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Genome-wide analysis and identification of TIR-NBS-LRR genes in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) reveal expression patterns to TuMV infection

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ABSTRACT

TIR-NBS-LRR (TNL) genes greatly affect plant growth and development. Ninety TNL-type genes were identified and characterized in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*). Tissue-expression profiling revealed different expression levels in different tissues. qRT-PCR analysis revealed the expression patterns of 69 genes challenged by *Turnip mosaic virus* (TuMV): 42 genes were up-regulated, and 11 genes down-regulated; genes were grouped according to their different expression patterns. Sixteen candidate genes were identified as responding to TuMV infection. This study supplies information on resistance genes involved in Chinese cabbage's response against TuMV, and furthers the understanding of resistance mechanisms in *B. rapa* crops.

Introduction

Plants are subject to attack from pathogens and herbivores during their growth and development. As sessile organisms, they have developed a series of sophisticated defense mechanisms, regulated by gene-for-gene disease resistance (*R*) genes, to resist infringement from diverse biotic challenges [1]. In many cases, plant–pathogen interactions are triggered by pathogen avirulence (*avr*) genes and corresponding plant disease *R* genes. If relevant *R* and *avr* genes appear in hosts and pathogens simultaneously then plants display disease resistance, but if not, disease occurs [2].

Currently, over 100 reference resistance genes and 104,310 putative genes make up the super *R* gene family (Plant Resistance Genes database; PRGdb; http://prgdb.org) [3]. Based on putative protein domains, *R* genes are divided into five classes: CNL (CC-NBS-LRR), TNL (TIR-NBS-LRR), RLK (Receptor like kinases), RLP (Receptor like proteins), and Pot (Ser/Thr kinase protein) [4,5]. The 'nucleotide-binding site plus leucine-rich repeat' (NBS-LRR) is an *R* gene superfamily, which is involved in encoding putative intercellular responders [2,6]. This superfamily is abundant in plants, containing 149 genes in *Arabidopsis* [7], 535 related sequences in rice (*Oryza sativa*) [8], and approximate 400 genes in *Populus trichocarpa* [9]. The domains of NBS-LRR genes are characterized by two kinds of N-terminal ends: either a coiled-coil (CC) or a Toll/Interleukin-1 receptor (TIR) domain, referred to as TIR-NBS-LRRs (TNLs) or CC-NBS-LRRs (CNLs), respectively [5,10]. The NBS domain is essential for ATP/GTP binding activity, and consists of kinase-2α, kinase-3α, P-loop, and GLPL motifs [11,12]. The LRR domain can mediate interactions directly or indirectly with pathogen molecules [13]. The CC domain may be related to protein–protein interaction and signaling [5]. The function of the TIR domain is mainly related to resistance specificity and signaling;

Abbreviations: avr gene, avirulence gene; CC, coiled-coil; CNL, CC-NBS-LRR; CP, coat protein; dpi, days post-inoculation; LF, least fractionated blocks; MF1, medium fractionated blocks; MF2, most fractionated blocks; ML, maximum-likelihood; MW, molecular weight; MYA, million years ago; pI, proteins' isoelectric point; PRGdb, plant resistance genes database; qRT-PCR, quantitative reverse transcription PCR; RFLP, restricted fragment length polymorphisms; R gene, resistance gene; RL, resistant line; RLK, receptor like kinases; RLP, receptor like proteins; RPKM, reads per kilobase per million; SL, susceptible line; SSR, simple sequence repeat; TNL, TIR-NBS-LRR; TIR, toll/interleukin-1 receptor; TuMV, turnip mosaic virus; WGT, whole genome triplication.

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plant resistance proteins use their TIR domain in the detection of pathogens [14]. CNLs are more common in dicotyledon and cereal crops, and their relevant functions are similar to TNLs domains [15].

A feature of TNLs and CNLs is their species-specific distribution. TNL genes are numerous in dicots, yet few exist in cereals [8]. Furthermore, TNL gene sequences are more easily amplified from dicot genomes than cereal genomes. The truncation of TIR-NBS (TN) or TIR-X (TX) type protein domains in domesticated cereal plants may have led to loss of TNL genes in monocot plants such as rice, wheat (*Triticum* spp.), and maize (*Zea mays*) [16,17]. TNL genes are mainly involved in recognition of species-specific pathogens. Non-TIR-NBS genes (including CNLs) are derived from at least two species, demonstrating their ancestors originated before the divergence between species (before monocot and dicot); non-TIR-NBS genes function in basic defense, it may be due to the adaptation to biotic habitats [18].

Brassica crops provide food, oilseed, fodder, and condiments. Numerous materials have been developed for the investigation of genome evolution; these are largely based on *Arabidopsis thaliana* and *Brassica rapa* [19]. Chinese cabbage (*B. rapa* ssp. *pekinensis*) is an important member of the Brassicaceae family. Completion of the Chinese cabbage *Chiifu-401-42* draft genome sequence allows genome-wide identification analyses [20,21]. Chinese cabbage quality and yield are susceptible to constant barrages from biotic stresses. Among these, the *Turnip mosaic virus* (TuMV) belongs to the *Potyvirus* genus, it affects numerous plants in Asia, North America, and Europe, and does serious harm to *Brassica* crops and numerous other types of vegetables [22]. The host range of TuMV is wide, with the virus known to infect 318 kinds of dicotyledoneae; harm to *Brassica* plants is particularly acute, causing severe systemic vein clearing, necrosis, stunting, and plant death [23,24]. It is difficult to avoid TuMV infection because of the wide range of host species and its transmission by aphids, furthermore, the efficiency of pesticides used against it is declining [25]. Thus, natural plant resistance may prove an effective and environmentally friendly method to control TuMV.

