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Genome-Wide Analysis Revealed *NBS-LRR* Gene Candidates Associated with Bacterial Wilt Resistance in Eggplant (*Solanum melongena* L.)

Yaolan Jiang [1,2,†], Zhiliang Li [2,†], Wenxiang Li [3], Hefen Cheng [1,2], Wei Zhao [2], Tao Li [2], Baojuan Sun [2], Qian You [2,*] and Dinggang Zhou [1,*]

1   Hunan Key Laboratory of Economic Crops Genetic Improvement and Integrated Utilization, School of Life and Health Sciences, Hunan University of Science and Technology, Xiangtan 411201, China; yaolanae@163.com (Y.J.)
2   Guangdong Key Laboratory for New Technology Research of Vegetables, Vegetable Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China
3   College of Horticulture, South China Agricultural University, Guangzhou 510642, China
*   Correspondence: kuaileyouqianmeng@163.com (Q.Y.); dgzhoucn@163.com (D.Z.)
†   These authors contributed equally to this work.

Abstract: *NBS-LRR* genes constitute one of the largest resistance gene families in plants, which play key roles in resistance to pathogens. Although the identification and characterization of the *NBS-LRR* gene family has been extensively reported in various species, a comprehensive analysis in eggplant has not been previously documented. In this study, a total of 269 *SmNBS* genes were identified in the eggplant genome. Based on domain classification and phylogenetic analysis, *SmNBSs* were divided into three subgroups 231 CNLs (CC-NBS-LRR), 36 TNLs (TIR-NBS-LRR), and 2 RNLs (RPW8-NBS-LRR). Chromosomal mapping analysis revealed an uneven distribution of *SmNBSs* in clusters across chromosomes, with a predominant presence on chromosomes 10, 11, and 12. Structural analysis identified eight conserved motifs previously reported in *SmNBSs*, exhibiting high conservation in both amino acid sequences and their order. Evolutionary analysis demonstrated that tandem duplication events mainly contributed to the expansion of *SmNBS*. Subsequently, qRT-PCR analysis demonstrated that nine *SmNBSs* exhibited differential expression patterns in response to *R. solanacearum* stress, with *EGP05874.1* potentially involved in the resistance response. In conclusion, this study provides a comprehensive insight into *SmNBSs*, which will enhance the research on eggplant disease resistance and facilitate the breeding of new disease-resistant varieties.

Keywords: eggplant; *NBS-LRR*; disease resistance; genome-wide investigation; bacterial wilt resistance

1. Introduction

Plants are constantly threatened by a wide range of pathogens in the process of growth and reproduction. During the long-term evolutionary process, plants have evolved effective immune systems capable of both recognizing and responding to infections caused by pathogens [1,2]. Plant disease resistance genes (*R* genes) are a significant component of the plant immune system, which can detect the invasion of bacterial, fungal, or viral pathogens, and then trigger a series of immune responses, playing a key role in resistance to pathogens [3]. The nucleotide-binding site leucine-rich repeat (*NBS-LRR*) genes constitute the largest class of *R* genes in plants, which accounts for over 60% of cloned functional *R* genes in angiosperms [4–6].

The *NBS-LRR* genes possess a central NBS and a C-terminal LRR domain as well as an N-terminal variable domain [4]. The *NBS-LRR* genes are categorized into three subgroups according to the presence or absence of the Toll/interleukin-1 receptor (TIR), coiled coil (CC), and resistance to powdery mildew8 (RPW8) in the N-terminal region.

These subgroups are known as TIR-NBS-LRR (TNL), CC-NBS-LRR (CNL), and RPW8-NBS-LRR (RNL), respectively [7]. The activation of *NBS-LRR* genes occurs by detection of modifications in effector target proteins [4], or by direct interaction with pathogen effectors [8], or by modification within the *NBS-LRR* genes themselves [9,10], triggering immune responses that inhibit the spread and proliferation of pathogens [11,12].

