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

Wen Xu¹, Tengsheng Zhou², Bo An², Baojiang Xu³, Genyi Li¹*²

1 Crop Designing Center, Henan Academy of Agricultural Sciences, Zhengzhou, 450002, China
2 Department of Plant Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada
3 Henan Academy of Agricultural Sciences, Zhengzhou, 450002, China

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

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

1. Introduction

Plant chitinases (EC 3.2.1.14) are enzymes that hydrolyze the N-acetyl glucosamine polymer chitin, a major component of fungal cell walls and exoskeleton of insects (Collinge *et al.* 1993) and are considered as one group of pathogenesis-related (PR) proteins (Legrand *et al.* 1987), which can be induced in response to the infection of various pathogenic micro-organisms. In the light of classification of glycosyl hydrolases based on amino acid sequence similarities, plant chitinases have been put in glycoside hydrolase family 18 (GH-18) and 19 (GH-19) (Henrissat 1991). According to the CAZy database ([http://www.cazy.org/Glycoside-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, 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 1991). The details of plant chitinase classification are described as follows. Class I chitinases have an N-terminal chitin-binding domain and a GH-19 catalytic domain. Class II chitinases consist of only a catalytic domain with a high level of sequence and structure similarity to class I chitinases but lack the chitin-binding domain and linker regions. Class IV chitinases show high homology with class I chitinases but are smaller due to one deletion in the chitin-binding domain and three deletions in the catalytic domain (Xu *et al.* 2016). Both class III and V chitinases have a GH-18 catalytic domain and a consensus sequence DXDXE, but there is no
homology for other amino acids (Umemoto et al. 2015). GH-18 chitinases are widely distributed in plants, animals, fungi, bacteria and viruses whereas GH-19 members almost exclusively exist in higher plants (Passarinho and de Vries 2002).

In higher plants, the expression of chitinase genes is involved in defense against biotic and abiotic stress as well as in growth and developmental processes (Collinge et al. 1993; Punja and Zhang 1993). For instance, a class III chitinase gene, Mtchitinase III-3, has been found to be induced upon the infection of fungi Glomus mosseae and Glomus intraradices in cortical root (Bonanomi et al. 2001). PSCHI4, a putative extracellular class II chitinase, is up-regulated in pine seedlings infected with the necrotrophic pathogen Fusarium subglutinans f. sp. Pini (Davis et al. 2002). In Arabidopsis thaliana, a class IV chitinase gene AtchitIV accumulated very rapidly in leaves after inoculation with Xanthomonas campestris and reached maximum mRNA accumulation after one hour infection (Gerhardt et al. 1997). In addition, several transgenic studies showed that enhanced levels of chitinase genes in transgenic plants can indeed improve resistance against pathogens and reduce the damage caused by fungi 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). There are several reports of induced expression of plant chitinases when plants were exposed to abiotic stresses such as heavy-metal stress (BekesiOva et al. 2008), 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 and wounding (Brederode et al. 1991). Furthermore, some chitinases are essential in physiological processes like somatic embryo development (Dejong et al. 1992) and formation of root nodules (Ovtsyna et al. 2000). In conclusion, chitinases play important roles in plant defense and plant health.

Rapeseed (Brassica napus) is an important oilseed crop worldwide. This crop is affected by various fungal pathogens, especially blackleg caused by Leptosphaeria maculans and sclerotinia stem rot by Sclerotinia sclerotiorum, which are the most destructive rapeseed diseases in Canada, Australia, Europe and many other regions around the world (West et al. 2001). Recently, a few studies have been conducted on chitinase genes responding to some pathogens infection in B. napus (Rasmussen et al. 1992a; Rasmussen et al. 1992b; Grison et al. 1996; Wang et al. 2005; Ahmed et al. 2012). For example, constitutive overexpression of a chimeric chitinase gene in rapeseed had been shown to exhibit an increased resistance to three fungal pathogens compared with their nontransgenic parental plants (Grison et al. 1996). Co-expression of defensin gene Rs-AFPI from R. sativus and chimeric chitinase gene chit42 from T. atroviride in rapeseed via Agrobacterium-mediated transformation demonstrated enhanced resistance against sclerotinia stem rot disease (Zarinpanieh et al. 2016). In addition, global studies of transcriptome dynamics of defense responses to L. maculans and S. sclerotiorum in B. napus presented that pathogen responsive genes including chitinases were rapidly induced during early infection (Low et al. 2014; Haddadi et al. 2016; Joshi et al. 2016; Wu et al. 2016). However, to date, the chitinase genes in rapeseed have not been systematically identified and thus the genetic resistance to L. maculans and S. sclerotiorum has been not yet studied. Recently, the availability of the whole genome sequence and RNA-seq sequencing enable further investigations into chitinase genes and their response to L. maculans and S. sclerotiorum infection on a genome-wide scale (Chalhoub et al. 2014; Woodhouse et al. 2014).