There have been numerous studies on the NBS-LRR domain [6,7,15,26]. However, none of these analyzed the TNL family in Chinese cabbage, an important model plant for the genomic study of *Brassica* crops. Nor has the challenge by TuMV been specifically investigated. In this study, genome-wide analysis identified 90 putative TNL encoding genes using the Chinese cabbage genome database. Deeper analysis was performed on these genes, including identifying gene locations on 10 chromosomes, subgenome distribution, phylogenetic analysis, and transcript profiling of different organs. Quantitative Real-Time PCR (qRT-PCR) analysis was used to reveal gene expression patterns. Sixteen genes were identified as significantly responding to TuMV infection. These results provide a foundation for further identification and more efficient screening of resistance genes, and will prove crucial for further investigations of relevant underlying mechanisms.

Materials and methods

Identification and chromosome locations of TNL genes

To analyze the genome-wide TNL encoding genes, we retrieved the genome-wide sequences of Chinese cabbage from the *Brassica* database (Version 1.5) (BRAD; http://brassicadb.org/brad/) [20,21]. Then, hmmbuild in HMMER v3.0 (http://hmmer.janelia.org/) was used to screen the whole TNL encoding gene family according to the specific hidden Markov model (HMM) profile. SMART v7.0 [27] and Pfam database (http://pfam.sanger.ac.uk/) analyses identified 90 putative genes. Chromosome size and centromere position from BRAD, and least fractionated (LF), medium fractionated (MF) and most fractionated (MF2) blokes subgenome data from the diploid ancestral genome of mesohexaploid *B. rapa* [28] were used to map TNL encoding gene loci on the ten chromosomes and three subgenomes, using the method described previously [29].

Conserved motif and phylogenetic analysis

Pepstats (http://www.ebi.ac.uk/Tools/seqstats/emboss_pepstats) was used to identify putative TNL characteristics, including isoelectric point (pI) and molecular weight (MW). MEME Suite [30] was used to identify conserved motifs in TNL proteins. The conserved motifs were used for phylogenetic analysis using the maximum-likelihood (ML) method, with the bootstrap value set as 1000 by MEGA v5.0 [31].

Expression analysis of TIR-NBS-LRR genes in different tissues

Illumina RNA-Seq data obtained from BRAD [32] provided the groundwork for analyses of TNL expression patterns in Chinese cabbage. Gene expression patterns were obtained as previously described [29]. Heat map and hierarchical clustering were employed to display the different expression levels using the RPKM (reads per kilobase per million) value in Cluster v3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm).

Plant materials, growth conditions, and virus treatments

Seeds of the Chinese cabbage (*B. rapa.* ssp. *pekinensis*) cultivar "Baotoulian", which is less susceptible to TuMV, were used for this study. They were planted in pots, and grown in an artificial climate chamber under the following cultural conditions: 25°C/18 °C day/night temperature; photoperiod, 16 h light/8 h dark; relative humidity, 70%. Four-week-old plants, at the four-leaf stage, were inoculated with TuMV. The TuMV (pathotype C4) came from viruliferous Chinese cabbages. Leaves showing obvious disease symptoms were ground in a porcelain mortar in phosphate buffer (0.02 mol/L, pH 7.0). The virus was mechanically transmitted to three leaves of each plant using a friction inoculation method, and mock leaves simultaneously inoculated by phosphate buffer alone. Inoculated plants were exposed in the same growth conditions as described above, as were the control group. Inoculated leaf samples were collected under a continuous time process (0, 1, 3, 7, 14, and 21 days post inoculation (dpi)) using three biological repetitions for each time point, and stored at −80 °C for further analysis.

RNA isolation and fluorescence quantitative real-time PCR

Total RNA was extracted from samples using an RNA extraction kit (TaKaRa RNAiso Reagent, Takara, Dalian, China) in accordance with manufacturer's instructions, and 2 μg total RNA were reverse transcribed using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time; Takara, Dalian, China). Products used as templates for qRT-PCR were diluted 10 times with ddH_2O. SYBR® *Premix Ex Taq*™ (Takara, Dalian, China) was employed to identify target gene expression, using a Fluorescent Quantity PCR Detecting System (Bio-Rad ICycler iQ5 Hercules, CA, USA), in accordance with manufacturer's protocols. Specific primers were designed based on the TNL encoding gene sequences using Beacon Designer v7.9 (Supplementary Table 1). *Actin* (*Bra028615.1*) was used as the reference gene [33]. PCR conditions and the calculation method of gene expression were as described previously [34]. The relative expression levels of TNL encoding genes in response to TuMV were analyzed using Cluster v3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm); gene and array clusters were created using the Euclidean Distance method, and visualized using JavaTree View v3.0 software (http://jtreeview.sourceforge.net/).

