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REVIEW ARTICLE

Identification and expression profiling analysis of NBS–LRR genes involved in *Fusarium oxysporum* f.sp. *conglutinans* resistance in cabbage

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Abstract

As one of the most important resistance (*R*) gene families in plants, the NBS–LRR genes, encoding proteins with nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains, play significant roles in resisting pathogens. The published genomic data for cabbage (*Brassica oleracea* L.) provide valuable data to identify and characterize the genomic organization of cabbage NBS–LRR genes. Ultimately, we identified 105 TIR (N-terminal Toll/interleukin-1 receptor)-NBS–LRR (TNL) genes and 33 CC (coiled-coil)-NBS–LRR (CNL) genes. Further research indicated that 50.7% of the 138 NBS–LRR genes exist in 27 clusters and there are large differences among the gene structures and protein characteristics. Conserved motif and phylogenetic analysis showed that the structures of TNLs and CNLs were similar, with some differences. These NBS–LRRs are evolved under negative selection and mostly arose from whole-genome duplication events during evolution. Tissue-expression profiling of NBS–LRR genes revealed that 37.1% of the TNL genes are highly or specifically expressed in roots, especially the genes on chromosome 7 (76.5%). Digital gene expression and reverse transcription PCR analyses revealed the expression patterns of the NBS–LRR genes upon challenge by *Fusarium oxysporum* f.sp. *conglutinans*: nine genes were upregulated, and five were downregulated. The major resistance gene *Foc1* probably works together with the other four genes in the same cluster to resist *F. oxysporum* infection.

Keywords *Brassica oleracea* · Resistance gene · Bioinformatics · Gene expression · Gene evolution

Abbreviations

DGE Digital gene expression
R Resistance
NBS Nucleotide-binding site
LRR Leucine-rich repeat
TIR N-terminal Toll/interleukin-1 receptor
CC Coiled-coil

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Introduction

Plants are constantly confronted by pathogens that alter their growth, metabolism, and reproduction. To resist invasion, plants have evolved numerous defense mechanisms (Pennisi 2009; Głowacki et al. 2011; Fujita et al. 2006). Resistant plants have multiple disease-resistance (*R*) genes, which confer resistance to different pathogens and insects (Van and Kamoun 2008). *R* proteins can sense the invasion of pathogens by detecting effector molecules generated during infection (Martin et al. 2003; Dangl and Jones 2001). The largest known class of *R* genes includes those containing a nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Deyoung and Innes 2006; Yue et al. 2012). To date, over 150 *R* genes have been cloned, about 80% of which encode NBS and LRR domains (Shao et al. 2014; Guo et al. 2016). Based on the structure of their N-termini, these

NBS–LRR proteins can be further divided into N-terminal Toll/interleukin-1 receptor (TIR)-NBS–LRR (TNL) and non-TNL types. The TNL type possess a domain homologous to the interleukin-1 receptor (TIR) and Toll, while most of the non-TNL proteins having a coiled-coil (CC) are commonly referred to as CC–NBC–LRR (CNL) proteins (Meyers et al. 2010; Blake et al. 2003). Despite both the TIR and CC domains being related to signaling and resistance specificity, their pathways are divergent (Deyoung and Innes 2006; Meyers et al. 2010). The TIR domain plays an important role in pathogen detection (Luck et al. 2000), while the CC domain is associated with protein–protein interactions (Van et al. 2007). The NBS domain consists of a P-loop, Gly–Leu–Pro–Leu (GLPL), kinase-2a, and kinase-3a motifs, and is essential for ATP/GTP binding activity (Saraste 1990; Miller et al. 2008). The LRR domain can interact with pathogens directly or indirectly (Jia et al. 2014). Earlier studies revealed that TNL genes are abundant in dicots, but absent in monocots. Recent whole-genome sequencing data have made it possible to comprehensively analyze NBS–LRR genes in economically important plants.

Cabbage (*Brassica oleracea* L.) is one of the major members of the *Brassicaceae* family. However, it is susceptible to infection by numerous fungal and bacterial pathogens, such as *Fusarium* wilt (FW), *Turnip mosaic virus* (TuMV), *Alternaria brassicicola* (Schweinitz) Wilts, and *Peronospora parasitica* (Pers.) Fr. The FW caused by *Fusarium oxysporum* f.sp. *conglutinans* (FOC) is particularly severe. Recently, the TNL-type resistance gene *Foc1* (Bo7g104800) was cloned (Lv et al. 2013); however, the defense mechanism involving *Foc1* remains unclear (Shimizu et al. 2014). In addition, many cloned *R* genes (e.g., *CRa*, *CRb*, and *Crr1a*) in *Brassica* crops are also TNL genes. However, there have been few studies on the cabbage NBS–LRR family (Shazia et al. 2018; Kim et al. 2015), and none of them analyzed the relationship between FOC infection and the NBS–LRR family. Consequently, comprehensive analyses of the relationships between NBS–LRR (especially the TNL genes) and FOC infection are indispensable.

To identify the NBS–LRR genes, their genome-wide encoded protein sequences were analyzed using the Hidden Markov Model (HMM). The distribution, gene cluster, and gene structure of identified genes were compared. Furthermore, their nuclear localization signal (NLS) and cis-elements were predicted. Expression profiles of these genes in six tissues were analyzed. Finally, the TNL family was chosen to elucidate the genes' response to FOC infection in different stages because the TNL family is much bigger than the CNL family and *Foc1* is a TNL-type gene. Fourteen TNL genes were identified as responding significantly to FOC infection in different stages, according to RNA sequencing (RNA-seq) and reverse transcription PCR (RT-PCR) analyses. These results will provide a basis for further identifying, screening, and mapping of cabbage NBS–LRR-encoding genes.