Due to the highly conserved NBS domain of *NBS-LRR* genes, the NBS domain was used for the identification of *NBS-LRR* genes (referred to as *NBS* genes) in various plants [13]. Since the genome-wide analysis of *NBS* genes in *Arabidopsis thaliana* and rice (*Oryza sativa*) at the beginning of the 21st century [14–16], *NBS* genes have been identified and analyzed in numerous other species, including cabbage [17], soybean [18], wheat [19,20], maize [21], pepper [22], potato [23], and tomato [24]. In most of these studies, plant genomes typically encode tens to thousands of *NBS* genes to combat various pathogens, and the total number of *NBS* genes differ greatly in different species and may indicate the complex evolutionary pattern in different plant genomes [25]. Large-scale evolutionary studies of angiosperms have drawn the basic outlines of *NBS* genes [7], and tandem replication events appear to be responsible for recent *NBS* gene increases [26,27].

Numerous functional *NBS-LRR* genes associated with disease and pest resistance have been identified in different angiosperm species [28]. In terms of disease resistance, the *Arabidopsis RPS2* and *RPM1* are reported to combat the bacterial pathogen *Pseudomonas syringae* through the protection of the host protein RIN4 [29,30]. *Pi64* provides broad-spectrum resistance to leaf and neck blight in rice, and *RppM* enhances resistance to southern corn rust in maize [31,32]. Then, the tobacco *N* mediates virus resistance through a Toll-IL-l-like pathway [33], and the potato *Rx2* protects resistance against potato virus X (PVX) [34]. Furthermore, the pepper *Bs2* provides resistance to bacterial spot disease (*Xanthomonas campestris*) [35], and the tomato *Sm* and *SLNLC1* confer resistance to gray leaf spot disease (*Stemphylium lycopersici*) [36,37]. In terms of pest resistance, there are many studies on nematodes. For example, the tomato *Hero* gene is a broad-spectrum resistance gene, displaying strong resistance to all pathogenic types of the potato cyst nematode *Globodera rostochensis* and has partial resistance to *Globodera pallida* [38]. Moreover, the eggplant *SacMi* may be associated with resistance to root-knot nematode (*Meloidogyne incognita*) [39]. These studies indicated that *NBS* genes play a crucial role in mediating plant resistance to various bacterial, fungal, and viral pathogens and pests, respectively. Moreover, a large number of *NBS* functional genes have been identified from Solanaceae plants, of which many have great significance for studying pathogens in the mechanisms of pathogen–host crop interaction and breeding disease-resistant varieties.

Eggplant (*Solanum melongena* L.) is a widely cultivated and important vegetable crop in the Solanaceae family. With the advancement and application of whole-genome high-throughput sequencing technology, the identification and analysis of *NBS* gene family members have been conducted in numerous species. However, a comprehensive identification and systematic study of the eggplant *NBS* gene family have not yet been performed, thus impeding our understanding of the role of *NBS* genes in eggplant resistance. In the current study, we identified 269 *SmNBS* genes in eggplant and analyzed their categorization, chromosomal distribution, gene structure composition, phylogenetic relationship, conserved motifs, gene duplications, and predicted *cis*-acting regulatory elements (CAREs). In addition, we analyzed *SmNBS* expression patterns under the stress of bacterial wilt pathogen with quantitative real-time PCR (qRT-PCR). This study successfully screened a batch of eggplant *R* gene resources, which will contribute to further research on the functional characteristics of *SmNBSs* in disease resistance and accelerate the breeding of resistant eggplant varieties.

2. Materials and Methods

2.1. Plant Growth Conditions and Treatments

Eggplant 'R76' and 'S91', which are highly resistant and susceptible to eggplant bacterial wilt, were provided by the Institute of Vegetables, Guangdong Academy of

Agricultural Sciences (Guangzhou, China). After germination, the seedlings were cultured under a day/night temperature of 28 °C/25 °C, with a photoperiod of 16 h of light and 8 h of darkness. When the seedlings were at the four-true-leaves stage, they were infected with *Ralstonia solanacearum* using root-dipping inoculation. The *R. solanacearum* strain GMI1000 was grown on nutrient broth medium at 28 °C overnight and suspended in sterile distilled water. The inoculum concentration of the suspension was 10^8 cfu/mL. The roots of seedlings were cut at 0–1 cm from the root tip and inoculated in a 30 mL suspension [40]. Roots for sample collection were collected at 0, 24, and 48 h post-inoculation with three replicates, respectively.