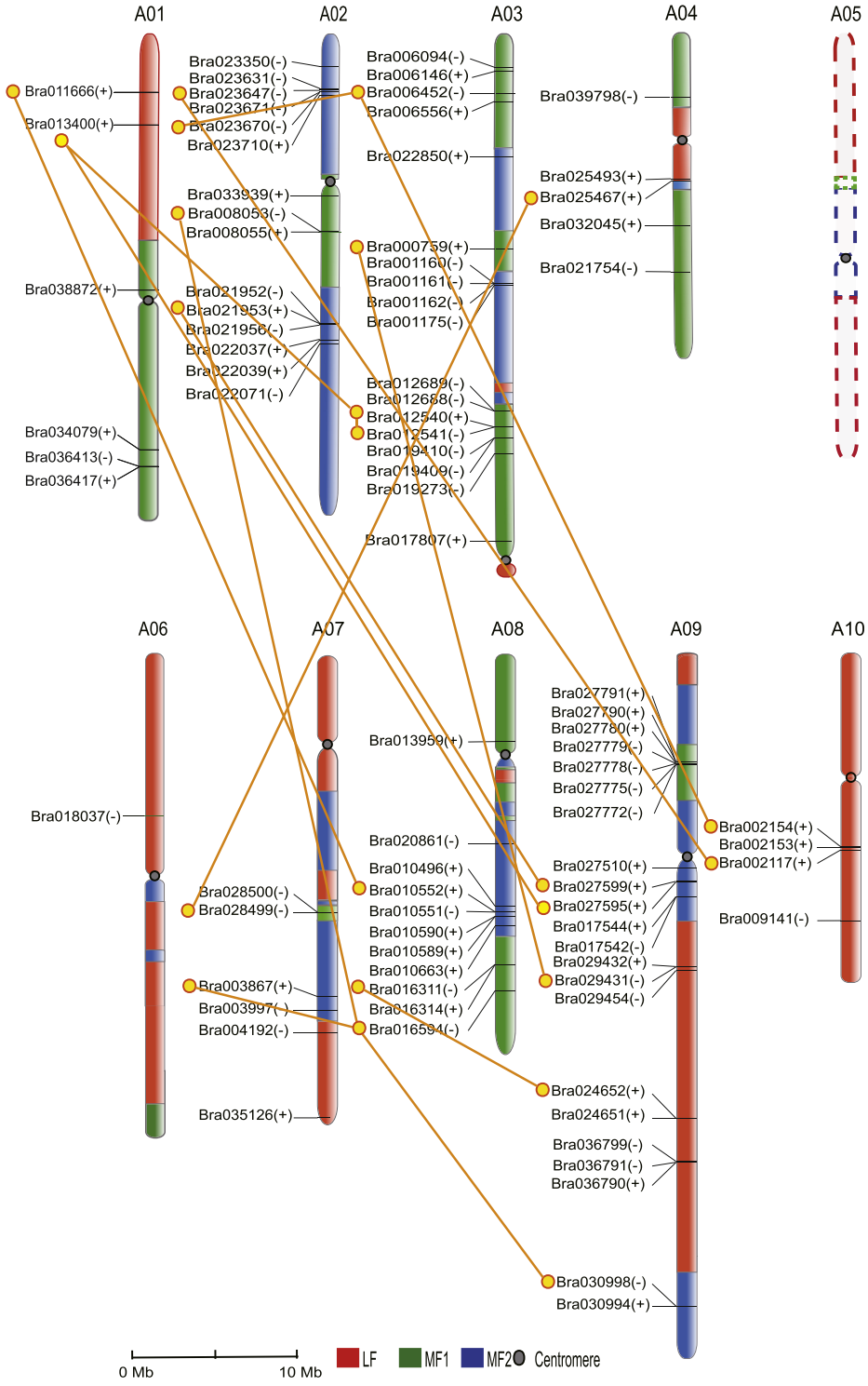

Fig. 1. Distribution of 88 TNL encoding genes and duplication events on 10 Chinese cabbage chromosomes as well as 3 subgenomes. There was no gene mapped on chromosome A05 (with dotted line). The centromeres were shown based on the analysis result of Chinese cabbage genome sequencing and two genes (*Bra011666*, *Bra000759*) on the scaffold cannot be located onto certain chromosomes. The size of each chromosome was indicated by the scale in megabases (Mb) on the bottom. Different colors bars represented different subgenomes (LF, MF1, and MF2). 16 pairs of duplicated TNL encoding genes were connected by orange straight lines on duplicated chromosomal segments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Results

Identification, chromosome distribution, and duplication events of TNL family genes in Chinese cabbage

Ninety genes, comprising all TNL domains in the whole Chinese cabbage genome, were identified. Characteristics, including chromosomal position, gene molecular weight, isoelectric point, domains and corresponding positions, and homologous genes in *A. thaliana* were analyzed (Supplementary Table 2). Of the 90 predicted TNL encoding genes, 88 annotated genes physically mapped to nine of the ten chromosomes. The remaining two genes, *Bra036019* and *Bra040210*, were located on two unanchored scaffolds, Scaffold000111 and Scaffold000191, respectively (Fig. 1). The chromosomal distribution of Chinese cabbage TNL encoding genes was non-random; this is similar to *A. thaliana* and rice [7,35]. In numerous plants, NBS-LRR (containing TNL encoding genes) loci have been characterized with isolated genes and gene clusters. NBS-LRR gene clusters mainly result from different heterogeneous clusters. A large number of genes (66/88, 76%) were located on chromosomes A02, A03, A08, and A09 (Fig. 1), with the remaining 24% on the other chromosomes. Chromosome A09 had the most TNL encoding genes (22/88, 25%), while no TNL encoding genes were present on chromosome A05 (Fig. 1). This is thought to be a result of *R*-gene evolution, as clusters facilitated for trending to mispair and produce frequent haplotypic diversity [36].

In addition, having been reported to be relative to *A. thaliana*, the almost complete triplication of the *B. rapa* genome was divided into triplicate segments, and orthologous divergence traced back to between 5 and 9 million years ago (MYA). The triplicate segments contained LF, MF and MF2 [20]. In this study, 88 genes could be mapped to the three fractionated blocks: MF1 contained 36 genes (42%); MF2, 34 genes (39%); and LF, 18 genes (20%; Fig. 1).

Furthermore, 16 pairs (involving 23 genes) of duplicated TNL encoding genes (Fig. 1) were identified on the chromosomal segments (Table 1). Most of these genes (20/23, 87%) were located on six chromosomes (A01, A02, A03, A08, A09, and A10). However, there was no significant difference among the three subgenomes (26%, 35%, and 39%, respectively). Some duplicated genes only contained two members, e.g., *Bra011666* and *Bra010552*; *Bra023647* and *Bra002117*; and *Bra025467* and *Bra028499*, while others included up to four genes, e.g., *Bra008053*, *Bra003867*, *Bra016594*, and. *Bra030998*. These results demonstrated TNL encoding gene retention and loss in Chinese cabbage at the chromosomal and subgenome level, thus genes of no use must have been lost, while useful genes were retained in the lineage following the whole genome triplication (WGT) event [37,38].

