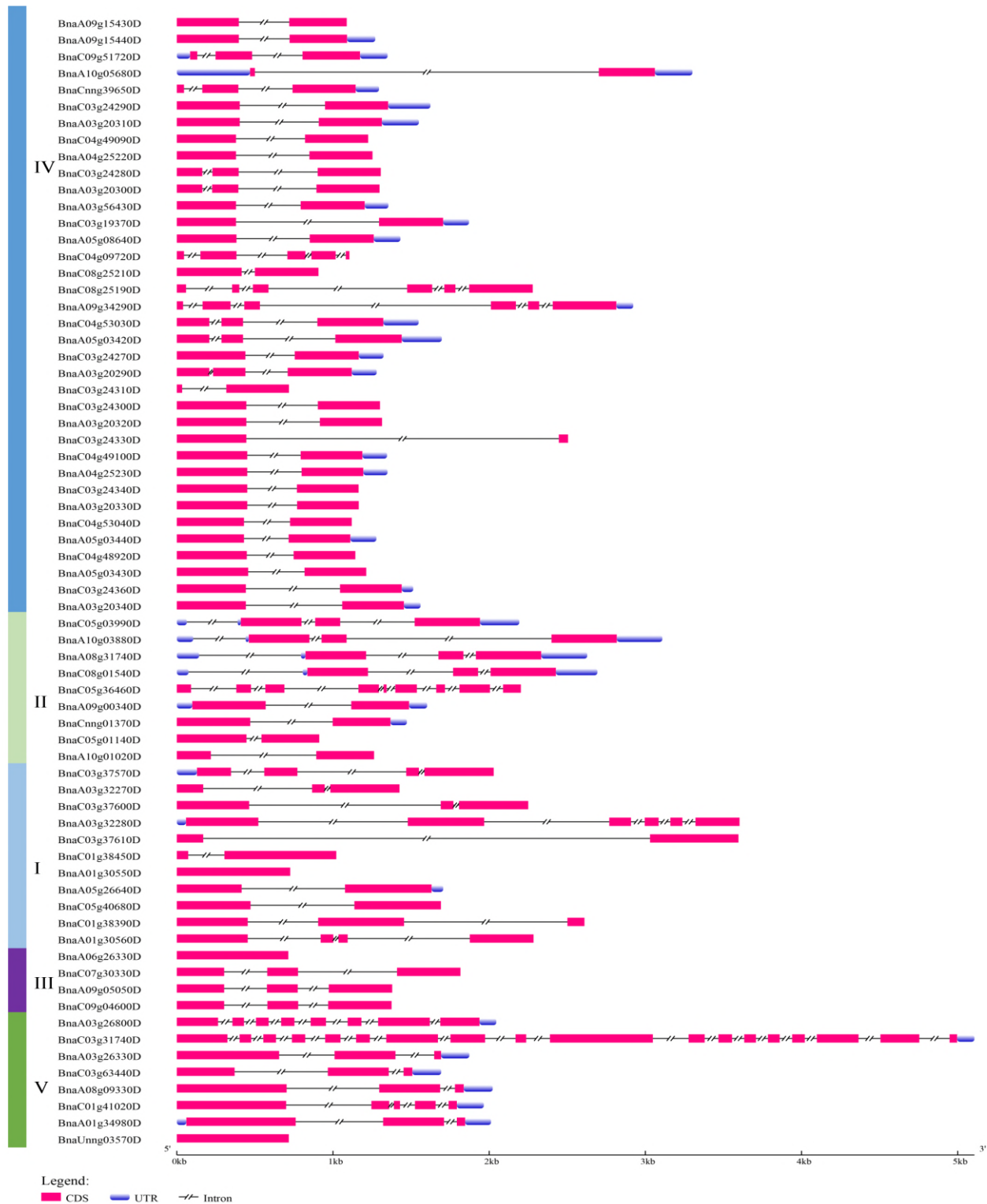


Figure 2 Phylogenetic relationships and motif compositions of chitinase genes. The phylogenetic tree was created in MEGA7.0 software. Five major phylogenetic groups designated as I to V were marked with different color backgrounds. Schematic representation of the conserved motifs of the chitinase gene family was elucidated by MEME software. Each motif was represented by a colored box numbered in the bottom. The details of individual motif were shown in Supplementary Figure 1 and 2.

To gain further insights into the structural diversity and functional evolution of chitinase genes, 10 motifs of all 68 chitinase genes are captured by MEME software and displayed schematically in Figure 2 and Supplementary Figure 1 and 2. As shown in Figure 2, most chitinases in the same class shared common motif compositions. Among the GH-19 family, motifs 1,3,4,9 were annotated as glycoside hydrolase catalytic domain and motif 7 was annotated as chitin-binding domain. Each member of chitinases in classes I, II and IV had at least one glycoside hydrolase catalytic domain and motif 4 was only detected in classes I and II. Most chitinase genes from subfamilies I and IV also harbored a chitin-binding domain whereas motif 7 did not exist in the subfamily II. In the GH-18 family, except for motif 8 and 9, other 8 motifs were annotated as glycoside hydrolase catalytic domains. Similarly, in the GH-19, at least one glycoside hydrolase catalytic domain was detected in every chitinase gene of class III and V. Interestingly, motif 7 and motif

115 10 are unique in class III whereas motif 2 can be only detected in class V. In addition, we analyzed the coding sequences with
 116 corresponding genome sequences of each chitinase genes in *B. napus*. A detailed illustration of the chitinase gene structure was shown
 117 in Figure 3. Most chitinase genes contained two or three exons, whereas three chitinase genes (BnaA01g30550D, BnaA0626330D and
 118 BnaUnng03570D) had no introns. In general, most chitinase genes in the same class showed similar conserved motifs and exon-intron
 119 structures. These findings revealed that motif compositions and gene structures of each class in the chitinase gene family were
 120 relatively conserved. The similar features of chitinase genes in the same class may fulfill similar functions and this claim need to be
 121 supported by their expression and related data.