Materials and methods

Identification of NBS genes

To identify the NBS–LRR genes, we downloaded the whole-genome protein sequence of cabbage from the Ensembl Plant database (http://plants.ensembl.org/index.html). Then the sequences were scanned using HMMER v3.1b2 (http://hmmer.org/) with the raw Hidden Markov Model (HMM) corresponding to the Pfam NBS (NB-ARC) family (PF00931) (Finn et al. 2016). Proteins with *E* value $< 1e^{-10}$ were selected. High scoring hits were used to construct a cabbage NBS HMM-profile, using HMM-Build to check for any missing hits. The TIR, NBS, and LRR domains of the identified NBS–LRR proteins were confirmed using Pfam (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) (Zhang et al. 2016a, b; Letunic et al. 2012). CC domains could not be analyzed through Pfam and SMART; therefore, we used Paircoil2 (http://cb.csail.mit.edu/cb/paircoil2/paircoil2.html) with a P-score cutoff of 0.025.

Genomic distribution on chromosomes

According to the gene position, Mapinspect software was used to map the physical location of the NBS–LRR genes. A gene cluster was defined as being present when the distance between two neighboring NBS–LRR genes was < 200 kb and contained ≤ 8 non-NBS genes between the two NBS–LRR genes (Richly et al. 2002; Blake et al. 2003).

Gene characteristics and structure

Information concerning the NBS–LRR genes, including open reading frames (ORFs) and exon numbers were retrieved from Ensembl Plants. The pI (isoelectric point) and MW (molecular weight) of the identified genes were calculated using Pepstats (http://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats). Furthermore, we performed NLS (nuclear localization signal) (http://cello.life.nctu.edu.tw/) analysis, and the promoter sequence (2000 bp upstream of the start codon) of each gene was submitted to the PlantCARE (http://bioinformatics.psb.ugen.be/webtools/plantcare/html/) for cis-element prediction (Lescot et al. 2002). Finally, the gene structure was analyzed using GSDS2.0 (http://gsds.cbi.pku.edu.cn/index.php) by comparing the cDNA and corresponding genomic sequence of each gene (Guo et al. 2007).

Phylogenetic and conserved motif analyses

To determine underlying relationships among family members of various NBS–LRR genes, MEGA 6.0 (http://www.megasoftware.net) was used to construct a phylogenetic tree using the maximum likelihood (ML) method with 1000 bootstrap replicates; excessively short sequences were excluded (Tamura et al. 2011). The conserved motifs were identified using MEME (http://meme-suite.org/tools/meme) and TBtools (http://www.cjchen.name) software (Bailey et al. 2009; Chen et al. 2018).

Gene duplication and K_a/K_s ratio analysis

Tandem duplication and segmental duplication genes were analyzed using CoGe (https://genomevolution.org/coge/) (Lyons and Freeling 2008). The Circos software (http://circos.ca/) was used to draw a circle map of the segmental duplicated genes (Krzywinski et al. 2009). Furthermore, DnaSP (6.12.01) was used to calculate the synonymous (K_s) and non-synonymous (K_a) substitution rates, and duplication events (Yang et al. 2008). A K_a/K_s calculator was used to estimate the K_a/K_s rates of evolution, K_a/K_s values of > 1 and < 1 were deemed to represent positive and purifying selection, respectively. To estimate the evolutionary time, the K_s values were converted to duplication time in millions of years, based on the ratio of one substitution per synonymous site per year. The calculation formula for the duplication events time was $T = K_s/2\lambda \times 10^{-6}$ Mya ($\lambda = 6.5 \times 10^{-9}$) (Librado and Rozas 2009; Gaut et al. 1996).

Tissue-expression analysis

The expression data of the 138 NBS–LRR genes in 6 tissues (flower, flower-bud, fruit, leaf, root, and stem) were obtained from the Expression Atlas (https://www.ebi.ac.uk/gxa/home) submitted by Liu et al. (2014). Then, the gene expression data were compiled to construct a heatmap using TBtools to display the expression levels of these NBS–LRR genes.

Plant materials and FOC treatments

The cabbage line ‘R4P1’, which is resistant to FOC, was sown in an artificial growth chamber under a photoperiod of 16-h light/8-h dark at 25 °C/18 °C day/night temperatures. Seedlings at the three-leaf stage were infected with FOC strain GLHW1 (race 1) using the root dip inoculation method (Tian et al. 2009). The suspension inoculation concentration was 1×10^6 spores/ml. Simultaneously, control plants were mock inoculated with distilled water. After inoculation, the seedlings were placed back into the original pots. Both inoculated and mock-inoculated plant roots were collected separately under a various time points (0, 4, 12, 24 and 48 h) using three replications. Briefly, root samples were frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

RNA isolation, RNA sequencing, and reverse transcription PCR

Total RNA was isolated using a kit (Cat: DP432, TIANGEN, China) following the manufacturer’s instructions. The quality and quantity of the RNA was confirmed using agarose gel electrophoresis and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA), and then sequenced using the Illumina Hiseq 2000 platform (Illumina, San Diego, CA, USA) (Wang et al. 2010). RT-PCR was conducted to measure the expression levels of TNL genes using SYBR Green 1 (Cat: FP205-01, TIANGEN, China) and a Roche LightCycler 480 system (Roche Branchburg, NJ, USA). Contaminating gDNA removal and RNA reverse transcription processes were performed using kits (Cat: AU311-02, Transgen, China). The 20 μL volume contained 10 μL of 2 × SuperReal PreMix Plus, 2 μL of cDNA, 1 μL of each primer, and 6 μL of ddH_2O, the constitutively expressed *GAPDH* gene was used as an internal reference. The amplification procedure comprised: 95 °C for 5 min; followed by 40 cycles of 95 °C for 10 s, then 60 °C for 20 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 7 min. All reactions were performed with three technical and biological replicates. Relative gene expression was calculated using the comparative CT method (Livak and Schmittgen 2001).

Results

Identification and distribution of NBS–LRR genes

In total, 236 NBS and 172 NBS–LRR genes were identified using HMMER search and verified using Pfam and SMART databases. These NBS–LRR genes could be roughly divided into three classes: TNL, CNL, and others, according to their structure. To facilitate comparative analyses, only the 105 TNL and 33 CNL genes were used in the following study. Among the 138 NBS–LRR genes, 133 were mapped onto the 9 chromosomes of cabbage while other 5 were located on different scaffolds. The ratio of cabbage TNL:CNL genes was almost 3:1 (105:33), which was higher than that in *B. rapa* (2:1) (90:41) and in *A. thaliana* (2:1) (83:51) (Yu et al. 2014). According to the criteria defining a gene cluster, we identified 70 (50.7%) genes belonging to 27 clusters (Table S1). Most of them (20) are TNL gene clusters, and it was less common to find TNL and CNL genes in the same cluster. The members in each cluster ranged from 2 to 5, and the cluster sizes ranged from 10,421 to 125,034 bp.