Conserved motifs and phylogenetic analysis of TNL encoding genes in Chinese cabbage

The 90 Chinese cabbage putative TNL encoding proteins were classified into four different groups (I–IV) based on conserved domain and protein motif structures (Fig. 2) using MEME suite and MEGA v5.0. The resultant phylogenetic tree harbored 32, 14, 29, and 15 members in groups I, II, III, and IV, respectively. The logos and regular expression sequence of protein motifs are given in Table 2. Every gene contained at least eight motifs, indicating that motifs are pivotal for gene function. Of the 15 motifs, motifs 1 and 3 were found in the TIR domain of every gene. Motif 1 was located in front of motif 3 in groups I and II but was reversed in groups III and IV, with the exception of *Bra027772*. The two genes adjacent to *Bra027772* in the phylogenetic tree were *Bra027779* (upper) and *Bra019409* (lower) (Fig. 2). *Bra019409* is located on chromosome 3, while both *Bra027772* and *Bra027779* are located on chromosome 9. Thus, the *Bra027772* and *Bra027779* duplication might have resulted from tandem duplication, whereas *Bra027772* and *Bra019409* likely resulted from whole genome duplication after truncation.

Expression patterns of TNL family genes in Chinese cabbage in response to TuMV

To select genes for expression pattern analysis and screening in response to TuMV infection, TNL encoding genes were analyzed in different tissues using Illumina mRNA-seq data available from BRAD (http://brassicadb.org/brad/genomeDominanceData.php). Among these, 12 genes (*Bra002117*, *Bra017542*, *Bra017544*, *Bra010663*, *Bra028499*, *Bra022037*, *Bra036799*, *Bra038872*, *Bra016594*, *Bra039798*, *Bra027595*, and *Bra030998*) were deemed not integral as a result of non-expression, or from their spatial and temporal expression patterns. Several TNL encoding genes showed up-regulation of expression in a tissue specific manner, e.g., *Bra008055* was highly expressed in leaves (RPKM = 55.6), while expression levels of *Bra006146* and *Bra027772*, were high in stems (RPKM = 64.2) and roots (RPKM = 32.1), respectively. Furthermore, many genes displayed spatial up-regulated expression patterns. Roots contained most up-regulated TNL encoding genes (50, 60%), followed by stems (48, 53%), while leaves had the lowest number (42, 47%). Some TNL encoding genes expressed highly in the same

Table 1
Colinearity of duplicated TNL family genes in Chinese cabbage.

Gene pairs	Gene names	Chromosome number	Subgenome[a]	Gene names	Chromosome number	Subgenome[a]
1	Bra010552	A08	MF2	Bra011666	A01	LF
2	Bra012541	A03	MF1	Bra013400	A01	LF
3	Bra013400	A01	LF	Bra027595	A09	MF2
4	Bra008053	A02	MF1	Bra016594	A08	MF1
5	Bra008053	A02	MF1	Bra030998	A09	MF2
6	Bra021953	A02	MF2	Bra027599	A09	MF2
7	Bra002117	A10	LF	Bra023647	A02	MF2
8	Bra006452	A03	MF1	Bra023670	A02	MF2
9	Bra002154	A10	LF	Bra023670	A02	MF2
10	Bra025467	A04	MF2	Bra028499	A07	MF1
11	Bra000759	A03	MF1	Bra029431	A09	LF
12	Bra002154	A10	LF	Bra006452	A03	MF1
13	Bra012540	A03	MF1	Bra027595	A09	MF2
14	Bra003867	A07	MF2	Bra016594	A08	MF1
15	Bra016311	A08	MF1	Bra024652	A09	LF
16	Bra016594	A08	MF1	Bra030998	A09	MF2

[a] LF: fractionated blocks, MF1: the medium fractionated blocks, and MF2: the most fractionated blocks.