122

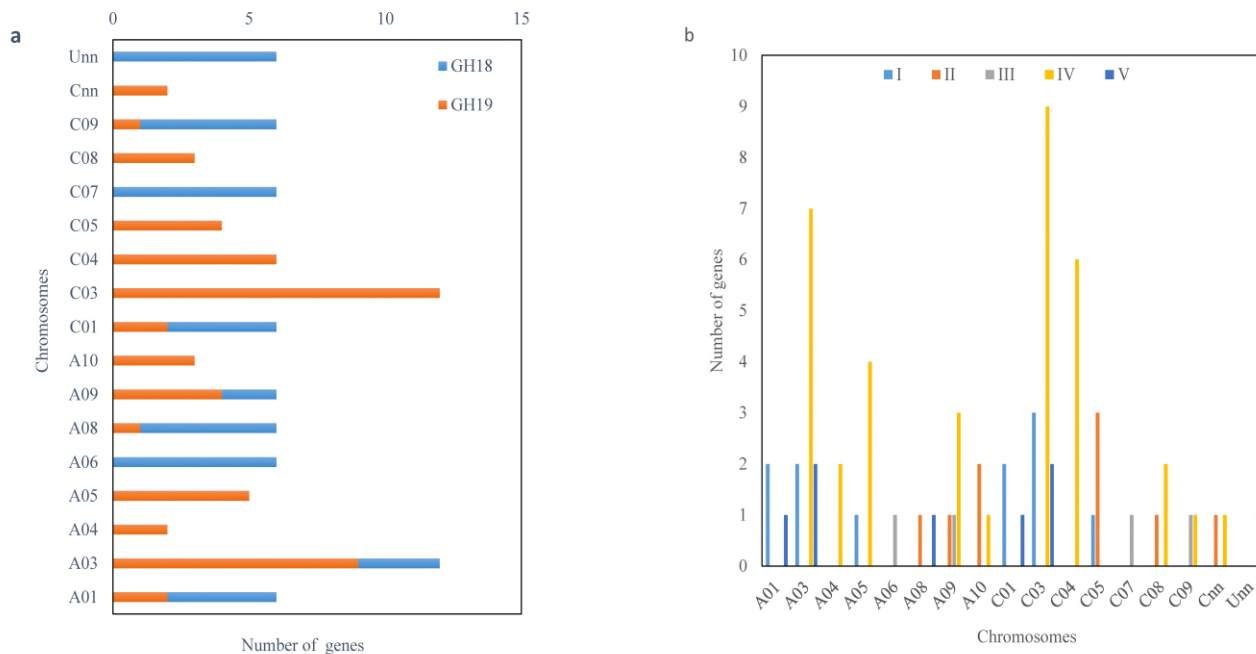


123

124 **Figure 3 Exon-intron structures of all chitinase genes in *Brassica napus*.** Schematic diagram represents the gene structure of all 68
 125 chitinase genes identified in this study using Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). CDS are shown as red boxes;
 126 introns are indicated by double slashes on the bar; UTR sequences are shown as blue boxes.

127 2.3 Chromosomal distribution and evolution patterns of chitinase genes in *B. napus*

128 The chromosomal distribution of 68 chitinase genes was analyzed based on the available gene annotation and genome sequence
 129 assembly. The results revealed that all 68 chitinase genes were distributed among 15 out of 19 chromosomes with the exception of
 130 chromosomes A02, A07, C02 and C06 in the *B. napus* genome (Figure 4a). There were 32 genes mapped in the A genome, and 35
 131 genes located in the C genome while one gene BnaUnng03570D was not assigned to a chromosome. GH-19 family presented on 13
 132 chromosomes except chromosomes A06 and C07 while GH-18 family were absent from chromosomes A04, A05, A10, C04, C05,
 133 C08. The number of chitinase genes varied considerably among different chromosomes and the large numbers of chitinase genes were
 134 found on chromosomes A03 and C03, harbouring 11 and 14 genes, respectively (Figure 4a). Furthermore, the classes of the chitinase
 135 gene family were distributed in different chromosomes and up to four classes in A03 and C03 chromosomes were identified (Figure
 136 4b).



137 **Figure 4 Distribution of the chitinase gene family on *Brassica napus* chromosomes** (a) Gene distribution of GH-18 family and
 138 GH-19 family on *Brassica napus* chromosomes. (b) Distribution of five subfamilies of chitinase genes on *Brassica napus*
 139 chromosomes. (c) Distribution of five subfamilies of chitinase genes on *Brassica napus* chromosomes.

140 To understand the evolution of the chitinase gene family, twenty-six pairs of paralogs were detected in 68 chitinase genes based
 141 on criteria for both coverage $\geq 70\%$ and identity $\geq 70\%$ (Table 2). The phylogenetic relationship analysis of chitinase genes also
 142 showed that most pairs of paralogs could be clustered together (Figure 1). For example, the four members in two pairs of paralogs
 143 (BnaA09g05050D and BnaC09g04600D, BnaA06g26630D and BnaC07g30330D) in Class III subfamily were clustered into two parts
 144 in a single clade. As genome duplication was considered, one member in the A subgenome would correspond to one homologous gene
 145 in the C subgenome in *B. napus*. In fact, 50 members of 68 chitinase genes showed such a one-to-one correspondence.
 146 Table 2 Table listing the chitinase genes/paralog sets among A and C subgenomes of *B. napus* and their orthologs in *A. thaliana*,
 147 *B. rapa*, *B. oleracea*.