Thirty-three cloned functional NBS–LRR genes from other species were mapped on the cabbage chromosomes according to their orthologous genes, which could provide a reference for follow-up gene mapping and the identification of gene function in cabbage (Fig. 1). Among them, three cloned resistance genes (*Foc1*, *CRa*, and *CRb*) in cabbage and Chinese cabbage are all located on chromosome 7. To date, we also mapped a probable *Xanthomonas campestris* pv. *campestris* resistance gene on chromosome 7.

Gene characteristics and structure

The genomic and coding sequence (CDS) sequences, protein length, MW, and pI of the 138 NBS–LRR genes were comprehensively analyzed (Table S2). The sequence lengths of genomic and CDS ranged from 2036 bp (Bo2g130100) to 28,414 bp (Bo2g127000) and 1644 bp (Bo9g094020) to 8988 bp (Bo3g153660), respectively. The protein lengths ranged from 203 AA (Bo9g094020.1) to 3429 AA (Bo3g153660.1). In addition, there were also significant variations in the MW and pI, which ranged from 30.23 kDa (Bo5g148350.1) to 378.09 kDa (Bo3g153660.1) and from 4.48 to 8.85, respectively. The average MW of TNL (136.39 kDa) and CNL (94.55 kDa) genes were markedly different. Over 88.7% of the NBS–LRR proteins were predicted to be located in the cytoplasm (Table S2), among which the percentage of CNL proteins (93.9%) was higher than that of TNLs (82.9%). The average exon number of the NBS–LRR genes was 5.96, with TNL genes having more exons (6.62) than CNL genes (3.88) (Table S2, Fig. 2). These results suggested that the number of introns may have increased and decreased during the structural evolution of the two types of NBS–LRR resistance genes in cabbage (Sharma et al. 2017).

Cis-element analysis

Cis-elements are usually involved in gene regulation; therefore, we performed cis-elements prediction for the 138 genes, over 66 CAREs (cis-acting regulatory elements) involved in gene regulation were identified; the conventional elements (TATA-box, CAAT-box) identified in *Arabidopsis* and common bean were also detected (Meyers et al. 2003; Wu et al. 2017). The remaining CAREs were mainly associated with light responsiveness, hormones, tissue-specific expression, and abiotic stress response (Table S3). The light-responsive elements, such as ACE, Box4, and AE-box were found in many genes. Furthermore, hormone-related CAREs such as abscisic acid (ABRE), auxin (TGA-box and TGA-element), and salicylic acid response (SARE and TCA-element) were also detected. In addition, we found CAT-boxes, RY-elements, AACA_motifs, and As1, which are associated with tissue-specific expression. Among them, As1, which is involved in root-specific expression, was detected in 99 NBS–LRR genes.

Fig. 1 Physical map of the 133 NBS–LRR genes on chromosomes C1–C9. The sizes of the nine chromosomes are represented by the scale in megabases (Mb) on the left. The TNL, CNL, and certain other types of NBS–LRR genes are distinguished in red, green, and blue, respectively. Gene clusters are marked with red frames. The 33 cloned NBS–LRR genes from other species are shown on the right corresponding to orthologous genes of cabbage

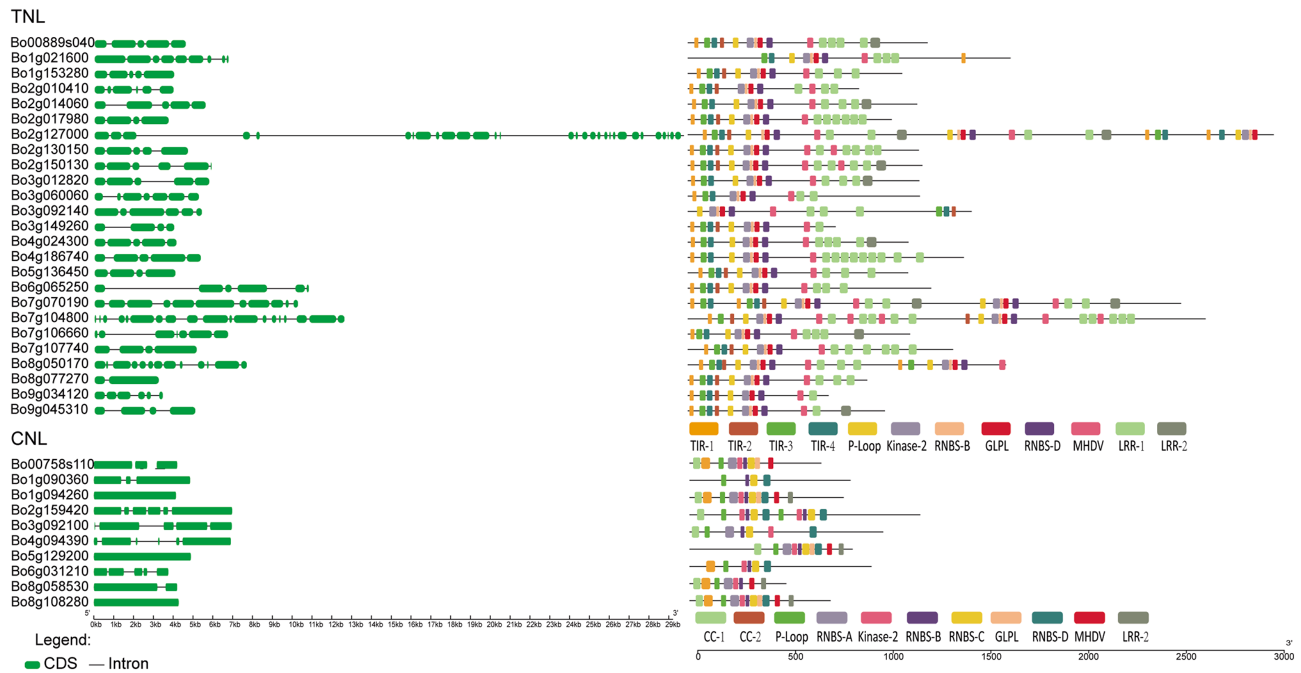

Fig. 2 The exon–intron structures and the corresponding conserved motifs of certain TNL and CNL genes. The green bars indicate the exons, and the black lines indicate the introns. Motifs are represented by different colored boxes