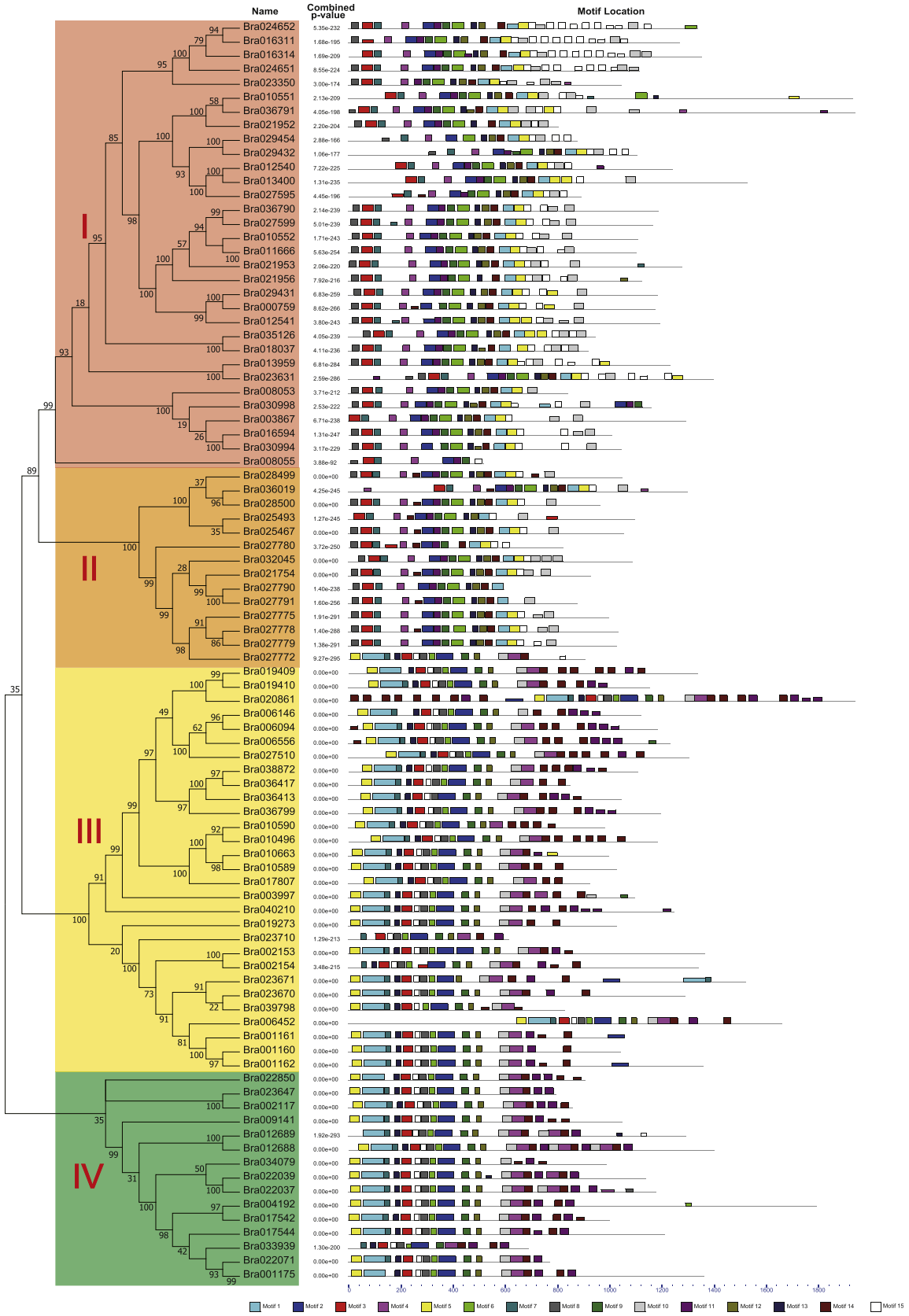

Fig. 2. Phylogenetic tree and conserved motifs distribution of TNL proteins. The left part exhibited the multiple alignments of 90 full-length TNL proteins generated by MEGA and the combined *P-values* were shown on the leaf side. The tree was divided into four groups named as I–IV. The right side was schematic conserved motifs and the sizes of motifs were indicated by the scale. Different motifs were indicated by different colors LOGOs from 1 to15 at the bottom and the detailed information of them was shown in Table 2.

tissue, demonstrating functional homoousia, while expression of some TNL encoding genes was diversified in different tissues, showing functional dissimilarity (Supplementary Fig. 1, Supplementary Table. 3).

Among the 90 genes, 69 genes specifically expressed in leaves, and were subjected to expression profile analysis via qRT-PCR. The $-\Delta\Delta CT$ data was analyzed using cluster software and visualized via a heat map that depicted overall variation tendency (Fig. 3, Supplementary Table. 4). In accordance with the gene cluster and expression level results at different sampling times (especially, early stages), genes were divided into three categories: i, up-regulated genes; ii, mediumly expressed genes; iii, down-regulated genes (Supplementary Table. 5, Fig. 3). In group i, 59.4% (42/69) of genes were up-regulated at most stages. Eleven genes belonged to group ii (15.9%); these were mainly down-regulated at early stages (1 dpi, 3 dpi, and 7 dpi). Group iii contained 16 genes (23.2%, 16/69); these were downregulated at most stages.

The expression level of *coat protein* (CP) gene was analyzed at different sampling times to measure the *in vitro* virus content change trend (Fig. 4). Virus content fluctuated significantly from 0 dpi to 21 dpi, with peak ($2^{-\Delta\Delta CT} = 955.4$) and valley values ($2^{-\Delta\Delta CT} = 1.9$) occurring at 1 dpi and 21 dpi, respectively. The reason for the rapid drop after 1 dpi was that the cultivar "Baotoulian" was less susceptible to TuMV. As a ground Chinese cabbage organ solution was used to infect plants, the initial value (0 dpi) was not zero ($2^{-\Delta\Delta CT} = 4.3$). Thus, small amounts of the virus were derived from the inoculation solution itself. Virus content at 14 dpi and 21 dpi was very similar, it was at a low level and tended to be stable ($2^{-\Delta\Delta CT} = 2.0$; Fig. 4). When comparing these groups with the expression tendency closely related to virus content variation, each group had a different correlation; representative genes are shown in Fig. 4. Group i contained two kinds of correlations: the first, including the four genes *Bra022039*, *Bra028499*, *Bra019273*, and *Bra021754*, was initially lowly expressed, then expression raised, with a steep rise correlating to virus content. Expression levels returned to their initial low and stable levels at the final stages (Fig. 4, i left). Therefore, these genes responded to the virus at early stages of infection, and furthermore they showed uniformity with the virus, showing the same change tendency at almost every stage. This indicated that these genes were likely to be involved in virus propagation. The second correlation in group i, contained three genes, *Bra011666*, *Bra023350*, and *Bra023647*, genes from this group behaved similar to the first, but also showed a steep rise at 7 dpi, excepting 1 dpi day (Fig. 4, i right). Thus, these genes can respond to a virus not only at the early stage, but also at later stages. As plant samples after 7 dpi were from uninoculated leaves, they are expected to be involved in virus propagation and transportation. Group ii, which included *Bra012689*, *Bra016594*, and *Bra002154* contained one correlation; expression descended at 1 dpi, and showed peak values at 3 dpi (Fig. 4, ii). They exhibited obvious hysteresis in expression levels compared with the virus. They therefore may have participated in viral defense as their expression ascended when virus content was high, and dropped when virus content was lower, and were close to normal levels at the end. The last group (iii) belonged to the correlation which exhibited peak values at 1 dpi, the correlations were similar to group i, but their expression values were much lower at 1 dpi.