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

2. Results

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           |
| BnaC01g38090D       | Glyco_hydro_19 | I     | CO1 | 37477744 | 37480353 | -      | 216               | 3               | YES           |
| BnaC01g38450D       | Glyco_hydro_19 | I     | CO1 | 37494610 | 37495630 | -      | 227               | 2               | NO            |
| BnaC03g35750D       | Glyco_hydro_19 | I     | CO3 | 23003071 | 23005099 | -      | 231               | 4               | YES           |
| BnaC03g37600D       | Glyco_hydro_19 | I     | CO3 | 23016407 | 23018656 | -      | 239               | 3               | YES           |
| BnaC03g37610D       | Glyco_hydro_19 | I     | CO3 | 23021408 | 23025003 | -      | 242               | 2               | YES           |
| BnaC05g40680D       | Glyco_hydro_19 | I     | CO5 | 38780461 | 38782151 | +      | 245               | 2               | YES           |
| BnaA08g31740D       | Glyco_hydro_19 | II    | A08 | 2107446 | 2110073 | +      | 245               | 3               | YES           |
| BnaA09g03430D       | 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           |
| BnaC05g01410D       | Glyco_hydro_19 | II    | CO5 | 600283  | 601194  | -      | 256               | 2               | YES           |
| BnaC05g03990D       | Glyco_hydro_19 | II    | CO5 | 1957654 | 1959846 | -      | 257               | 3               | YES           |
| BnaC05g36460D       | Glyco_hydro_19 | II    | CO5 | 35739352 | 35741554 | +      | 261               | 9               | YES           |
| BnaC08g08040D       | Glyco_hydro_19 | II    | CO8 | 12906975 | 1299667 | -      | 261               | 3               | YES           |
| BnaCnmg1030D        | Glyco_hydro_19 | II    | Cnn | 1527030 | 1528502 | +      | 263               | 2               | YES           |
| BnaC07g30330D       | Glyco_hydro_18 | III   | CO7 | 34864788 | 34866603 | +      | 263               | 3               | YES           |
| BnaC09g04600D       | Glyco_hydro_18 | III   | CO9 | 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           |
| Gene | Protein | Accession | pValue | Significance | pValue | Significance | pValue | Significance |
|------|---------|-----------|--------|-------------|--------|-------------|--------|-------------|
| BnaA03g56430D | Glyco_hydro_19 | IV | A03 | 711494 | 712848 | + | 275 | YES |
| BnaA04g25220D | Glyco_hydro_19 | IV | A04 | 18233599 | 18234851 | - | 275 | NO |
| BnaA04g25230D | Glyco_hydro_19 | IV | A04 | 18235520 | 18236868 | - | 279 | NO |
| BnaA05g03420D | Glyco_hydro_19 | IV | A05 | 1888936 | 1890632 | - | 279 | YES |
| BnaA05g03430D | Glyco_hydro_19 | IV | A05 | 1893397 | 1894609 | - | 280 | YES |
| BnaA05g03440D | Glyco_hydro_19 | IV | A05 | 1904615 | 1905892 | - | 280 | YES |
| BnaA05g08640D | Glyco_hydro_19 | IV | A05 | 4802795 | 4804226 | + | 281 | YES |