Conserved motifs and phylogenetic analysis

To derive the evolutionary relationships of the NBS–LRR genes, a phylogenetic tree was built using the maximum likelihood (ML) method, as shown in Fig. 3, NBS–LRR genes were clearly divided into two (TNL and CNL) major groups and six subgroups: TNL (1–4), CNL (1–2). TNL-1 was the largest subgroup (31 genes) and accounted for 29.5% of TNL genes, mostly on chromosome 2. In contrast, CNL-2 only harbors 11 members. Using MEME analysis, 12 and 11 different types of common motifs were identified as existing widely in most TNL and CNL proteins. In addition, GLPLA and kinase-3 motifs were detected in some NBS–LRR genes' NBS-subdomains, LDL was detected in some NBS–LRR genes' LRR-subdomains, and EDVID was detected in some CNL genes. The eight major motifs (P-loop, Kinase-2, RNBS (A-D), MHDV, and GLPL) found in the *Arabidopsis* NBS–LRR genes were also detected. P-loop, RNBS, and Kinase-2 were present in all the 138 NBS–LRR genes, and P-loop, kinase-2, and GLPL were highly similar between the CNL and TNL genes (Fig. 4). The NBS motifs (A-D) were identified in all the CNL genes, while there were no NBS-A and NBS-C in the TNL genes. Furthermore, the order of the TIR (1–4) motifs detected for the cabbage TNL proteins was consistent with that in *Arabidopsis* and common bean (Meyers et al. 2003; Wu et al. 2017). Among the TNL genes, TIR-1 and TIR-4 were missing from the proteins encoded by Bo1g021600, Bo1g021620, and Bo2g127000. TIR-1 was missing from eight proteins (Bo3g092140, Bo3g153660, Bo7g106700, Bo8g081470, Bo9g059350, Bo9g061240, Bo9g061310, and Bo00889s060), while the rest of the TNL proteins contained the four types of TIR motifs (Fig S3). Moreover, we identified two types of CC (CC-1 and CC-2) motifs from 33 CNL proteins, and more than half of these proteins contained both types (Fig S4). These TIR (1–4) and CC (1–2) motifs were also observed in *Populus trichocarpa* and *A. thaliana* (Kohler et al. 2008; Meyers et al. 2003). In addition, two (LRR-1 and LRR-2) and one (LRR-2) motifs were detected in the LRR region of the TNL and CNL genes, respectively.

Gene duplication analysis

In cabbage, 33 tandem duplicated genes are distributed in 15 tandem arrays (Table S4). Thirty-one genes are distributed on seven chromosomes unevenly, and the remaining two were unanchored on scaffolds. Interestingly, none of the CNL genes were duplicated. Single tandem duplicated genes containing two genes were identified on chromosomes 1, 5, 6 and 8, and no duplicated genes were found on chromosomes 3 and 4 (Table S4, Fig. 5). The highest number of tandem arrays (5), with 12 genes, is located on chromosome 7. The K_a/K_s values of most the 39 and 21 pairs of tandem and segmental duplicated genes were < 1, indicating that these genes have evolved under negative selection (Table S4, Fig. 6). The duplication events of the two types

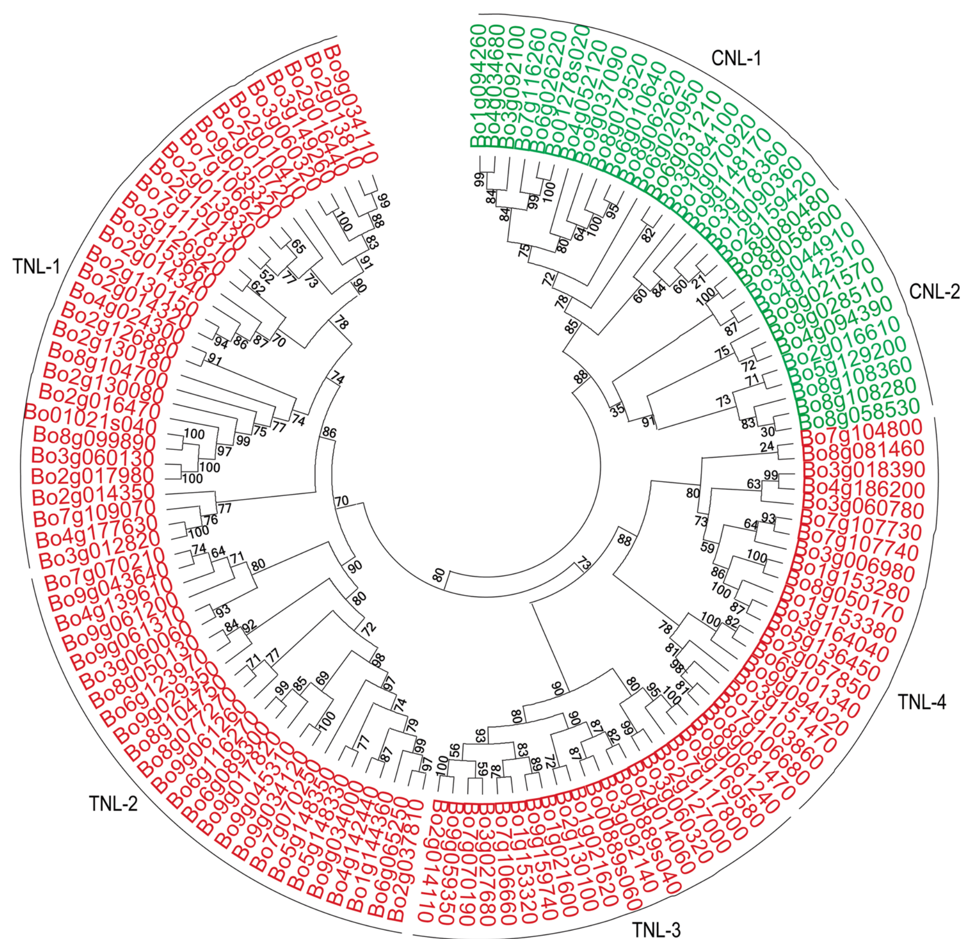

Fig. 3 Phylogenetic analysis of NBS–LRR proteins using the maximum likelihood method

of duplicated genes occurred between 0.62 ($K_s = 0.008$) and 221.43 Mya ($K_s = 2.88$), with an average of 104.23 Mya, the tandem duplication events occurred from 2.52 Mya ($K_s = 0.033$) to 194.93 Mya ($K_s = 2.53$), with average 97.52 Mya. These results indicated that the expansion of the cabbage NBS–LRR genes mostly arose from whole-genome duplication events during their evolution (Haron et al. 2016; Gaut et al. 1996).