<i>B.napus_C</i> vs <i>A. thaliana</i>	<i>B.napus_A</i> vs <i>A.</i> <i>thaliana</i>	<i>B.napus_C</i> vs <i>B.oleracea</i>	<i>B.napus_A</i> vs <i>B.rapa</i>	<i>B.napus_A</i>	<i>B.napus_C</i>
				BnaA03g32270D	BnaC03g37570D
AT2G43610.1	AT2G43610.1	Bo3g036710.1	Bra000314.1	BnaA03g20330D	BnaC03g24340D
AT2G43620.1	AT2G43620.1	Bo3g036730.1	Bra000315.1	BnaA03g20340D	BnaC03g24360D
AT2G43590.1	AT2G43590.1	Bo3g036660.1	Bra000311.1	BnaA03g20310D	BnaC03g24290D
			Bra027940.1	BnaA09g15430D	BnaA09g15440D
			Bra027940.1	BnaA09g15440D	BnaA09g15430D
AT4G01700.1	AT4G01700.1	Bo9g004220.1	Bra036316.1	BnaA09g00340D	BnaCnng01370D
AT4G19810.1	AT4G19810.1	Bo1g021980.1	Bra020951.1	BnaA08g09330D	BnaC03g63440D
AT5G24090.1	AT5G24090.1	Bo9g013820.1	Bra026469.1	BnaA09g05050D	BnaC09g04600D
		Bo3g036670.1	Bra000312.1	BnaA03g20320D	BnaC03g24300D
		Bo3g028270.1	Bra023009.1	BnaA03g56430D	BnaC03g19370D
AT3G12500.1	AT3G12500.1	Bo5g133420.1	Bra034754.1	BnaA05g26640D	BnaC05g40680D
AT2G43580.1	AT2G43580.1	Bo3g036650.1	Bra000311.1	BnaA03g20300D	BnaC03g24280D
	AT2G43590.1	Bo4g194450.1	Bra037699.1	BnaA04g25220D	BnaC04g49090D
		Bo5g003080.1	Bra033318.1	BnaA10g01020D	BnaC05g01140D
		Bo4g020930.1	Bra004773.1	BnaA05g03440D	BnaC04g53040D
		Bo1g139190.1	Bra038727.1	BnaA01g30550D	BnaC01g38450D
AT2G43610.1	AT2G43610.1	Bo4g194460.1	Bra037698.1	BnaA04g25230D	BnaC04g49100D
AT2G43570.1	AT2G43570.1	Bo3g036640.1	Bra000310.1	BnaA03g20290D	BnaC03g24270D
AT5G24090.1	AT5G24090.1	Bo7g097210.1	Bra009731.1	BnaA06g26630D	BnaC07g30330D
AT1G05850.1	AT1G05850.1	Bo8g005830.1	Bra030628.1	BnaA08g31740D	BnaC08g01540D
AT4G19810.1	AT4G19810.1	Bo1g021960.1	Bra013426.1	BnaA01g34980D	BnaC01g41020D
		Bo8g083650.1		BnaA09g34290D	BnaC08g25190D
AT1G05850.1	AT1G05850.1	Bo5g007040.1	Bra015457.1	BnaA10g03880D	BnaC05g03990D
	AT3G12500.1	Bo1g139210.1	Bra038726.1	BnaA01g30560D	BnaC01g38390D
AT2G43570.1		Bo4g020920.1	Bra004771.1	BnaA05g03420D	BnaC04g53030D
AT2G43620.1	AT2G43620.1	Bo3g036730.1	Bra004772.1	BnaA05g03430D	BnaC04g48920D
	AT4G19750.1		Bra000892.1	BnaA03g26330D	
	AT4G01040.1		Bra000939.1	BnaA03g26800D	
			Bra001454.1	BnaA03g32280D	
	AT2G43590.1		Bra005345.1	BnaA05g08640D	
			Bra039597.1	BnaA10g05680D	
AT2G43590.1		Bo3g024590.1			BnaCnng39650D
		Bo9g055720.1			BnaC09g51720D
AT3G54420.1		Bo8g083670.1			BnaC08g25210D
		Bo5g123590.1			BnaC05g36460D
					BnaC04g09720D
		Bo3g064970.1			BnaC03g37610D
					BnaC03g37600D
		Bo3g054420.1			BnaC03g31740D
		Bo3g036700.1			BnaC03g24330D
AT2G43600.1		Bo3g036680.1			BnaC03g24310D
AT4G19810.1		Bo1g021980.1	Bra020951.1		BnaUnng03570D