| BnaA09g15430D | Glyco_hydro_19 | IV | A09 | 8977793 | 8978880 | + | 281 | YES |
| BnaA09g15440D | Glyco_hydro_19 | IV | A09 | 8979421 | 8980691 | + | 281 | NO |
| BnaA09g34290D | Glyco_hydro_19 | IV | A09 | 25164422 | 25167344 | + | 281 | YES |
| BnaA10g05680D | Glyco_hydro_19 | IV | A10 | 3646850 | 3650151 | - | 282 | YES |
| BnaC03g19370D | Glyco_hydro_19 | IV | C03 | 10063312 | 10065182 | - | 282 | YES |
| BnaC03g24270D | Glyco_hydro_19 | IV | C03 | 13607893 | 13609216 | - | 282 | YES |
| BnaC03g24280D | Glyco_hydro_19 | IV | C03 | 13614270 | 13615575 | - | 282 | YES |
| BnaC03g24290D | Glyco_hydro_19 | IV | C03 | 13628566 | 13630189 | - | 283 | YES |
| BnaC03g24300D | Glyco_hydro_19 | IV | C03 | 13641790 | 13643090 | - | 284 | YES |
| BnaC03g24310D | Glyco_hydro_19 | IV | C03 | 13644193 | 13644910 | - | 302 | YES |
| BnaC03g24330D | Glyco_hydro_19 | IV | C03 | 13646581 | 13649085 | - | 302 | YES |
| BnaC03g24340D | Glyco_hydro_19 | IV | C03 | 13654943 | 13656106 | - | 302 | NO |
| BnaC03g24360D | Glyco_hydro_19 | IV | C03 | 13695822 | 13697335 | - | 318 | YES |
| BnaC04g09720D | Glyco_hydro_19 | IV | C04 | 7384173 | 7385277 | + | 319 | YES |
| BnaC04g08920D | Glyco_hydro_19 | IV | C04 | 47325431 | 47326573 | + | 320 | YES |
| BnaC04g09090D | Glyco_hydro_19 | IV | C04 | 47408585 | 47409809 | - | 322 | NO |
| BnaC04g09100D | Glyco_hydro_19 | IV | C04 | 47411832 | 47413177 | - | 322 | YES |
| BnaC04g53030D | Glyco_hydro_19 | IV | C04 | 680771 | 682319 | - | 322 | YES |
| BnaC04g53040D | Glyco_hydro_19 | IV | C04 | 688006 | 689125 | - | 322 | NO |
| BnaC08g25190D | Glyco_hydro_19 | IV | C08 | 27018000 | 27020278 | + | 323 | NO |
| BnaC08g25210D | Glyco_hydro_19 | IV | C08 | 27024727 | 27025633 | + | 329 | NO |
| BnaC09g55720D | Glyco_hydro_19 | IV | C09 | 948730 | 950079 | - | 334 | YES |
| BnaCnn39650D | Glyco_hydro_19 | IV | Cnn | 38235388 | 38236682 | + | 342 | YES |
| BnaCnn39640D | Glyco_hydro_19 | IV | Cnn | 38235388 | 38236682 | + | 342 | YES |
| BnaA01g34980D | Glyco_hydro_18 | V | A01 | 304675 | 306686 | + | 363 | YES |
| BnaUmmg03570D | Glyco_hydro_18 | V | Umm | 5067935 | 5068651 | - | 371 | NO |
| BnaA03g26800D | Glyco_hydro_18 | V | A03 | 13187584 | 13189629 | + | 381 | YES |
| BnaA03g26330D | Glyco_hydro_18 | V | A03 | 12888937 | 12890809 | + | 383 | YES |
| BnaC03g31740D | Glyco_hydro_18 | V | C03 | 19525415 | 19530521 | + | 424 | YES |
| BnaA08g09330D | Glyco_hydro_18 | V | A08 | 8944530 | 8946552 | - | 513 | YES |
| BnaC01g41020D | Glyco_hydro_18 | V | C01 | 240723 | 242688 | + | 1005 | YES |
Figure 1 Phylogenetic tree of the chitinase gene family in *Brassica napus*. The chitinase protein sequences were used to construct multiple sequence alignments using MUSCLE program within MEGA 7.0 software. Phylogenetic analysis was performed using MEGA 7.0 with the neighbor-joining method with 1,000 bootstrap replications.