Tissue-specific expression of NBS–LRR genes

Based on the published transcriptome data, we analyzed the expression levels of 106 NBS–LRR genes (expression data for 25 TNL and 7 CNL genes were not found) in six tissues (Table S5). Among the 80 TNL genes, 39 showed relatively high or specific expression in roots, while only 7 CNL genes were highly or specifically expressed in the roots (Fig. 7). We concluded that over one-third of TNL genes are highly expressed in roots, suggesting functional homology. Combined with the chromosome analysis, we found that, except for four TNL genes (*Bo7g059500*, *Bo7g070250*, *Bo7g106660*, and *Bo7g107710*), almost all of the 18 NBS–LRR genes on chromosome 7 are highly or specifically expressed in roots. Considering *Foc1*, *Bra012688*, *CRa,* and *CRb* are the FOC and club-root *R* genes, suggesting that these genes probably play specific roles in root disease resistance. Meanwhile, there were more TNL genes than CNL genes; therefore, DGE and RT-PCR were conducted to verify the TNL gene expression patterns after FOC infection.

Responses to FOC pathogen inoculations

The expression data of 88 out of 105 TNL genes were available, and the expression level of 14 genes significantly decreased or increased after FOC inoculation (Table S6, Fig. 8). These genes could be placed into two categories: upregulated (Fig. 9a, b) and downregulated (Fig. 9c) genes. Overall, three genes (*Bo1g153280*, *Bo6g123970*, and *Bo8g099890*) exhibited higher expression at 24 h after inoculation, whereas four genes (*Bo1g153320*, *Bo2g014060*, *Bo2g014320*, and *Bo9g043640*) were highly expressed at 48 h. In addition, five genes (*Bo6g089300*, *Bo7g106620*, *Bo7g106630*, *Bo7g106660*, and *Bo7g106680*) were initially upregulated after infection, but were then downregulated after 4 h. The expression level of *Foc1* after FOC infection was irregular.

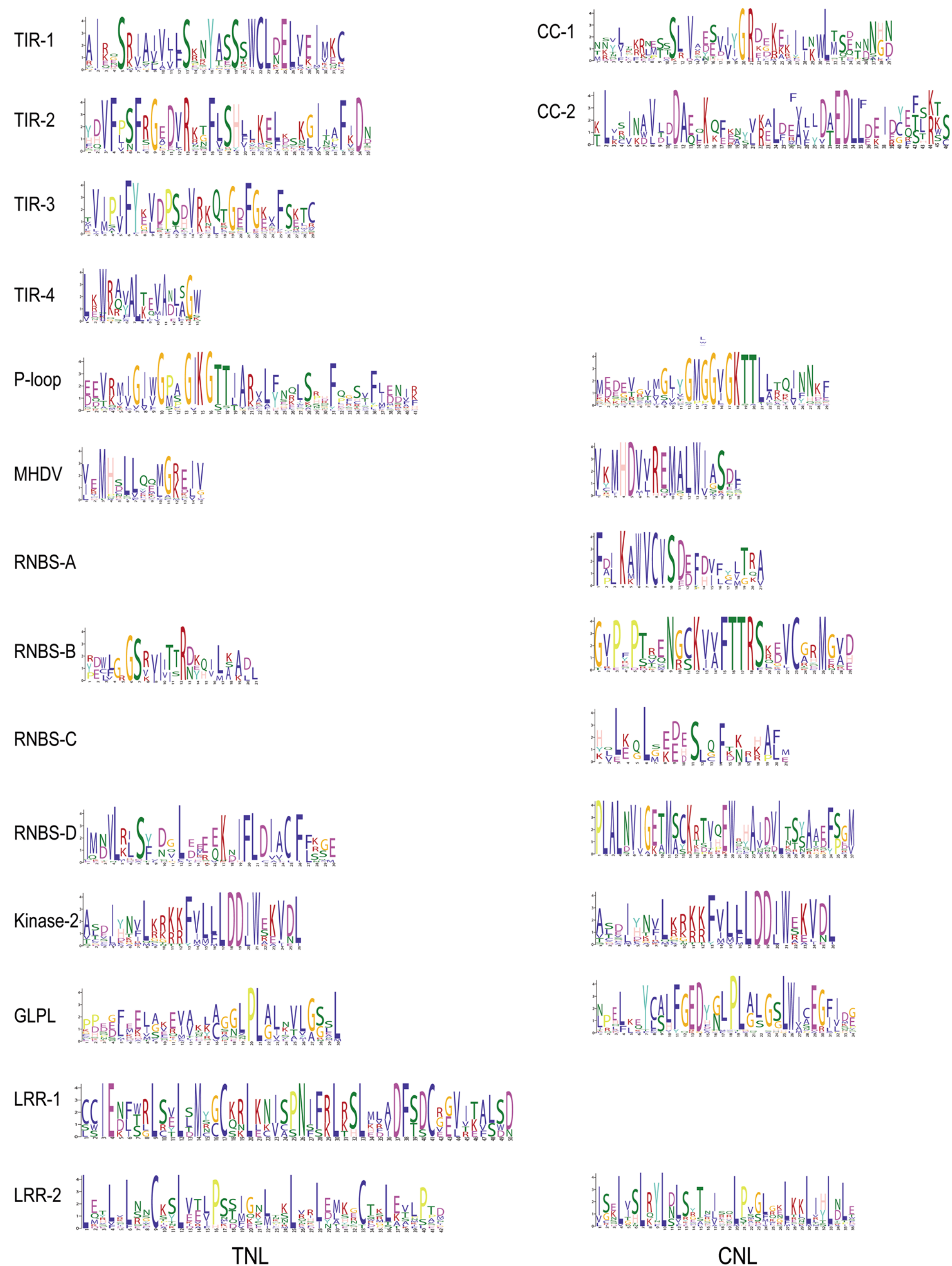

Fig. 4 MEME analysis of the TNL and CNL proteins. Different colored letters represent amino acids belonging to the different families