2.2. Identification and Categorization of NBS Genes in Eggplant

The 'GUIQIE-1' genome sequence was used as a reference genome for genome-wide mining of *NBS* genes [41]. The Hidden Markov Model (HMM) of the NB-ARC domain (PF00931) was used as a query to identify the candidate *NBS* genes in eggplant with threshold E-values of 10^{-4} using two methods, including the HMMsearch (http://hmmer.org/ (accessed on 2 March 2023)) and BLAST [42]. High-score genes were obtained from the HMMsearch with E-values $< 10^{-20}$ and used to construct the specific eggplant NBS HMM-profile. This specific eggplant HMM was used to check for any missing *NBS* genes with E values < 0.01. Subsequently, all obtained candidate genes were combined, and redundant genes were removed. HMMscan was conducted for non-redundant candidate genes (E-value set to 10^{-4}) and to eliminate genes without the conserved NBS domain. Then, the remaining candidate genes were submitted to the Pfam database (http://pfam-legacy.xfam.org/ (accessed on 5 March 2023)) and SMART (http://smart.embl-hei-delberg.de/ (accessed on 5 March 2023)) to verify the presence of LRR (PF13855.9), TIR (PF01582), and RPW8 (PF05659) domains using default parameter settings, while CC domains were identified using COILS (http://toolkit.tuebingen.mpg.de/pcoils (accessed on 12 March 2023)) with an E-value of 0.9.

2.3. Gene Characteristics, Structure, and Chromosomal Distribution Analysis

The ExPASy tool (https://web.expasy.org/ttools (accessed on 5 March 2023)) was applied to predict the chemical–physical properties with default parameters, including protein length, isoelectric point (pI), and molecular weights (MW) of the detected NBS proteins. The detailed information of gene structure was extracted from the GFF3 file of the 'GUIQIE-1' reference genome and visualized utilizing TBtools (https://github.com/CJ-Chen/TBtools/releases (accessed on 12 March 2023)) [43]. TBtools was also utilized to visualize the chromosomal distribution of all identified *SmNBS* genes in the eggplant genome.

2.4. Phylogenetic, Conserved Motifs, and Cis-Acting Elements Analysis

The NBS protein sequences from eggplant were extracted and then sequence alignment was performed using MUSCLE v5.1 program with default parameters [44]. A phylogenetic tree was constructed with the maximum likelihood (ML) method and 1000 bootstrap using IQ-TREE v2.2.2.6 and embellished by iTOL (https://itol.embl.de/itol.cgi (accessed on 12 March 2023)) [45,46]. The conserved motifs were identified using the MEME online tool (http://meme-suite.org/tools/meme (accessed on 12 March 2023)) and visualized using TBtools [47]. The 2 kb upstream sequences of the *SmNBS* genes were extracted and submitted to the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 12 March 2023)) for prediction of *cis*-acting regulatory elements in their promoter region.

2.5. Gene Duplication and Syntenic Analysis

The duplication events of *SmNBSs* were analyzed utilizing MCScanX software [48]. The non-synonymous (Ka)/synonymous (Ks) analysis of *SmNBS* gene pairs was performed using the Simple Ka/Ks Calculator in TBtools. To demonstrate the synthetic relationships

of *NBS* genes between *Arabidopsis* and tomato (*Solanum lycopersicum*), syntenic analysis was performed utilizing MCScanX.