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

The above phylogenetic tree highlighted that the 68 chitinase genes could be divided into five well-supported subfamilies, which were consistent with Class I, II, III, IV, and V. As expected, the chitinase genes of Glyco_hydro_19 and Glyco_hydro_18 families were clustered into two relatively distinct branches. Chitinase genes from subfamilies Class III and Class V are in the Glyco_hydro_18 clade, whereas subfamilies Class I, II, and IV that belong to Glyco_hydro_19 clade were clustered together and showed close relationships (Figure 2). According to the phylogenetic tree, chitinases in different subfamilies had various characteristics. Among the five subfamilies, Class IV was found to be the biggest group with 36 members, accounting for almost a half of the chitinase gene 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
10 are unique in class III whereas motif 2 can be only detected in class V. In addition, we analyzed the coding sequences with corresponding genome sequences of each chitinase genes in B. napus. A detailed illustration of the chitinase gene structure was shown in Figure 3. Most chitinase genes contained two or three exons, whereas three chitinase genes (BnaA01g30550D, BnaA0626330D and BnaUnng03570D) had no introns. In general, most chitinase genes in the same class showed similar conserved motifs and exon-intron structures. These findings revealed that motif compositions and gene structures of each class in the chitinase gene family were relatively conserved. The similar features of chitinase genes in the same class may fulfill similar functions and this claim need to be supported by their expression and related data.
Figure 3 Exon-intron structures of all chitinase genes in *Brassica napus*. Schematic diagram represents the gene structure of all 68 chitinase genes identified in this study using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). CDS are shown as red boxes; introns are indicated by double slashes on the bar; UTR sequences are shown as blue boxes.

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

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

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

To understand the evolution of the chitinase gene family, twenty-six pairs of paralogs were detected in 68 chitinase genes based on criteria for both coverage ≥70% and identity ≥70% (Table 2). The phylogenetic relationship analysis of chitinase genes also showed that most pairs of paralogs could be clustered together (Figure 1). For example, the four members in two pairs of paralogs (BnaA09g05050D and BnaC09g04600D, BnaA06g26630D and BnaC07g30330D) in Class III subfamily were clustered into two parts in a single clade. As genome duplication was considered, one member in the A subgenome would correspond to one homologous gene in the C subgenome in *B. napus*. In fact, 50 members of 68 chitinase genes showed such a one-to-one correspondence.

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

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

Blackleg, also known as stem canker caused by L. maculans and sclerotinia stem rot caused by S. sclerotiorum are two major rapeseed diseases in most major rapeseed growing areas. The expression profiling of all chitinase genes in response to L. maculans and S. sclerotiorum infection in resistant and susceptible B. napus accessions were investigated using whole-transcriptome sequencing data to understand the roles of chitinase genes at early stages of infection. The results showed that these two biostresses caused a significant expression induction of some members in the chitinase gene family. The differentially expressed genes (DEGs) of chitinases in response to L. maculans were identified. Among all 68 chitinase genes in the genome, 31 and 25 chitinase genes were up-regulated in the resistant lines and susceptible lines inoculated with the pathogen compared with their water control, respectively. Of these up-regulated chitinase genes, 24 were overlapped in the resistant lines and susceptible accessions. Combined data of all resistant and susceptible lines revealed that 15 chitinase genes were upregulated and no chitinase gene was dramatically downregulated during the infection of pathogen L. maculans (Figure 5).
**Figure 5 Expression profiles of chitinase genes in response to *Leptosphaeria maculans* (lma) and *Sclerotinia sclerotiorum* (sel) 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.
In a previous report, the abundance of transcripts in resistant and susceptible *B. napus* accessions at the 4 day post-inoculation treatments were analyzed to understand the differential defense response to *S. sclerotiorum* (WU et al. 2016). Using these data and all 68 chitinase genes identified in this study, 23 and 20 chitinase genes were up-regulated while 6 and 11 members were downregulated in the resistant accession and susceptible accession compared with water control, respectively. Compared with the expression of chitinase genes in response to blackleg pathogen infection, 16 up-regulated and 4 down-regulated chitinase genes were overlapped respectively. Compared with the S accession, the analyses showed that 13 and 7 chitinase genes were the same as those upregulated and downregulated ones against *S. sclerotiorum* attack, respectively.