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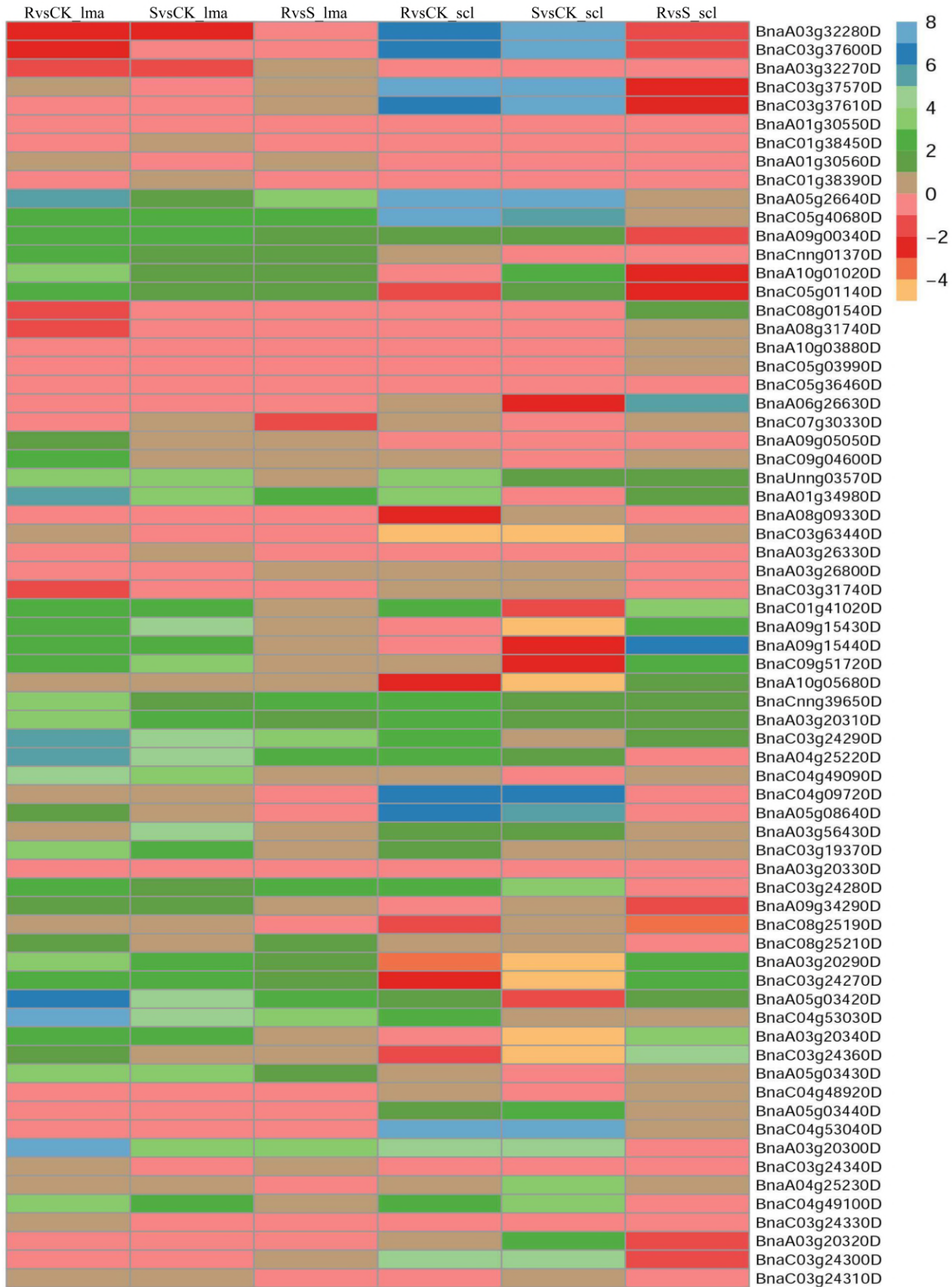
151

The distribution of chitinase genes indicated a relatively deep evolutionary origin of these chitinase genes as well as gene duplication. Previous research suggests that the evolution of a plant disease resistance gene family is usually mediated by

152 recombination, tandem duplication, and segmental duplication (LEISTER 2004). The allotetraploid *B. napus* is a spontaneous
153 hybridisation of *B. rapa* (A genome) and *B. oleracea* (C genome). To understand the origin and duplication patterns of these
154 chitinase genes, putative orthologs of chitinase genes in *B. napus* were also identified in *A. thaliana*, *B. rapa* and *B. oleracea* (Table
155 2). The results demonstrated that 30 orthologs of 32 chitinase genes in the A subgenome were identified in *B. rapa* and 32 orthologs of
156 35 chitinase genes in the C subgenome in *B. oleracea*. The gene BnaUnng03570D had both orthologs (Bo1g021980.1 and
157 Bra020951.1) in *B. rapa* and *B. oleracea*, respectively, but it had much higher identities with Bo1g021980.1. Furthermore, the order
158 and synteny of chitinase genes BnaA03g20290D, BnaA03g20300D, BnaA03g20310D, BnaA03g20320D, BnaA03g20330D,
159 BnaA03g20340D in chromosome A03 and BnaC03g24270D, BnaC03g24280D, BnaC03g24290D, BnaC03g24300D,
160 BnaC03g24340D, BnaC03g24360D in chromosome C03, as well as those genes for both subgenomes of the allopolyploid (the A and
161 C subgenomes in *B. napus* vs. the A and C genomes in *B. rapa* and *B. oleracea*) revealed that there were a few structural
162 rearrangements and genomic collinearity in *B. napus* with regard to the chitinase gene family evolution and expansion. These findings
163 showed that most chitinase genes (63/68) of allotetraploid *B. napus* were inherited from their diploid ancestors by recombination or
164 segmental duplication. It is interesting to observe that the best hit of chitinase gene BnaA09g15430D was BnaA09g15440D which
165 were tandem repeats on the A09 chromosome. It was also observed that five chitinase genes in *B. napus* had no orthologs in their
166 diploid ancestors. Of these, three genes (BnaA09g34290D, BnaC04g09720D and BnaC03g37600D) and one paralog of two genes
167 (BnaA03g32270D and BnaC03g37570D) did not have orthologs in *A. thaliana*, *B. rapa* and *B. oleracea*, which might result from
168 incomplete and error-filled genome assemblies and gene annotation errors or gene structure rearrangement in the evolutionary process.
169 Perhaps these five genes might be the new members of the chitinase gene family during the evolution in *B. napus*.