2.6. Expression Analysis of SmNBSs

The expressions of *SmNBSs* in different infection periods were analyzed, and the transcriptome data were available at GenBank: PRJNA837016 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA837016/ (accessed on 28 March 2023)) [40]. Firstly, low-quality reads were removed from the RNA-seq data using Fastp v0.23.2 to obtain clean data [49], and Hisat2 v2.2.1 was used to map the clean data to the 'GUIQIE-1' reference genome [50]. Then, the mapped reads were quantified using featuresCounts V2.0.6 [51]. Finally, the transcripts per million (TPM) values were calculated with counts values by R, and TBtools was utilized to construct a heatmap. The data with low expression were filtered out, and 186 *SmNBSs* data were retained for heat mapping. The differentially expressed genes (DEGs) were identified with DESeq2.

Additionally, the expressions of *SmNBSs* under *R. solanacearum* inoculation were analyzed using qRT-PCR in Bio-Rad CFX Manager Software v3.1. The total RNA was extracted using the MagicPure® Total RNA Kit (TRAN®, Beijing, China), and the cDNA synthesis was performed using the TransScript® First-Strand cDNA Synthesis SuperMix (TRAN®, Beijing, China). The TransStart® Top Green qPCR SuperMix (TRAN®, Beijing, China) was applied for qRT-PCR following the manufacturer's instructions. The qRT-PCR reaction system had a total volume of 10 µL, containing 5 µL Green qPCR SuperMix, 1 µL cDNA template, 0.4 µL forward primer, 0.4 µL reverse primer, and 3.2 µL nuclease-free water. The amplification procedure comprised 94 °C for 30 s, 39 cycles of 94 °C for 5 s, and 60 °C for 30 s. Three replicate experiments were conducted for each root sample to calculate the average Ct values. Relative gene expression level was calculated using the $2^{-\Delta\Delta Ct}$ method [52]. *SmActin* was chosen as a reference gene [53]. All primers used in this study were designed with primer3 plus, and the primer sequences are shown in Table S1 [54].

3. Results

3.1. Identification and Categorization of the NBS Gene Family

A total of 269 *SmNBS* genes were identified in the eggplant genome, accounting for 0.88% of the total genome (Table 1). The proportion of *SmNBSs* in the eggplant genome was within the range previously reported for other species (0.22–1.99%) [20,55]. According to the combination of the C-terminal domain (LRR) and N-terminal domain (CC, TIR, or RPW8) and phylogeny analysis, 269 *SmNBSs* were divided into three subgroups (231 CNLs, 36 TNLs, or 2 RNLs) (Table 2, Figure S1). Among the CNLs subgroup, six *SmNBSs* in the CNLs contained complete domains (CC, NBS, and LRR), while 225 *SmNBSs* in this subgroup lacked partial domains, categorized as follows: CN (111, CC and NBS, but lacking LRR), N_{CC} (112, NBS, but lacking both CC and LRR), and NL_{CC} (2, NBS and LRR, but lacking the CC). Similarly, TNLs were classified as TNL (6), TN (24), and N_{TIR} (6), and the rest of the genes belonged to the RNLs and were classified as RN (2).

The results showed that CNLs (85.87%) accounted for the overwhelming majority, followed by TNLs (13.38%) and RNLs (0.65%). Moreover, not all genes possessed the complete structure of these three domains. Among these genes, only 12 *SmNBSs* had complete structures and contained all three domains (CC/RPW8/TIR-NBS-LRR), while the remaining 257 *SmNBSs* lacked the LRR domain and 118 *SmNBSs* only contained the NBS domain (Table 2).

Table 1. Summary of the number of identified *NBS* genes in 11 plant genomes.

Species	*NBS* Gene Subgroups			Total *NBS* Genes	Total Genes	Proportion of *NBS* Genes	Source
	CNL	TNL	RNL				
Akebia trifoliata	50	19	4	73	24,138	0.30%	[56]
Arabidopsis thaliana	55	94	–	149	25,498	0.58%	[13]
Carica papaya	33	20	1	54	24,746	0.22%	[55]
Dioscorea rotundata	166	–	1	167	26,198	0.64%	[57]
Manihot esculenta	181	146	–	327	30,666	1.07%	[58]
Oryza sativa	467	63	–	535	37,544	1.42%	[16]
Populus trichocarpa	279	123	–	402	45,555	0.79%	[59]
Secale cereale	581	–	1	582	86,991	0.67%	[60]
Solanum melongena	231	36	2	269	30,518	0.88%	In this study
Solanum lycopersicum	222	31	2	255	34,074	0.75%	[22]
Triticum aestivum	2148	5	3	2151	107,891	1.99%	[20]

Table 2. Summary of the number of *SmNBSs* in each subgroup.