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

Table 3 Fold change (log2) of chitinase gene expression in response to *Leptosphaeria maculans* (lma) and *Sclerotinia 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   |
|       | BnaA05g20300D | 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   |
|       | BnaCng39650D  | 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  |
|       | BnaA05g20290D | 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    |
### 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 Huang 2002; Lee et al. 2008), salt (Hong and Huang 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; Zarinpajeh 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 (TaRa 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 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; TaRa et al. 2005). The above results indicate that some members of the chitinase gene family have developed as powerful basal defense 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 htsq-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
Wen Xu and Genyi Li designed the study. Tengsheng Zhou and Bo An performed the experiments. Wen Xu and Baojiang Xu analyzed the data and drafted the manuscript. Genyi Li and Wen Xu finished the manuscript. All of the authors carefully checked and approved this version of the manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

**References**

Ahmed, N. U., J. I. Park, M. S. Seo, T. S. Kumar, I. H. Lee et al., 2012 Identification and expression analysis of chitinase genes related to biotic stress resistance in Brassica. Mol Biol Rep 39: 3649-3657.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang et al., 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

Anders, S., P. T. Pyl and W. Huber, 2015 HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31: 166-169.

Bekesiova, B., S. Hraska, J. Libantova, J. Moravcikova and I. Matusikova, 2008 Heavy-metal stress induced accumulation of chitinase isoforms in plants. Molecular Biology Reports 35: 579-588.

Bonanomi, A., A. Wiemken, T. Boller and P. Salz, 2001 Local induction of a mycorrhiza-specific class III chitinase gene in cortical root cells of Medicago truncatula containing developing or mature arbuscules. Plant Biology 3: 194-199.

Brederode, F. T., H. J. M. Linthorst and J. F. Bol, 1991 Differential Induction of Acquired-Resistance and Pr Gene-Expression in Tobacco by Virus-Infection, Ethephon Treatment, Uv-Light and Wounding. Plant Molecular Biology 17: 1117-1125.

Cantarel, B. L., P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard et al., 2009 The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Research 37: D233-D238.

Chalhoub, B., F. Denoeud, S. Liu, I. A. Parkin, H. Tang et al., 2014 Plant genetics. Early allopolyploid evolution in the post-Neolithic Brassica napa oisled genome. Science 345: 950-953.

Chen, P. J., R. Senthilkumar, W. N. Jane, Y. He, Z. Tian et al., 2014 Transplastomic Nicotiana benthamiana plants expressing multiple defence genes encoding protease inhibitors and chitinase display broad-spectrum resistance against insects, pathogens and abiotic stresses. Plant Biotechnol J 12: 503-515.

Cheng, F., S. Liu, J. Wu, L. Fang, S. Sun et al., 2011 BRAD, the genetics and genomics database for Brassica plants. BMC Plant Biol 11: 136.

Collinge, D. B., K. M. Kragh, J. D. Mikkelsen, K. K. Nielsen, U. Rasmussen et al., 1993 Plant chitinases. Plant J 3: 31-40.

Davis, J. M., H. G. Wu, J. E. K. Cooke, J. M. Reed, K. S. Luce et al., 2002 Pathogen challenge, salicylic acid, and jasmonic acid regulate expression of chitinase gene homologs in pine. Molecular Plant-Microbe Interactions 15: 380-387.

Dejong, A. J., J. Cordewener, F. Loschiavo, M. Terzi, J. Vandenkerckhove et al., 1992 A Carrot Somatic Embryo Mutant Is Rescued by Chitinase. Plant Cell 4: 425-433.

Ding, X., B. Gopalakrishnan, L. B. Johnson, F. F. White, X. Wang et al., 1998 Insect resistance of transgenic tobacco expressing an insect chitinase gene. Transgenic Res 7: 77-84.

Dong, X., Y. Zhao, X. Ran, L. Guo and D. G. Zhao, 2017 Overexpression of a New Chitinase Gene EuCHIT2 Enhances Resistance to Erysiphe cichoracearum DC in Tobacco Plants. Int J Mol Sci 18.

Finn, R. D., J. Clements and S. R. Eddy, 2011 HMMER web server: interactive sequence similarity searching. Nucleic Acids Res 39: W29-37.

Gerhardt, L. B. D., G. Sachetto-Martins, M. G. Contarini, M. Sandroni, R. D. Ferreira et al., 1997 Arabidopsis thaliana class IV chitinase is early induced during the interaction with Xanthomonas campestris. Febs Letters 419: 69-75.