Overall, the DGE data for the 14 TNL genes were basically consistent with RT-PCR results. Besides, through comparative expression profile analysis of these genes, we hypothesized that different genes might have different functions after FOC infection. Taken together, the RNA-seq and RT-PCR expression analyses support the hypothesis that TNL genes are involved in FOC infection at different times.

Discussion

In our research, 172 cabbage NBS–LRR genes were identified, revealing a much higher number compared with the 70 described by Yu et al. (2014). Similar numbers of NBS–LRR genes were also observed in *A. thaliana* (207)

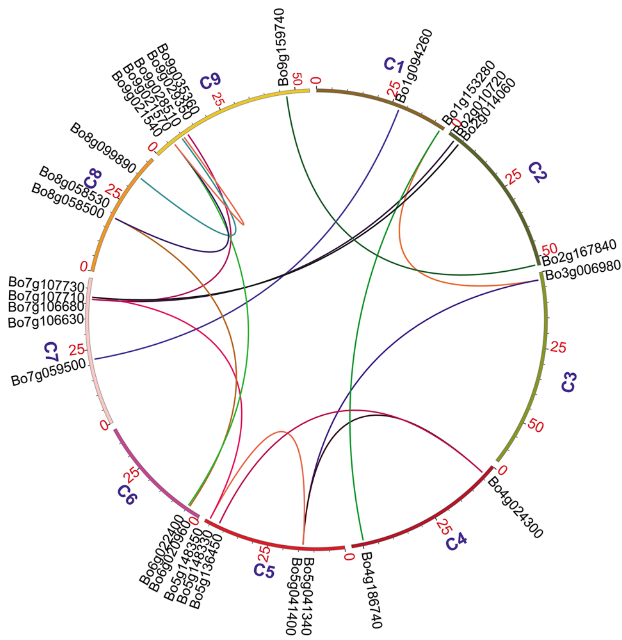

Fig. 5 Circos diagram of segment duplicated NBS–LRR genes. C1–C9 represent nine chromosomes; the black lines on the chromosomes stand for the location. Colored lines stand for the relationship of the segmental duplicated genes

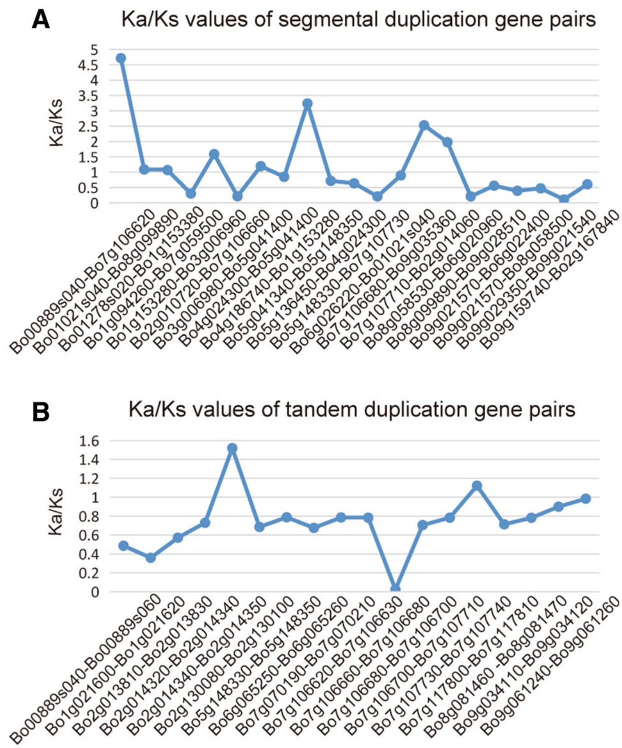

Fig. 6 The K_a/K_s values of two types of duplicated genes. **a**, **b** Represent K_a/K_s values of segmentally and tandemly duplicated gene pairs, respectively