Subgroup	Predicted Domains	Number (Proportion)
CNL subgroup		231 (85.87%)
CC-NBS (CN)	CC, NBS	111
CC-NBS-LRR (CNL)	CC, NBS, LRR	6
NBS (N_{CC})	NBS	112
NBS-LRR ($N_{CC}L$)	NBS, LRR	2
TNL subgroup		36 (13.38%)
TIR-NBS (TN)	NBS, LRR	24
TIR-NBS-LRR (TNL)	TIR, NBS, LRR	6
NBS (N_{TIR})	NBS	6
RNL subgroup		2 (0.65%)
RPW8-NBS (RN)	RPW8, NBS	2
Total		269

3.2. Gene Characteristics and Structure

The predicted NBS proteins of eggplant varied greatly in length and MV. The protein sequence length of *SmNBSs* ranged from 64 (*EGP31000.1*) to 3636 (*EGP06795.1*) with an average of 704 amino acids, and the MW varied from 7.03 (*EGP31000.1*) to 415.00 (*EGP06795.1*) kDa, and their PI ranged from 4.33 (*EGP31000.1*) to 9.55 (*EGP17686.1*) (Table S2).

A gene structure map was constructed to compare the number and position of exons of *SmNBSs* (Figure S2). The number of exons of the *SmNBSs* ranged from 1 to 14, with a total of 709 exons and an average of 2.64 exons. The average number of exons in the RNLs (5.50) was higher than those in the TNLs (4.44) and CNLs (2.33). RNLs contain two genes with exon numbers of five and six. A few TNLs (13.89%) had less than three exons, while more than half of the CNLs (66.23%) had less than three exons.

3.3. Genomic Distribution on Chromosomes

The chromosomal distribution map shows the specific position of *SmNBSs* on 12 chromosomes (Figure 1). Visually, *SmNBSs* are unevenly located across different chromosomes, mainly concentrated on chromosomes 10, 11, and 12. Among them, chromosome 11 contains the maximum number of *SmNBSs* (65 genes, 24.16%). Conversely, chromosomes 3 and 7 contained the fewest number of *SmNBSs* (6 genes, 2.23%). Furthermore, three genes were identified to be placed on separate scaffolds. The map shows that the majority of *SmNBSs* are situated in the two terminal regions of the chromosomes and usually distributed in clusters.
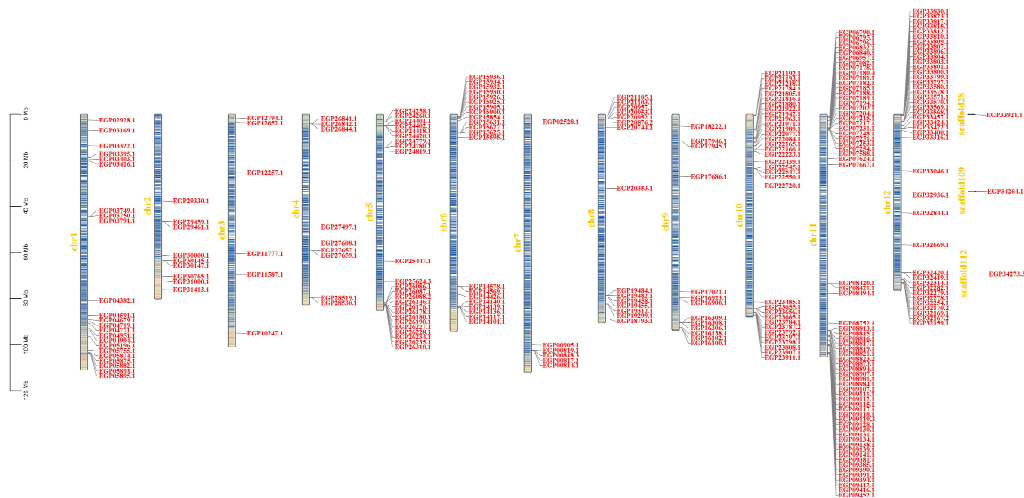
Figure 1. The chromosomal distribution of *SmNBSs*. The scale bar on the left represents the chromosomal distance (120 Mb). The chromosomal number is provided on the left of each chromosome. *SmNBS* gene numbers are indicated on the right of each chromosome.