Grison, R., B. Grezes-Besset, M. Schneider, N. Lucante, L. Olsen et al., 1996 Field tolerance to fungal pathogens of Brassica napus constitutively expressing a chimeric chitinase gene. Nat Biotechnol 14: 643-646.

Haddadi, P., L. Ma, H. Wang and M. H. Borhan, 2016 Genome-wide transcriptomic analyses provide insights into the lifestyle transition and effector repertoire of Leptosphaeria maculans during the colonization of Brassica napus seedlings. Mol Plant Pathol 17: 1196-1210.

Henrissat, B., 1991 A Classification of Glycosyl Hydrolases Based on Amino-Acid-Sequence Similarities. Biochemical Journal 280: 309-316.

Hong, J. K., and B. K. Hwang, 2002 Induction by pathogen, salt and drought of a basic class II chitinase mRNA and its in situ localization in pepper (Capsicum annuum). Physiologia Plantarum 114: 549-558.
Woodhouse, M. R., F. Cheng, J. C. Pires, D. Lisch, M. Freeling et al., 2014 Origin, inheritance, and gene regulatory consequences of genome dominance in polyploids. Proc Natl Acad Sci U S A 111: 5283-5288.

Wu, J., Q. Zhao, Q. Yang, H. Liu, Q. Li et al., 2016 Comparative transcriptomic analysis uncovers the complex genetic network for resistance to Sclerotinia sclerotiorum in Brassica napus. Sci Rep 6: 19007.

Xu, F., C. Fan and Y. He, 2007 Chitinases in Oryza sativa ssp. japonica and Arabidopsis thaliana. J Genet Genomics 34: 138-150.

Xu, G., C. Guo, H. Shan and H. Kong, 2012 Divergence of duplicate genes in exon-intron structure. Proc Natl Acad Sci U S A 109: 1187-1192.

Xu, J., X. Xu, L. Tian, G. Wang, X. Zhang et al., 2016 Discovery and identification of candidate genes from the chitinase gene family for Verticillium dahliae resistance in cotton. Sci Rep 6: 29022.

Yamamoto, T., H. Iketani, H. Ieki, Y. Nishizawa, K. Notsuka et al., 2000 Transgenic grapevine plants expressing a rice chitinase with enhanced resistance to fungal pathogens. Plant Cell Reports 19: 639-646.

Yeh, S., B. A. Moffatt, M. Griffith, F. Xiong, D. S. C. Yang et al., 2000 Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiology 124: 1251-1263.

Zarinpanjeh, N., M. Motallebi, M. R. Zamani and M. Ziaei, 2016 Enhanced resistance to Sclerotinia sclerotiorum in Brassica napus by co-expression of defensin and chimeric chitinase genes. J Appl Genet 57: 417-425.
Supplementary Table 1  Amino acid sequences of 68 chitinase genes in *B. napus*

| Accession | Sequence |
|-----------|----------|
| >BnaA06g26630D | MSNIKNFKPVSFFISCCCFCKPSHSRAGIAIYWQNGFGSLSSSTCATGRYAVNIALKFQNGQTPELNLAGHCNPAAN TCTHGFAGVKTTCQLRGIKVFLITRLLSLGGAIGNYSIRSDAKMVADYLWNFLGKKSSAPRLGDAVLDGIYELGSP QHWDDLYRFLSNFSGRGRKYYGITAP0CPFDNLGSLTGLKTLRDFVYVWMFYNPCQYTSCTQSLFHSWKWTTSVT AQKIFGGLPAAPEAEoggles 
| >BnaA05g26640D | MKTCLLFLIFSLLSSFAEEQGRCQAGGALCPNLCCSEFGWCGNTEPYCKQPGCSQCGTGGPGPTGDLSGIIIRSFQDD MLKHRRDNSACAPGFTYDADFIAAKSFPFGGTGTDATRRKKEIAAFFQGQSTHTGIGWATAPDGPSWGYCFKQEQNPS SNYCSPSAEWPCASQKSYRGPPQMLSWYNYQGCGRAIGDLDLNNPDLVSNQVIAFKAAAIFWMTIQSPKPSCHAVIV GWWQPSDADRAAGRPGYGVINNIGGLECRGQPRDARVADRGFYQRCNIIILGVNPGGNLDCYNQSRFASVNFELDAAX |
| >BnaC03g63440D | MASNPTSRKSFIDSSRLARSGNFHGLDLDWEPSSATEMNNFGTLLREWRSAVVAAEASSTRPRLLLAAAVFYSSDVYSL YPVQAVASSLDWVNLMAYDFYGPGWSTVTGPPAALNSLNAAGPSGDAGVRAWQAGLPATQQLVLGFPYGGYAWRLSNAPSPSYAAATTGSAIPDGSIGYGQIKFIVDNAGATTYNTSVTVDGICYAGT5SWIGYDDNQSVT5KVRRAKQKGLRNYFSWH |
RVNDRVGYFQRYAKLFNVTGPYLDENCQPRFSSX