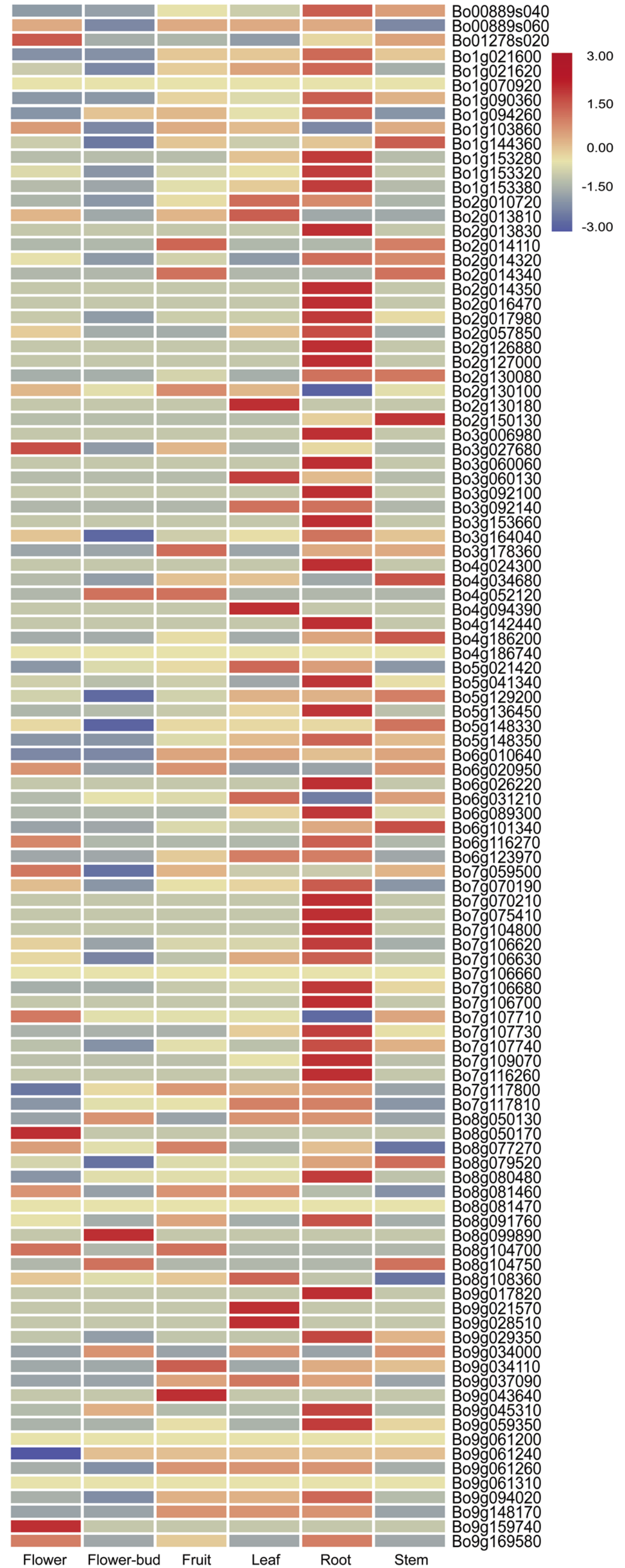

Fig. 7 Tissue-specific digital expression profiles of 106 genes. Higher expression of each gene is presented in red; otherwise, blue was used. The genes with a TPM equal to 0 were not used in this array

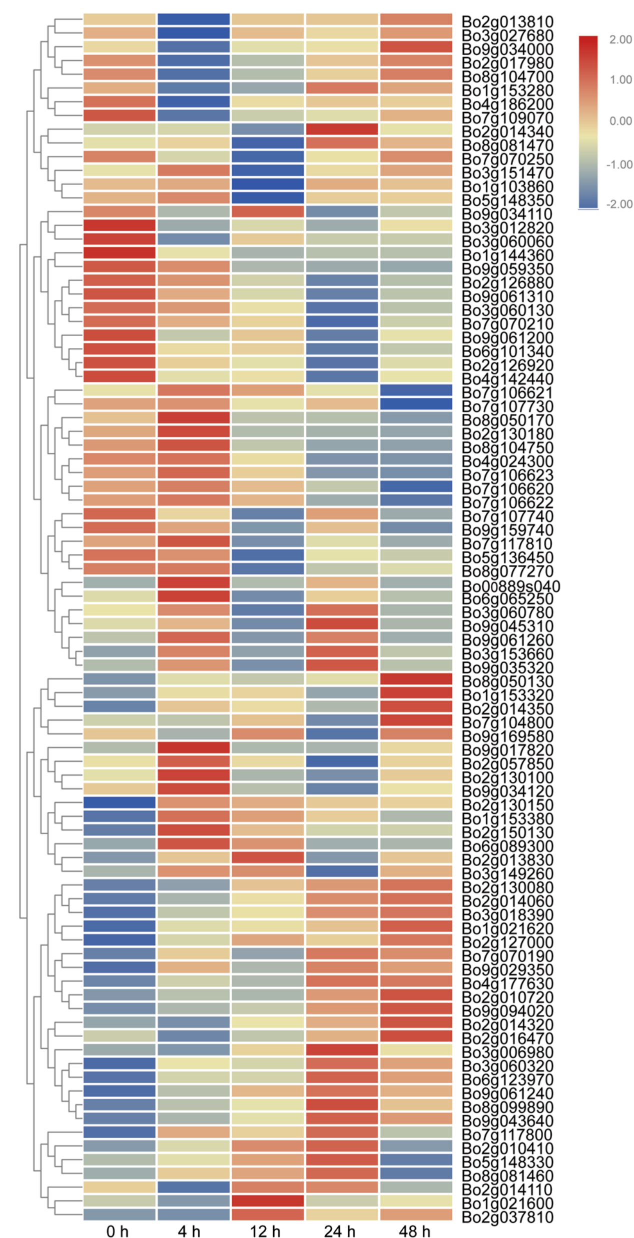

Fig. 8 Expression profiles of 88 TNL-encoding genes under FOC challenge. Cabbage roots were selected in different times (0, 4, 12, 24, and 48 h). The expression color scale is shown at the top right. Higher expression for each gene is presented in red; otherwise, blue was used. The genes with an RPKM equal to 0 were not used in this array

(Yu et al. 2014) and *Cucumis sativus* L. (233) (Zhang et al. 2016a, b). Compared with *B. rapa* and *A. thaliana*, cabbage has more NBS genes, possibly because the cabbage genome (630 Mb) (Liu et al. 2014) is larger than that of *B. rapa* (485 Mb) (Wang et al. 2014) and *A. thaliana* (123 Mb) (Dennis and Surridge 2000). The NBS proteins of cabbage account for 0.39% of the total proteome, which is similar to chickpea (0.36%) (Sharma et al. 2017).

NBS genes evolve in concert with pathogens, and rapid evolution of NBS genes among different cultivars of a species exposed to different biotic stresses is frequently observed. Many studies indicate that NBS proteins carrying a TIR domain originated earlier than the non-TIR type (Yue et al. 2012) and the TNL genes are less diversified in most monocots (Bai et al. 2003). The ratio of cabbage TNL to CNL genes is 3:1, which is higher than that in *B. rapa* (2:1), *A. thaliana* (2:1) (Yu et al. 2014), and in other monocot crops, indicating that cabbage NBS–LRR genes might have evolved more slowly (Tarr and Alexander 2009; Zhang et al. 2016a, b). The greater contribution of TNL genes than CNL genes to resistance might be one of the main explanations for the large difference in the number of TNL and CNL genes in cabbage.

Similar to other species, the distribution of cabbage NBS–LRR genes is uneven and they mainly exist in clusters, as a result of rapid gene evolution (Leister 2004; Friedman and Baker 2007; Wan et al. 2013; Lv et al. 2015; Lozano et al. 2015; Die et al. 2018). However, only 51% of the cabbage NBS–LRR genes are present in 27 clusters (Yang et al. 2008), whereas over 70% of the NBS–LRR genes in rice are present in 104 clusters. This small number of gene clusters may be another explanation for the slower evolution of cabbage compared with other monocots.