3.4. Phylogenetic Relationships and Conserved Motifs

We constructed a phylogenetic tree using the ML method to study the evolutionary relationships of *SmNBSs* (Figure 2). The phylogenetic tree is divided into three groups, each with a different color. The CNLs are clearly clustered and separated from the TNLs and RNLs. The CNL group is the largest branch in the phylogenetic tree, containing 231 genes, including three main subgroups, CNL-1 (yellow), CNL-2 (green), and CNL-3 (blue). The TNL group (red) covers 36 genes, including several incomplete genes (N_{TIR}). The RNL group (purple) only has two genes, which are located between the other two groups.
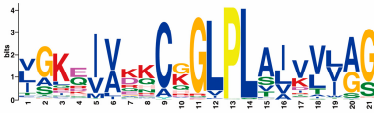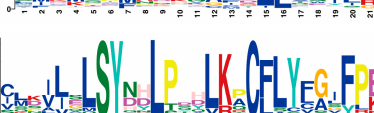

Figure 2. Phylogenetic analysis of *SmNBSs* using the maximum likelihood method. Different subgroups are represented by different colors. The size of the purple circle on the branch indicates the value of the bootstrap.

The NBS domain has been reported to consist of several functionally conserved and strictly ordered motifs [61]. Conserved motif analysis revealed that *SmNBSs* also contain eight conserved motifs (P-loop, GLPL, Kinase-2, RNBS-A, RNBS-B, RNBS-C, RNBS-D, and MHDL), which are consistent with other species such as *Arabidopsis* (Table 3, Figure S3) [14]. The more frequently a motif occurs, the more conserved it is. Among them, P-loop (distributed in 212 *SmNBSs*), GLPL (distributed in 213 *SmNBSs*), and Kinase-2 (distributed in 213 *SmNBSs*) are the most common and conserved motifs. Comparative analysis of *SmNBSs* motifs indicated that the amino acid sequences 'GKTTLA', 'GLPL', and 'DDVW' are highly conserved among P-loop, GLPL, and Kinase-2.

Table 3. Conservative motifs analysis of *SmNBSs*.

Motif	Sequence Tag	Conserved Sequence	Number
P-loop		VISIVGMGGJGKTTLARKVYN	212
GLPL		VGKZIVKKCGGLPLAJVVLAG	213
Kinase-2		LLKGKRYLIVLDDVWDTEA	213
RNBS-A		DERVRSHFDVRAWCTVSQEYBEREJ	173
RNBS-B		NGSRIJLTTRNKEVA	207
RNBS-C		PHELRLLSEEESWELFEKKAF	210
RNBS-D		CLKILSLSYNHLPDHLKPCFLYFGIFPED	188
MHDL		TCKMHDLLRDLCLRKAKEENF	181

3.5. Cis-Acting Regulatory Elements in the Promoters

Cis-acting regulatory elements (CAREs) in plants can interact with various transcription factors involved in gene regulation affecting gene expression [62]. We performed *cis*-acting regulatory element prediction on the upstream 2 kb sequences of the 269 *SmNBSs* to predict the potential biological functions and transcriptional regulation. Common promoter elements (TATA-box and CAAT-box) identified in *Arabidopsis*, cabbage, and bean were also identified in *SmNBSs*, which were the major CAREs in the promoter regions [14,17,18]. In addition, the remaining CAREs involved in various response processes, such as hormone-responsive elements, stress-responsive elements, light-responsive elements, growth and development elements, and others.