170 2.4 Transcriptomic profiles of the chitinase gene family in response to *L. maculans* and *S. sclerotiorum* during early infection in *B.* 171 *napus*

172 Blackleg, also known as stem canker caused by *L. maculans* and sclerotinia stem rot caused by *S. sclerotiorum* are two major
173 rapeseed diseases in most major rapeseed growing areas. The expression profiling of all chitinase genes in response to *L. maculans*
174 and *S. sclerotiorum* infection in resistant and susceptible *B. napus* accessions were investigated using whole-transcriptome sequencing
175 data to understand the roles of chitinase genes at early stages of infection. The results showed that these two biostresses caused a
176 significant expression induction of some members in the chitinase gene family. The differentially expressed genes (DEGs) of
177 chitinases in response to *L. maculans* were identified. Among all 68 chitinase genes in the genome, 31 and 25 chitinase genes were up-
178 regulated in the resistant lines and susceptible lines inoculated with the pathogen compared with their water control, respectively. Of
179 these up-regulated chitinase genes, 24 were overlapped in the resistant lines and susceptible accessions. Combined data of all resistant
180 and susceptible lines revealed that 15 chitinase genes were upregulated and no chitinase gene was dramatically downregulated during
181 the infection of pathogen *L. maculans* (Figure 5).



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Figure 5 Expression profiles of chitinase genes in response to *Leptosphaeria maculans* (lma) and *Sclerotinia sclerotiorum* (scl) infection. Relative fold change in as compared to control in resistant and susceptible *B. napus* lines and was used to generate heatmap. R and S were represented as resistant and susceptible lines, respectively. The colored scale for the relative expression levels is shown.

187 In a previous report, the abundance of transcripts in resistant and susceptible *B. napus* accessions at the 4 day post-inoculation
 188 treatments were analyzed to understand the differential defense response to *S. sclerotiorum* (WU *et al.* 2016). Using these data and all
 189 68 chitinase genes identified in this study, 23 and 20 chitinase genes were up-regulated while 6 and 11 members were downregulated
 190 in the resistant accession and susceptible accession compared with water control, respectively. Compared with the expression of
 191 chitinase genes in response to blackleg pathogen infection, 16 up-regulated and 4 down-regulated chitinase genes were overlapped
 192 respectively. Compared with the S accession, the analyses showed that 13 and 7 chitinase genes were the same as those upregulated
 193 and downregulated ones against *S. sclerotiorum* attack, respectively.

194 Chitinase genes which were induced after infection with *L. maculans* and *S. sclerotiorum* in rapeseed were further classified into
 195 three groups (Table 3). In the first group, 10 members of the chitinase gene family had stronger up-regulation in resistant accessions
 196 than susceptible counterparts with more than two values of log₂. These 10 chitinase genes showed a wide range of upregulation in
 197 resistant accession infected by *S. sclerotiorum* and the differences of the upregulation between resistant and susceptible accessions
 198 varied and were less than two in the log₂ values. There were 9 chitinase genes showing higher upregulation in the infection of
 199 resistant accessions with *L. maculans* with the log₂ values of more than three while most of these genes were less upregulated in
 200 susceptible accessions. In the infection of *S. sclerotiorum*, only few chitinase genes in the second group showed upregulated. Eight
 201 members of chitinase genes in the third group showed very high levels of upregulation with the log₂ values ranging from 4.45-7.96 in
 202 the infection of *S. sclerotiorum* while the differences in both resistant and susceptible accessions were little or even higher in
 203 susceptible accessions. The chitinase genes in the third group showed much less variation in the infection of *L. maculans* and the
 204 expression of some of these genes were not detected, indicating that these genes did not play a critical role in the defense against *L.*
 205 *maculans*. The results suggested that the cross-talk under different pathogen attack and redundant functions can be maintained over
 206 long evolutionary periods. On the other hand, eight chitinase genes were preferentially induced by *L. maculans* infection while other 8
 207 chitinase genes were preferentially increased by *S. sclerotiorum* infection respectively. The distinctive expression pattern of the
 208 chitinase gene family suggested that the functions of different members in the chitinase gene family have diverged during long-term
 209 evolution and might exhibit different roles against different biotic and abiotic stresses.

210 Table 3 Fold change (log₂) of chitinase gene expression in response to *Leptosphaeria maculans* (lma) and *Sclerotinia*
 211 *sclerotiorum* (scl) infection in resistant and susceptible *B. napus* lines.

Group	Gene	R/CK_lma	S/CK_lma	R/S_lma	R/CK_scl	S/CK_scl	R/S_scl
1	BnaA05g26640D	5.72	1.71	3.9	7.68	7.29	0.39
	BnaC04g53030D	7.99	4.26	3.83	2.6	0.87	0.82
	BnaA03g20300D	7.06	3.76	3.63	4.1	4.19	-0.48
	BnaC03g24290D	5.94	4.03	3.51	2.4	0.81	1.8
	BnaA05g03420D	6.45	4.33	2.88	1.09	-1.11	1.12
	BnaC05g40680D	2.05	2.78	2.62	7.1	5.97	0
	BnaA01g34980D	5.64	3.17	2.43	3.08	-0.41	1.78
	BnaCnng39650D	3.29	1.16	2.17	2.1	1.04	1.85
	BnaA04g25220D	5.96	4.2	2.15	2.08	1.85	-0.19
	BnaC03g24280D	2.51	1.36	2.08	2.88	3.21	-0.29
2	BnaC04g49090D	4.24	3.64	0.88	0.41	0	0.18
	BnaA05g03430D	3.93	3.21	1.82	0.12	-0.7	0.33
	BnaC04g49100D	3.69	2.08	0.87	2.13	3.71	-0.87
	BnaA10g01020D	3.64	1.66	1.3	-0.59	2.65	-2.82
	BnaA03g20290D	3.38	2.61	1.95	-3.54	-4.95	2.49
	BnaC03g19370D	3.16	2.23	0.61	1.25	0.93	0.2