> BnaA08g09330D
MSSTKPTSSLVISTFFTCCLLLQHSSAQTVVGVWFYFESPEFVTDINSSHTHTLFCAFADLNSQNQVTSSTNQKFPFSTFTQT
VQRNNPSTKLMIGGIANKSFASNASMPTRSRKFDSSSLRASNGHHGLDDLWYEPPSATEMNFMGTLLREWRSAVAA
EASTSRPLLAAAVAYSSDYSVLYPVPVVQAVASSLQLWNMLAYDFGPWSTGTVPAPAALNSNAPGSDGAVRAWI
QLAQATQVLGFYPPYYAWRLSNANSPSYAATGSAISPDSGGYQRKFIIVDNGATTVYNTSTVGDYCYATTGW
DDQNSIVTVKRYAKQKGLRGYFSWHVGADDNSGLRSASRAWDATVVTTRFFX

> BnaA09g34290D
MGQSVIYKLKFNSKILPIQPTSFPLVRDLSQKKIDLEKNNKLKCYSNGSNVILFWFWRQLQELEGGLSACRRDIDSRSLDGEQQ
TYLAKGDILPTTQATELVRSEYLCQGFNGNTSDYCVGGQCGQPFCAPPPANGVSDEVITQEFNGIIDQAEIGSVDV
SREIEAFAHVFHTHTHCFIEEINGPSRDTATQPYCNGNYAVGPILQSWNNFYPGPAGTAIGFDGLNAPETVATD
PVGSKFTALWYNTNRPVQIVSPQFGATARIANGAECDGANSATVQRVYYTDYCRQLVDPGNNLTCX

> BnaC03g19370D
MTLTKSTLVLCLCLLLGFYSETVKSQNCGCSPDLCSSQFQFCGTGNDYCGPGCQSPCPTTQAESLSITVSQFSSFDGITNRA
GDCAGKFYTRDFAFIENAAANTTPSANSVTSLLEATMTAHTFQVEYGFCYFIEEINGASQNYCNDKFDPQYPCAAGKNYYGRG
IQLSWNYNYAPCGQLGLDLQSPHELGSDPVTAVARTALWFWVNNPRVLPQFGATARAINGKVECDGASPDKVSNRIRY
YRECEQLELGDSNLCX

> BnaA05g34400D
MATQISILKNTLILFLFTLTILTKTVFSQHCSTTGCAGNLCCSRWGYCGTTNAYCGTGCRGCPSCRSTTPPTPSGGGGL
MADPRDTIANVTLSTNFSMKSYNGCPCAKFYTRQAFIABAASQFAPYRGTVAKREIAALMAFAQHSESFGFCCYKEEIAARG
CQASTYVCQPGKNNYYGRGIPQITWNNYNYAAGKFGLPLLLTDPMVARSEPFAKCAMFWFNEKVPRVPDQFGGATVR
RINGECENGCRCRAAPAVSQVRNYLERFCRQFGISGTSLCX

> BnaA05g34300D
MNOTKTSNLDLFFTLILTILTSTQHDCFQTGACSMCCSRYCGGTTADYCGTCGRCSGCPYESCQVGLNAAAPRDIAN
VVTAPAFAIGMSKVCNCGPACFKFYTRQAFIABAASQFAPYRGTVAKREIAALMAFAQHSESFGFCCYKEEIAARG
CQASTYVCQPGKNNYYGRGIPQITWNNYNYAAGKFGLPLLLTDPMVARSEPFAKCAMFWFNEKVPRVPDQFGGATVR
RINGECENGCRCRAAPAVSQVRNYLERFCRQFGISGTSLCX