Abundant hormone-responsive elements were discovered, including the abscisic acid response element (ABRE), ethylene response element (ERE), and auxin response element (AuxRE). For stress-response elements, a large number of CAREs were distributed throughout the promoter region, such as heat stress-related elements (STRE) and resistance response elements (WRE3). Light-responsive elements mainly consisted of ACE, G-box, I-box, and MRE. Numerous elements associated with plant growth and development were identified, such as CAT-box, HD-Zip, and RY-element. In addition, almost all *SmNBSs* contained *cis*-acting elements of MYB and MYC. These results indicated that *SmNBSs* can play a widespread role in various regulatory processes in plants. Therefore, the analysis and functional annotation of *cis*-acting elements of *SmNBS* family members will facilitate subsequent functional studies.

3.6. Gene Duplication and Synteny Analysis

A circus map was constructed to illustrate the tandem and segmental duplications of *SmNBSs* (Figure 3). The results revealed that 22 tandem duplication gene pairs of *SmNBSs* were observed on seven chromosomes (4, 5, 6, 9, 10, 11, and 12). In addition, both chromosomes 11 and 12 contain six tandem duplication genes pairs, which could be attributed to the abundance of clustered *SmNBSs* on these chromosomes. Nine segmental duplication gene pairs were identified on six chromosomes (2, 3, 4, 10, 11, and 12), with the majority located on chromosomes 10, 11, and 12. The results indicate that tandem duplication events were a major factor contributing to the expansion of *NBS* genes in eggplant. The Ka/Ks is usually used as an informative indicator of natural selection in the evolution process. All tandem and segmental duplicate genes exhibited Ka/Ks values of less than one, indicating that *SmNBSs* evolved under negative selection.

Figure 3. The gene duplication types of *SmNBSs* on 12 chromosomes in eggplant. The red lines are segmental duplication gene pairs, and the blue lines are tandem duplication gene pairs.

Synteny analysis was performed on eggplant, *Arabidopsis,* and tomato to visualize the collinear relationships of *NBS* genes among various plant species (Figure 4). A total of 87 *NBS* gene pairs in eggplant had a synteny relationship with tomato, while only 22 *NBS* gene pairs were syntenic to *Arabidopsis*. Clearly, the number of collinear gene pairs between eggplant and tomato was significantly higher compared to that between eggplant and *Arabidopsis*, thus indicating a strong collinear correlation between eggplant and tomato. The results showed that *SmNBSs* and *SlNBSs* exhibit compatible phylogenetic conservation. In addition, there were nine *NBS* genes with a collinear relationship in eggplant, *Arabidopsis,* and tomato, suggesting that these genes may be the result of retention of ancestral *NBS* genes from a common ancestor.
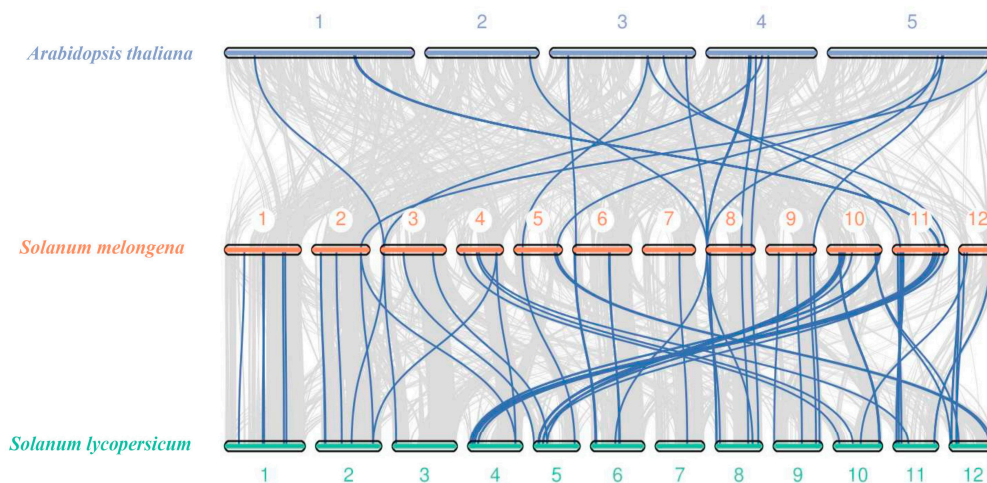

Figure 4. Synteny analysis of *NBS* genes in eggplant, tomato, and *Arabidopsis*. Gray lines show all collinearity relationships within the genomes of eggplant and other species, while blue lines show the collinearity relationships of NBS genes. The numbers represent the corresponding chromosome numbers in eggplant, tomato, and *Arabidopsis*.