Discussion

As one of most important vegetables cultivated worldwide and a model plant, Chinese cabbage is widely used in genetic research. Due to its importance, breeding targets of Chinese cabbage include numerous traits, such as high quality and yield, high nutrient content, tolerance to extreme temperatures, and resistance to pathogens. Considerable work has been carried out on TNL family genes involved in resistant to pathogens [7,16,39]. There have been several bioinformatical analysis studies on TNL family genes in *B. rapa* [17,38,40]. The release of the *B. rapa* genome has greatly enhanced this research. Since *A. thaliana* was first sequenced in 2000, numerous other species, including potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), and rice have been sequenced. The overall bioinformatical information provides an efficient means for analyzing a huge amount of sequence data to reveal genomic and underlying genetic mechanisms.

The distribution of Chinese cabbage TNL encoding genes on chromosomes was uneven, with many genes clustered: 25% (22/88) of genes were located on chromosome 9, while no gene located to chromosome 5. *A. thaliana* has undergone three duplication events [41]: a whole genome triplication event (WGT: γ) shared with most eudicots, and two more recent whole genome duplications (WGD: β then α) shared with species with Brassicales order lineages.

Table 2
Information of motif LOGOs shown in Fig. 2.

Motif ID	*e* value	Width	Sites	Regular expression sequence[a]	Description
Motif 1	3.5e−1891	50	50	[KR]NYASSSWCL[DN]ELVEI[ML]KC[RK]EELGQ[TI]V[MI][PT][IV]FY[EG]VDPS[DH]V[RK]KQTG[DE]FGK[AV]F	TIR domain
Motif 2	3.9e−1329	44	50	VDDL[VE]QL[ED]A[LM]A[KG]ETSWFG[PS]GSRII[VI]TT[EQK]D[KQR]K[LI]L[KR]AHGI[ND]H[IV]Y[EKH]V	
Motif 3	1.2e−2148	70	50	SS[LS][SP]RNWR[YH][DH]VFPSF[HR]G[EP]DVRK[TG]FLSH[LVFI][LR]KE[FL]K[RS]KGITAF[IK]D[NQ]EI[EK]R[SG][EQK]SI[GA]PEL[VIK][QR]AIR[GE]SR[IV][AS][IV]V[LV]LS	TIR domain
Motif 4	9.3e−1551	50	50	PS[RKN]DF[DE][GD][FL]VG[MI]EAH[IML][ERA][KE][MIL][KS]SLLCL[ED]S[DEN]EV[RK]M[IV]GIWGP[ASP]GIGKTTIAR[AV]L[FY]	
Motif 5	1.3e−1229	46	50	FE[EK]LAREVTKLAGNLPLGLRV[ML]GS[YS][LF]RG[MKR]SK[DEQ]EW[EI][NDE]ALPRLR[TS]SLD	
Motif 6	1.3e−1887	70	50	LRLLHWDx[YFC]P[ML][RTK]CLPSKF[NSR]PEFLV[EK]LIM[RP]NSKLEKLWEG[IVT][QK]PLT[NCS]LK[WK]MDLSGSxNLKE[IL]PDLS[TKN]AT[NS]LE	
Motif 7	3.8e−1046	36	50	W[RK]QAL[TA][DE]VA[NT]IAG[YE][HD]SSNWDNEA[DK][ML]IEK[IV]ATDVSNK	
Motif 8	1.0e−1205	50	50	RA[CG][LS]D[DE]Y[GS]LKL[RH]LQ[EK]Q[FL]LS[KQE][IL][LF]NQKD[IM]K[IV]xHLG[VA][AVI][KQ]ERLKD[KQ][KR]VL[IV][VI]LD[DE]	
Motif 9	3.3e−1091	50	50	S[VIT]L[RK][FV][SG]Y[DE]AL[SD][DE][EKN][DE][KQ][SA][LI]FLHIACFFN[GY]EK[VI][DE]xV[KE]E[MC]LA[KD]SFLDVR[HQ]GLK[VT]	
Motif 10	7.3e−1118	50	50	PSSI[GQR]NL[HN]KLKKLE[ML]xGC[ST][KN]LE[VT]LPT[NG]INL[KE]SL[ED][ER]L[ND]LS[GD]CS[RS]L[RK][ST]FP[DEQ]I	
Motif 11	1.5e−799	36	50	L[DN]LSGCSSLV[EK]LPSSIGN[AL][TI]NL[EK][KE]LNLSGCSSLVEL	
Motif 12	5.2e−540	28	50	KGC[RK][KN]LVSLP[QEP]LPDSLLFLDA[HE][GD]CESL[EK]	
Motif 13	5.5e−532	19	50	EAL[QE][IM][FL]C[RQ][YS]AF[GR]Q[KN][SY]P[PK]DG	
Motif 14	7.6e−441	19	50	EPG[KQ]RQFLVDAK[DE]IC[DE]VL[AE]	
Motif 15	4.7e−505	26	50	xKYA[VC][LF]PGEEVP[AS][YE]FT[HY][RQ]AT[GS][SGN]SLTI	

[a] Alternative amino acids.