	BnaUnng03570D	3.15	3.2	0.63	3.66	1.97	1.16
	BnaA03g20310D	3.01	2.55	1.74	2.27	1	1.22
	BnaC04g53040D	0	0	0	7.69	7.47	0.29
	BnaC03g37570D	0.01	-0.63	0.34	7.33	7.96	-2.73
	BnaC03g37610D	-0.21	-0.88	0.51	6.78	7.1	-2.75
3	BnaA03g32280D	-2.61	-2.03	-0.74	6.71	7.07	-1.68
	BnaC03g37600D	-2.3	-0.81	-0.54	6.62	7.26	-1.18
	BnaC04g09720D	0.3	0.75	-0.3	6.51	6.19	-0.84
	BnaA05g08640D	1.97	0.72	-0.25	6.27	5.62	-0.72
	BnaC03g24300D	-0.09	0	0.06	4.5	4.45	-1.5

3. Discussion

Plants have developed highly sophisticated immune mechanisms to respond to pathogen attack by the induction of expression of a large number of genes encoding pathogenesis-related (PR) proteins, such as chitinases. Chitinases are believed to play important roles in plant-pathogen interactions and catalyze the hydrolysis of the β -1-4-linkage in the N-acetyl-D-glucosamine polymer of chitin, which is a major component of many fungal cell walls, but absent in higher plants (LEGRAND *et al.* 1987; COLLINGE *et al.* 1993). The chitinase gene family has been widely characterized as excellent candidates to improve plants tolerance to stresses, including drought (HONG and HWANG 2002; LEE *et al.* 2008), salt (HONG and HWANG 2002), cold (YEH *et al.* 2000), heat (KWON *et al.* 2007), UV light, wounding (BREDERODE *et al.* 1991), fungal pathogens and some insect pests (LIN *et al.* 1995; DING *et al.* 1998; YAMAMOTO *et al.* 2000; WANG *et al.* 2005; PRASAD *et al.* 2013; CHEN *et al.* 2014). However, the genome-wide identification and expression pattern of the chitinase gene family in response to *L. maculans* and *S. sclerotiorum* infection has not been reported in *B. napus*.

In this study, a total of 68 chitinase genes were identified in *B. napus* genome. Of these, GH-18 family and GH-19 family have 12 and 56 chitinase genes, respectively, which was further supported by analysis of gene structure and conserved motifs (Figure 2, 3). GH-18 family was divided into Class III (4 genes) and Class V (8 genes). GH-19 family was composed of Class I (11 genes), Class II (9 genes) and Class IV (36 genes). However, there were 13 and 26 chitinase genes in GH-19 family and GH-18 family in para rubber tree, respectively (MISRA 2015). Class IV had the most members in *B. napus* whereas Class III posed the most genes of chitinase in Para rubber tree, which may reveal that there is evolutionary divergence of specific classes of chitinases in different species. The chitinase gene family has 24, 32, 35, and 68 members in *A. thaliana*, *B. rapa*, *B. oleracea*, and *B. napus*, respectively (XU *et al.* 2007), which suggested that chitinase genes in *B. napus* had expanded in comparison to its ancestors. Gene duplication events, such as tandem duplication and segmental duplication play important roles in the rapid expansion and evolution of gene families (XU *et al.* 2012). The AACC genome of *B. napus* was formed through recent allopolyploidy between the ancestors of *B. rapa* (AA) and *B. oleracea* (CC) (CHALHOUB *et al.* 2014). From our analysis, 30 orthologs of 32 chitinase genes in the A genome were identified in *B. rapa* and 32 orthologs of 35 chitinase genes in the C genome of *B. oleracea*. Most orthologous gene pairs in *B. rapa* and *B. oleracea* are still homeologous pairs in *B. napus*. Most chitinase genes in *B. napus* showed a close relationship to their ancestor chitinase genes, which suggested that segmental duplication or polyploidy events contributed to the expansion of the chitinase gene family in *B. napus*. Only one pair of tandemly duplicated genes (BnaA09g15430D and BnaA09g15440D) was identified. These findings suggest that segmental duplication and tandem duplication likely plays an important role in the expansion of the chitinase gene family in *B. napus*. Moreover, five genes (BnaA09g34290D, BnaC04g09720D, BnaC03g37600D, BnaA03g32270D and BnaC03g37570D) do not have orthologs in *A. thaliana*, *B. rapa* and *B. oleracea*, suggesting that they may be the new members of the chitinase gene family and coevolve with fungi in response to variation in pathogen defenses.

Chitinases play a major role in host defense by directly attacking fungal pathogens in *A. thaliana* (GERHARDT *et al.* 1997), rice (LIN *et al.* 1995), grapevine (YAMAMOTO *et al.* 2000), tobacco (DING *et al.* 1998; CHEN *et al.* 2014; DONG *et al.* 2017), peanut