Gene duplication is considered to be the source of plant diversity and complexity, allowing them to adapt to changing circumstances. Segmental duplication and tandem duplication are considered to represent the two principal evolutionary patterns that cause gene family expansion in plants. In many plants, segmental duplication appears to be the dominant process that generates gene families, including the NBS–LRR and other *R* gene families. Moreover, the proportion of gene clusters in cabbage is small, and the probability of generating new NBS–LRR genes through tandem duplications is low. Therefore, we concluded that most NBS–LRR genes in cabbage arose from whole-genome duplication events during evolution (Haron et al. 2016). Overall, the 33 tandemly duplicated genes are distributed in 15 arrays located in the identified 27 clusters. Similar to *B. rapa*, the K_a/K_s values of most tandemly and segmentally duplicated genes are < 1, indicating that these NBS–LRR genes have evolved under negative selection (Yu et al. 2014).

Although most tandem duplicated genes in the same clusters showed high similarity, some of them were located in different phylogenetic clades (Lozano et al. 2015). These NBS–LRR genes were clearly divided into two major groups and six main subgroups (four TNL and two CNL genes) with different numbers of members. The motif analysis indicated that four and two types of TIR and LRR motifs could be detected among the TNL genes, while only two and one types of CC and LRR motifs were detected among the CNL genes. Furthermore, the order of the four types of

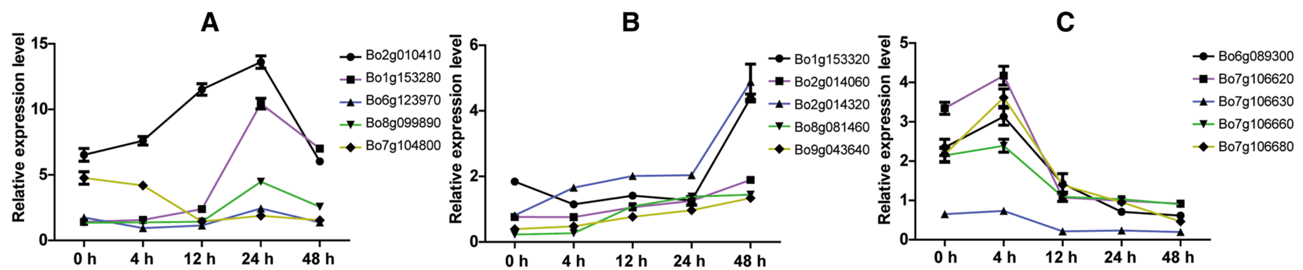

Fig. 9 The relative expression levels of 18 genes. The vertical axis represents the relative expression level and 0, 4, 12, 24, and 48 h (*x*-axis) indicate the treatment times. Different lines represent different treatment times. Error bars represent the standard error of the mean based on three replicates

TIR motifs in cabbage TNL genes was consistent with that in the common bean (Wu et al. 2017). The P-loop, RNBS, and Kinase-2 motifs were detected in all 138 NBS–LRR proteins. In addition, the P-loop, kinase-2, and GLPL motifs showed high similarity between the CNL and TNL proteins. Combined with the inference that NBS–LRRs with TIR domains may have originated earlier than non-TIR proteins, we hypothesized that some TIR motifs in TNL genes had been replaced by CC or other motifs via gene recombination and duplication.

Similar to the specific expression of NBS–LRR genes in *Arabidopsis*, the obtained public expression data of cabbage NBS–LRR genes also showed that numerous TNL genes were highly or specifically expressed in the roots, including *Foc1* and the homologous genes of *CRa* and *CRb,* which correlates with their designation as root disease *R* genes (Tan et al. 2007). Analysis of the cis-elements showed that As1, which is involved in root-specific expression, was detected in over 70% of NBS–LRR genes, including *Foc1* and the homologous genes *CRa* and *CRb*. Therefore, we inferred that the presence of As1 may be one reason explanation for the higher expression of these genes in the root than in other tissues.

To further study the resistance mechanism involving *Foc1* and other TNL genes, RNA was extracted at different times after infection to conduct DGE and qRT-PCR analyses. The results suggested that nine upregulated and five downregulated genes probably participate in FOC resistance (Conesa et al. 2016; Meyers et al. 2010) the expression of the *Foc1* was irregular during infection (Xing et al. 2016). In addition, four genes (*Bo7g106620*, *Bo7g106630*, *Bo7g106660*, and *Bo7g106680*) were all downregulated at 4 h after infection. Previous studies have shown that a series of different NBS–LRR genes work together to resist the invasion of the same pathogenic bacteria (Van and Kamoun 2008). The orthologous genes of *Foc1* and the four genes in *A. thaliana* were analyzed because genes in same cluster are often similar in structure and function (Landolfo et al. 2018; Kozák et al. 2018). We found that *Foc1*, *Bo7g106630*, and *Bo7g106680* are orthologous or have relatively high similarity with *AT4G19500*, while the orthologous gene of *Bo7g106620* and *Bo7g106660* is *AT4G19510*, and both *AT4G19500* and *AT4G19510* encode TNL type proteins (Peele et al. 2014; Iyer and Aravind 2012). In addition, the protein interaction relationship analysis in the STRING database (https://string-db.org/cgi/input.pl) showed that the products of *AT4G19500* and *AT4G19510* have known interactions and are co-expressed. The NLS prediction indicated that the proteins encoded by *Foc1*, *Bo7g106620*, and *Bo7g106680* are located in the cytoplasm, while the proteins of *Bo7g106630* and *Bo7g106660* are in the outer-membrane. Based on these results, we hypothesized that the four genes participate in FOC resistance with *Foc1*. Further experiments will be performed to verify this inference.

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Author contributions ZCL, JHY, JGK, JMX, and HPW designed the research. ZCL and HPW completed the experiments. JMX and HLL performed the data analysis and prepared the manuscript. ZCL, XHZ, JGK, and JX revised the manuscript. All authors had read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

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