> BnaA05g34200D
MSNIKFLKVLSFSSFIISSCFCKPSHSSRGIAYIWGQNFEGSLASTCAGTRGYAYVIAFLVFKNGQQTPELNLGHCNAPAN
TCHHTFGAQVKVCTQRGIGVMSLGGAI康养ISREDAKMVADYLLWNFLGGKSSARPGLDADVLDGDFNIELGSPQHWD
DLVRLFNSHFRSKRIYVITSQAPLPITQFPPDDLMSGALKLTRFLFYVMFYNNPQPCTYTSGDTQSFJSWSKWTWIIIATQKIF
GLLPAAPEAAGGYIPAPDLVYPLKVVSRKKSYYGVMLWSKFWDDKNGYSSSVIVARVX

> BnaA03g20340D
MATHQVLFLFLLTIIITKTFSQHCTTTCGACNLLCSSRYGCGTTAAYCGTGCRGCPSCSGGSPTPSPTTTGGLNAEPRD
TIAANVTQTSFSGDMKSYNGCPCAKFYTRQAFIABAASQFAPYRGTVAKREIAALMAFAQHSESFGFCCYKEEIAARG
CQASTYVCQPGKNNYYGRGIPQITWNNYNYAAGKFGLPLLLTDPMVARSEPFAKCAMFWFNEKVPRVPDQFGGATT
RINGECENGCRCRAAPAVSQVRNYLERFCRQFGISGTSLCX

> BnaA03g20300D
MATHNVLLKNALMIFLLFLTTIMTETAFSQNCGKTGCNNMCCSRCRSWNGYCGTTNAYCGTGCSQGCSKPKPTTPSGSGL
NAGPRGSIASVTFFAPPMGKSMKVCNCGPACFKFYTRQAFIABAASQFAPYRGTVAKREIAALMAFAQHSESFGFCCYKEEIAARG
CQASTYVCQPGKNNYYGRGIPQITWNNYNYAAGKFGLPLLLTDPMVARSEPFAKCAMFWFNEKVPRVPDQFGGATT
RINGECENGCRCRAAPAVSQVRNYLERFCRQFGISGTSLCX

> BnaA03g20320D
MSNRNATVENALVFFLLAFAVMAKTVFSQNCQSTTGCPGKLCESCSWGCSCIKDQCQFGCWCSGLCHLKNKSYGF NGNYSV
AGPRGPISVSITFFAPPMGKSMKVCNCGPACFKFYTRQAFIABAASQFAPYRGTVAKREIAALMAFAQHSESFGFCCYKEEIAARG
CQASTYVCQPGKNNYYGRGIPQITWNNYNYAAGKFGLPLLLTDPMVARSEPFAKCAMFWFNEKVPRVPDQFGGATT
RINGECENGCRCRAAPAVSQVRNYLERFCRQFGISGTSLCX

> BnaA03g20310D
MSNRNATVENALVFFLLAFAVMAKTVFSQNCQSTTGCPGKLCESCSWGCSCIKDQCQFGCWCSGLCHLKNKSYGF NGNYSV
AGPRGPISVSITFFAPPMGKSMKVCNCGPACFKFYTRQAFIABAASQFAPYRGTVAKREIAALMAFAQHSESFGFCCYKEEIAARG
CQASTYVCQPGKNNYYGRGIPQITWNNYNYAAGKFGLPLLLTDPMVARSEPFAKCAMFWFNEKVPRVPDQFGGATT
RINGECENGCRCRAAPAVSQVRNYLERFCRQFGISGTSLCX

> BnaA03g20300D
MALTNLSTVLFLCLFLGLYSETVKSNCGCPLLCSSQFQFYCGTADYCGTGCPGCSPGPTSPSSFGGSGVGSIVTQAFF
GIQNAQCGCGAKFKYTYRDFAIENAAANTTPNFSNVTREIALMFHAFHTGFCYFIEEINGASRDCDENRQYPCAPGK
YFRGPRQPLQSWNYNGAFCQISLNLNLQPLVSSNPTAVFTGTLFWFMSNVPRVLPQFGFATIRAINGMECNGNNGSA
VNARIYRYDYGQGLVDPGNLSCX

> BnaA03g20300D