(PRASAD *et al.* 2013) and pepper (HONG and HWANG 2002). Some chitinase members can be induced by fungal pathogens, such as *Cylindrosporium concentricum*, *Phoma lingam*, and *S. sclerotiorum*. The role of chitinase gene also had been studied in *B. napus* and overexpression of chitinase genes could increase tolerance in transgenic plants previously (GRISON *et al.* 1996; ZARINPANJEH *et al.* 2016). Transgenic plants of *B. napus* cv. ZS 758 carrying sporamin and chitinase PjChi-1 genes exhibited increased levels of resistance to *S. sclerotiorum* and reduced the size of leaf spot in transformants compared to untransformed wild-type plants (LIU *et al.* 2011). However, constitutive expression of pea chitinase gene showed little or no enhancement of resistance to *L. maculans* in transgenic rapeseed compared with non-expressing transgenic lines (WANG *et al.* 1999). Some chitinases such as pineapple leaf chitinase-A do not have any antifungal activity (TAIRA *et al.* 2005). Although many studies about the individual member of the chitinase gene family have been published, there is little information about analysis of their expression divergence of the chitinase gene family at a genome-wide level in rapeseed, especially under different fungal pathogen stresses. In this study, detailed expression pattern of the chitinase gene family against *L. maculans* and *S. sclerotiorum* infection in rapeseed was analyzed using RNA-seq data. The results showed that many chitinase genes could transcriptionally respond to *L. maculans* and *S. sclerotiorum* infection in rapeseed (Figure 5), implying possible function of chitinase genes in response to these two fungal pathogens in *B. napus*. The results reveal that the resistant accessions differentiate from the susceptible ones in pathogen defense so we hypothesized that the function of different chitinase genes has been diverged against pathogens in resistant and susceptible *B. napus* accessions. Previously, the expression of chitinase genes could be induced in response to all kinds of pathogens, such as *G. mosseae*, *F. subglutinans* f. sp. *Pini*, *X. campestris*, *L. maculans* and *S. sclerotiorum* (GERHARDT *et al.* 1997; BONANOMI *et al.* 2001; DAVIS *et al.* 2002; LOWE *et al.* 2014; WU *et al.* 2016). Next, to identify genes critically responsible for *L. maculans* and *S. sclerotiorum* resistance in resistant accessions, we compared the *L. maculans* and *S. sclerotiorum* responsive chitinase genes in both resistant and susceptible accessions, respectively. Furthermore, we compared the 18 up-regulated chitinase genes on pathogen *L. maculans* aggression with 18 up-regulated chitinase genes against *S. sclerotiorum* attack. Interestingly, the upregulation after infection with *L. maculans* was stronger in resistant accession than in susceptible accessions. In contrast, the upregulation after *S. sclerotiorum* attack showed higher levels in both resistant and susceptible accessions whereas there was much less differences in in both resistant and susceptible accessions. In addition, there were some chitinase members that no expression changes was detected, which may be accounted for that some chitinases showed little or no enhancement of resistance and do not have any antifungal activity (WANG *et al.* 1999; TAIRA *et al.* 2005). The above results indicate that some members of the chitinase gene family have developed as powerful basal defence against various pathogens attack and other individual members of the chitinase gene family have evolved different roles in response to different environmental stresses in *B. napus*.

In summary, our study provides a comprehensive analysis of the chitinase gene family in the rapeseed, including gene identification, sequence features, physical location, evolutionary relationship, and expression patterns of chitinase genes responding to *L. maculans* and *S. sclerotiorum* infection, which could facilitate further dissection of the function of the chitinase gene family in rapeseed.

4. Materials and Methods

4.1 Identification of chitinase genes in *B. napus*

The v4.1 genome sequences and annotations of *B. napus* were downloaded from the FTP site of the Brassica database (ftp://brassicadb.org/Brassica_napus/)(CHENG *et al.* 2011). To identify chitinase genes in *B. napus*, Glyco_hydro_18 (PF00704) and Glyco_hydro_19 (PF00182) domains were obtained from the Pfam website (<http://pfam.xfam.org/>). The HMMER software version 3.0 was employed to identify chitinase genes against all known protein sequences (FINN *et al.* 2011). All candidate genes were further submitted to Pfam analysis (<http://pfam.xfam.org/>) to confirm the presence of one of the above two domains with E-value 0.0001. For annotation, the identified protein sequences were aligned with NCBI nr database using BLAST alignment (E-value cut-off of 1e-5)

(ALTSCHUL *et al.* 1997). The identification of signal peptide was performed in the website (<http://www.cbs.dtu.dk/services/SignalP/>) (PETERSEN *et al.* 2011).

4.2 Phylogenetic tree construction, and sequence analysis

All identified chitinase genes were aligned using the MUSCLE program within MEGA 7.0 software (KUMAR *et al.* 2016). Subsequently, a neighbor-joining (NJ) method was then applied to construct a phylogeny of chitinase genes with a 1000 bootstrap replication. Motifs of chitinase proteins in *B. napus* were investigated statistically using online MEME software (<http://meme-suite.org/tools/meme>), which set the maximum number of motifs at 10. Subsequently, InterProScan (<http://www.ebi.ac.uk/interpro/search/sequence-search>) was employed to annotate the all identified motifs. In addition, the exon-intron structures of genes were performed with the gene structure display server program (<http://gsds.cbi.pku.edu.cn/>).

4.3 Chromosomal distribution and evolution patterns of chitinase genes

The chromosomal locations of chitinase genes were determined based on annotation data obtained from the *B. napus* database. The orthologous relationships between the chitinase genes in *B. napus* and *A. thaliana*, *B. rapa*, and *B. oleracea* genes were evaluated following the criteria: we used program BLAST to identify putative orthologues between chitinase genes in *B. napus* and one of *A. thaliana*, *B. rapa*, and *B. oleracea* species with both coverage over 70% and identity more than 70%, all chitinase sequences from *B. napus* was searched against all gene sequences from one of *A. thaliana*, *B. rapa*, and *B. oleracea* species. Tandem duplication was characterized as multiple genes of one family located within the same or neighboring intergenic region (LI *et al.* 2014).