B. rapa shares the same evolution process, including a WGT event between 13 and 17 MYA [20,37]. According to "two-step" genome triplication and differential subgenome methylation, Brassicaceae genomes formed from chromosomal rearrangements, and breaking and fusion of the polyploidy ancestor; this comprised 24 genomic blocks, which can be divided into three subgenomes and displayed along the five chromosomes of *A. thaliana* [20,32]. Only a short segment (MF2: *Bra039484–Bra025437*) comes from mid-chromosome 5 of *A. thaliana*. According to previous *A. thaliana* studies, approximately 37% of TNL encoding genes are located on *At*-chromosome 5, and these did not map to the middle part of the chromosome [7,17]. Wang et al. [20] showed there was almost no segmental collinearity of *B. rapa* and *A. thaliana* genomes in the mid-regions of Arabidopsis chromosome Chr5 and Chinese cabbage chromosome A05. Thus, few chromosome 5 genes from *B. rapa* are derived from *A. thaliana* chromosome 5 [20].

In recent years, many studies have made progress in the genetic breeding and molecular cloning of *R* genes, revealing that TNL encoding genes are involved in defence mechanisms; most genes mentioned in this work have been reported in Chinese cabbage or other plants. Two probable *R* genes (*Bol037156* and *Bol037157*) to Fusarium wilt were homologous to *Bra022039* and *Bra012689*, respectively [42]. Kover and Cheverud [43] identified a QTL associated with the bacterium *Pseudomonas syringae*, this QTL contained *At5g41540*, which is orthologous with *Bra028499*. The deduced amino acid sequence of *RPS6*, containing *At5g46450* orthologous to *Bra022071*, showed the highest similarity to the TNL resistance protein RAC1, which determines resistance to the oomycete pathogen *Albugo candida* [44]. *Bra013400* was orthologous to *At4g19500*, which was more transiently expressed in response to silverleaf whitefly feeding; although *At4g19500* showed decreased expression in *A. thaliana* [45], *Bra013400*

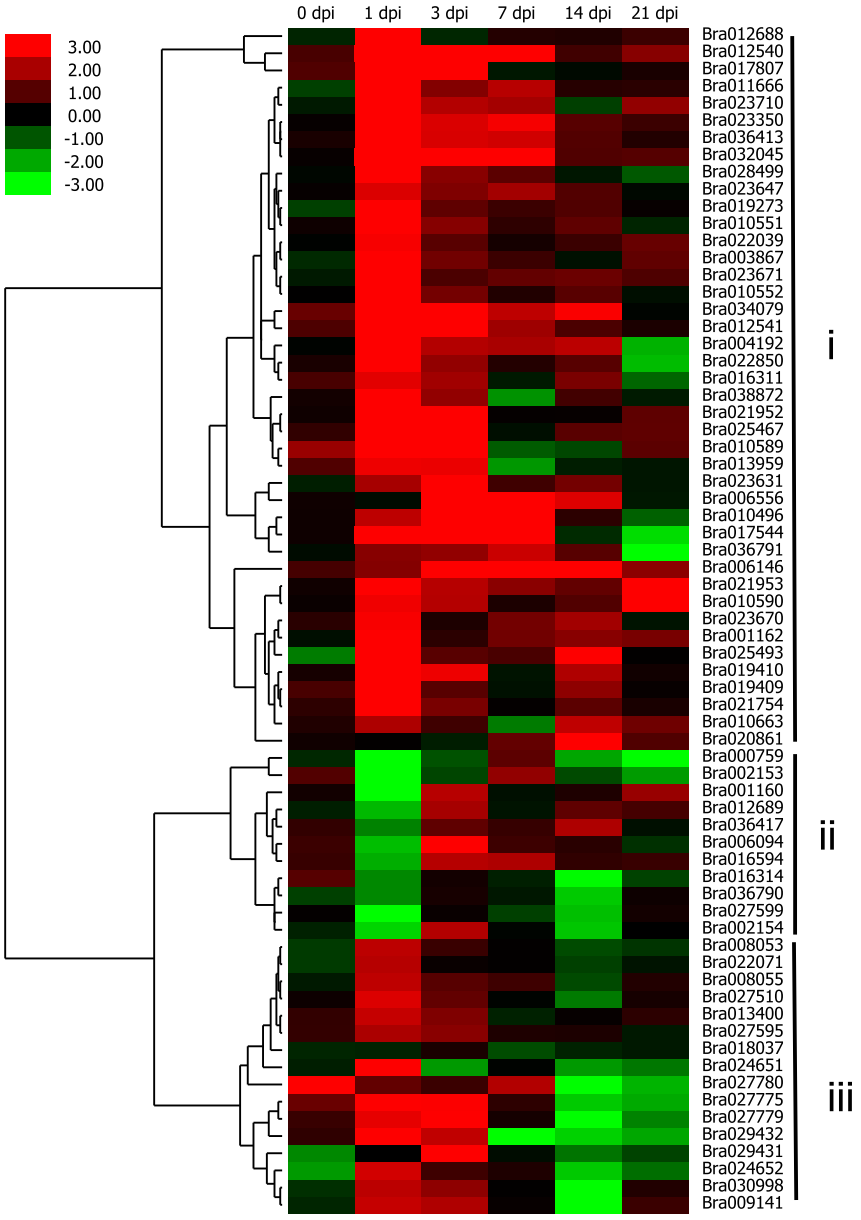

Fig. 3. Expression profiles of 69 TNL encoding genes under the challenge of TuMV. Four-week-old Chinese cabbage samples were selected in different times (0 dpi, 1 dpi, 3 dpi, 7 dpi, and 21 dpi). The qRT-PCR data was normalized by *Actin gene (Bra028615.1)*. The $-\Delta\Delta CT$ was used in drawing heat map and hierarchical clustering. Euclidean distance was used in gene cluster and the color bar was on the top left.