4.4 Analysis of transcriptome sequencing data

The sequence data responsive to *L. maculans* infection was deposited in the BioProject Database of the National Center for Biotechnology Information under accession number PRJNA378851. Transcriptome data under accession number PRJNA274853 publicly available on the NCBI SRA database were mined and analyzed for expression patterns of the rapeseed chitinase genes in response to *S. sclerotiorum* infection. Sequencing reads were then aligned to the *B. napus* reference genome sequence (ftp://brassicadb.org/Brassica_napus/) using TopHat, v2.1.1 (KIM *et al.* 2013). Mapping data was used to estimate expression values for annotated genes using htseq-count tool (ANDERS *et al.* 2015). Differential gene expression analyses were performed using the R/Bioconductor package, DESeq2 (LOVE *et al.* 2014). An absolute value of log₂ fold change >1.5 and the False Discovery Rate (FDR) < 0.05 was set to declare differentially expressed genes.

Supplementary Materials

Supplementary Figure 1 Details of the ten conserved motifs of chitinase GH-18 family as derived by MEME analysis.

Supplementary Figure 2 Details of the ten conserved motifs of chitinase GH-19 family as derived by MEME analysis.

Supplementary Table 1 Amino acid sequences of 68 chitinase genes in *B. napus*.

Acknowledgments

This study was financially supported by the Independent Innovation Special Fund of Henan Academy of Agricultural Sciences (2018ZC78), the Henan Fundamental and Frontier Research Fund (162300410153) and by the Natural Sciences and Engineering Research Council (NSERC) CRD project and the Growing Forward project of SaskCanola and Agriculture and Agri-Food Canada (AAFC).

Author Contributions

319 Wen Xu and Genyi Li designed the study. Tengsheng Zhou and Bo An performed the experiments. Wen Xu and Baojiang Xu
320 analyzed the data and drafted the manuscript. Genyi Li and Wen Xu finished the manuscript. All of the authors carefully checked and
321 approved this version of the manuscript.

322 **Conflicts of Interest**

323 The authors declare no conflict of interest.

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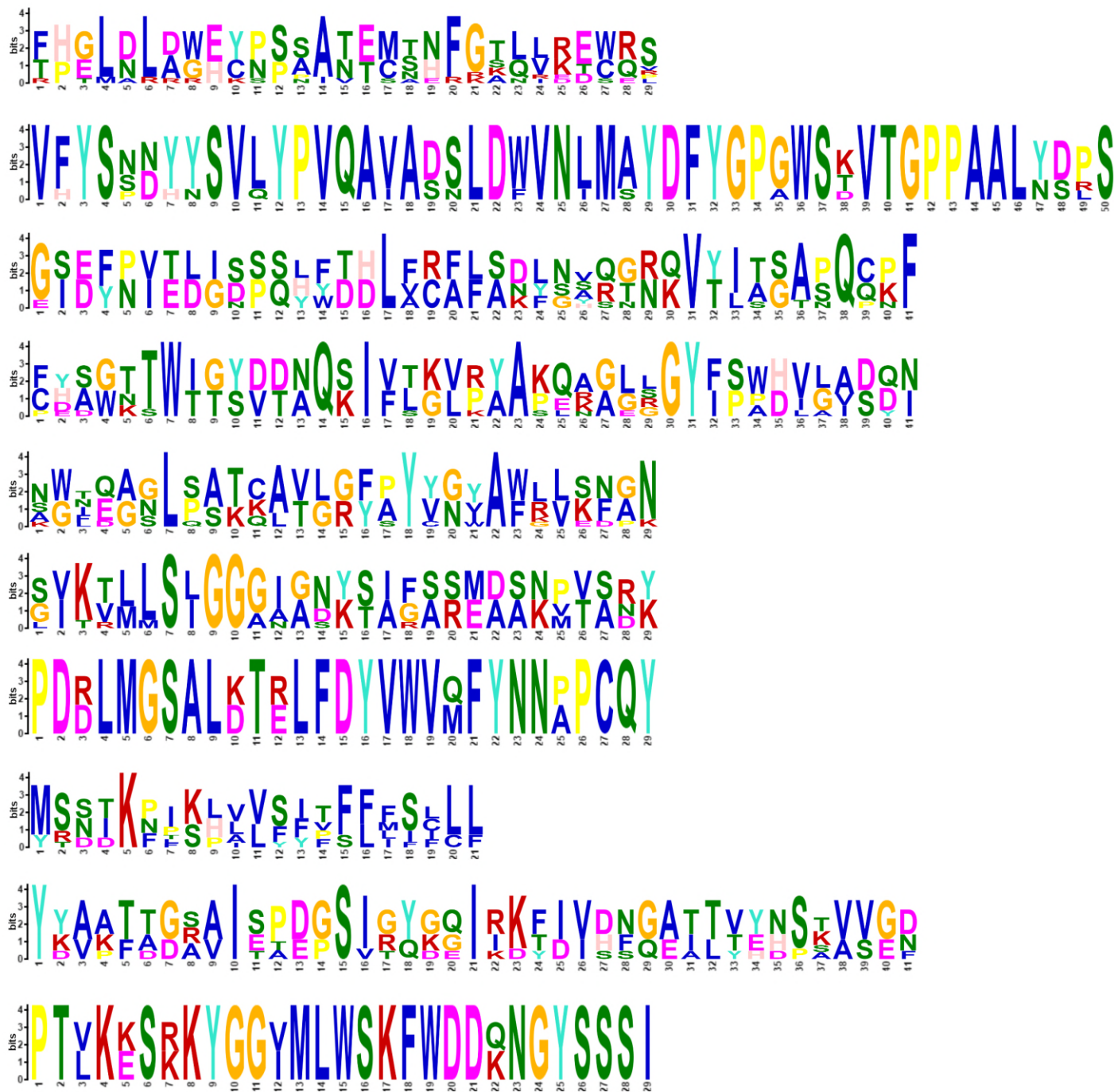
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Supplementary Figure 1



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482 Supplementary Figure 2



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Supplementary Table 1 Amino acid sequences of 68 chitinase genes in *B. napus*

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