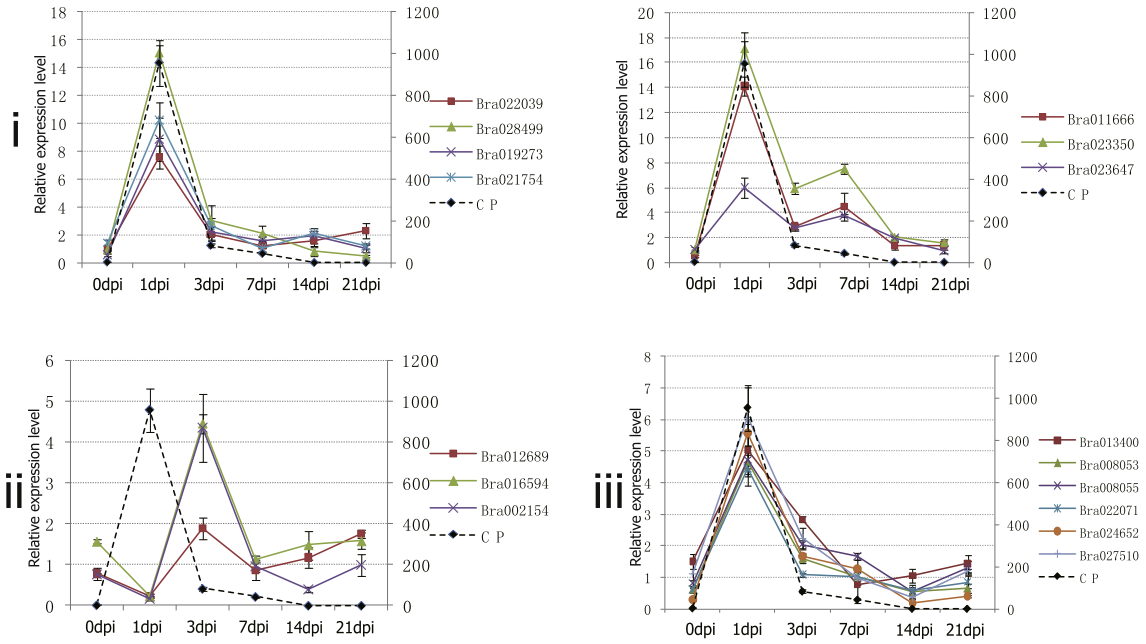

Fig. 4. The correlations between 16 genes expression levels and virus' content variation. i, ii, iii showed the expression correlations. The vertical axis represented the relative expression level and the 0 dpi, 1 dpi, 3 dpi, 7 dpi, 14 dpi, 21 dpi (*x*-axis) indicate the treatment times. Different colour lines represented different genes and CP gene was shown in black dotted line in all subgraphs. Error bars represent the standard error of the mean based on three replicates.

expression was found to be increased in this research. *At1g63730* was orthologous with *Bra021754*, its expression was up-regulated 6.59 fold in *A. thaliana* colonized by *Pseudomonas putida* MTCC5279 [46]. Shimizu et al. found that *Bra022071* and *Bra008053* showed resistant line > susceptible line expression when responding to *Fusarium* [47]. *AtTN15* (*AT4G09420*) was orthologous to *Bra008055*, this is known to interact with effectors from *Pseudomonas syringae*, *Ralstonia. solanacearum*, *Bremia. lactucae*, and *Hyaloperonospora. arabidopsidis*, and with plant NBS-LRR proteins [48]. *TAO1* is the Mt-0 allele of the Col-0 gene *At5g44510*, this gene is orthologous with *Bra027510* and contributes to disease resistance induced by the *P. syringae* effecter *AvrB* [49]. All the above Chinese cabbage genes and orthologous/homologous genes belonged to the sixteen genes that significantly responded to TuMV infection. Therefore, part of sixteen genes may be related to resistance or similar functions with viruses in Chinese cabbage.

Much genetic research into resistance against TuMV has been performed in Chinese cabbage, and recently some resistance genes have been mapped on the Chinese cabbage genome. Two resistance genes, *recessive TuMV resistance 01* (*retr01*) and *Conditional TuMV resistance 01* (*ConTR01*), were mapped on the upper portion of chromosomes A04 and A08, respectively using RFLP probes [50]. The *recessive Turnip mosaic virus resistance 02* (*retr02*) gene was mapped to scaffold000060 or scaffold000104 on chromosome A04 using microsatellite and indel marker assays [51]. In addition, *resistance and necrosis to tumv 1-2* (*rnt1-2*), *TuRB07* (*TuMV RESISTANCE in Brassica 07*), and a novel TuMV resistance locus (*TuMV-R*) were mapped to chromosome A06 [52–54]. Among these 13 candidate genes above, six (*Brao18862*, *Bra018863*, *Bra018835*, *Bra018834*, *Bra018810*, and *Bra018810*) belonged to the CNL family. Moreover, a novel microRNA induced by TuMV infection was reported to target TNL on chromosome A03 [55]. In this study, the sixteen predicted genes mainly mapped to chromosomes A02, A03, and A09 (61.5%).

Ninety TNL encoding genes were identified in the whole genome of Chinese cabbage, and these were anchored on 9 of the 10 chromosomes. Their characteristics, classification, and expression patterns were analyzed under normal and infectious conditions. Sixteen candidate genes were identified as responding to TuMV infection. These genes are expected to be very important in the response to pathogens, and their cloning and verification should be the focus of subsequent work. The expression pattern data, under normal and infectious conditions, can be applied to construction of a pathogen-defense regulation network model in Chinese cabbage. This work provides genetic resources for the genetic modification of plants and is conducive to the breeding of pathogen resistant varieties.

Authors' contributions

SL, CZ, YL, ZW, and XH designed the research. SL and YL completed the experiments. SL and JT performed data analysis and manuscript preparation. CZ, JT, DJ and XH participated in revising the manuscript. All authors had read and approved the final version of the manuscript.

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2015.04.001.

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