3.7. Expression Pattern of SmNBSs under Pathogen Treatment

Based on published transcriptome data on pathogen infections, the expression pattern of *SmNBSs* was analyzed. In this study, the RNA-seq data comprised the transcriptome data of eggplant roots infected with *R. solanacearum* at different stress time points (0, 24, and 48 h post-inoculation (hpi)). Heatmaps were constructed using transcripts per million (TPM) values (Figure 5A, Table S3). After *R. solanacearum* infection, the expression levels of 13 DEGs were significantly increased or decreased, which could be divided into two categories: up-regulated genes and down-regulated genes (Figure 5B). For example, the expression pattern of one gene was up-regulated at 24 and 48 hpi, and six genes were down-regulated at 24 and 48 hpi. However, six genes were up-regulated at 24 hpi, but down-regulated at 48 hpi.

3.8. Responses to R. solanacearum Pathogen Infection

The DEGs and highly expressed *SmNBSs* were selected, and the expression levels of these genes after *R. solanacearum* infection were validated using qRT-PCR. Nine *SmNBSs* were generally up-regulated in the resistant variety R76 and susceptible variety S91 (24 hpi or 48 hpi), indicating their potential specific roles in disease resistance. In R76, the expression levels of seven genes were mainly up-regulated at 24 hpi and down-regulated at 48 hpi. In contrast, the expression levels of six genes were mainly significantly down-regulated at 24 hpi and then up-regulated at 48 hpi in S91. There were two genes whose expressions were constantly up-regulated in R76.Moreover, *EGP05874.1* was dramatically increased in R76 compared with S91 at 24 hpi, indicating that this gene may play a positive regulatory role in the resistance response of eggplant plants to the *R. solanacearum* pathogen (Figure 6).
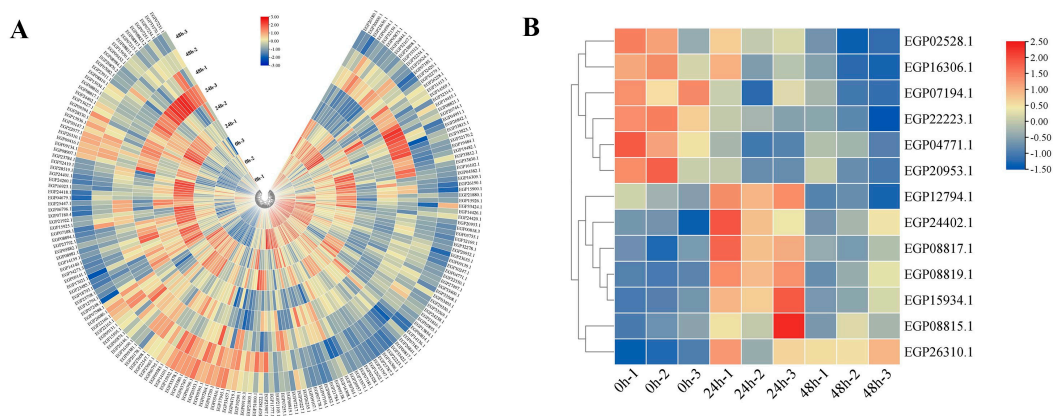
Figure 5. Expressions heatmap of *SmNBSs* under *R. solanacearum* pathogen infection. (**A**) Expression heatmap of the total 186 *SmNBSs*. (**B**) Expression heatmap of 13 DEGs. Higher expression for each gene is represented in red; otherwise, blue is used.
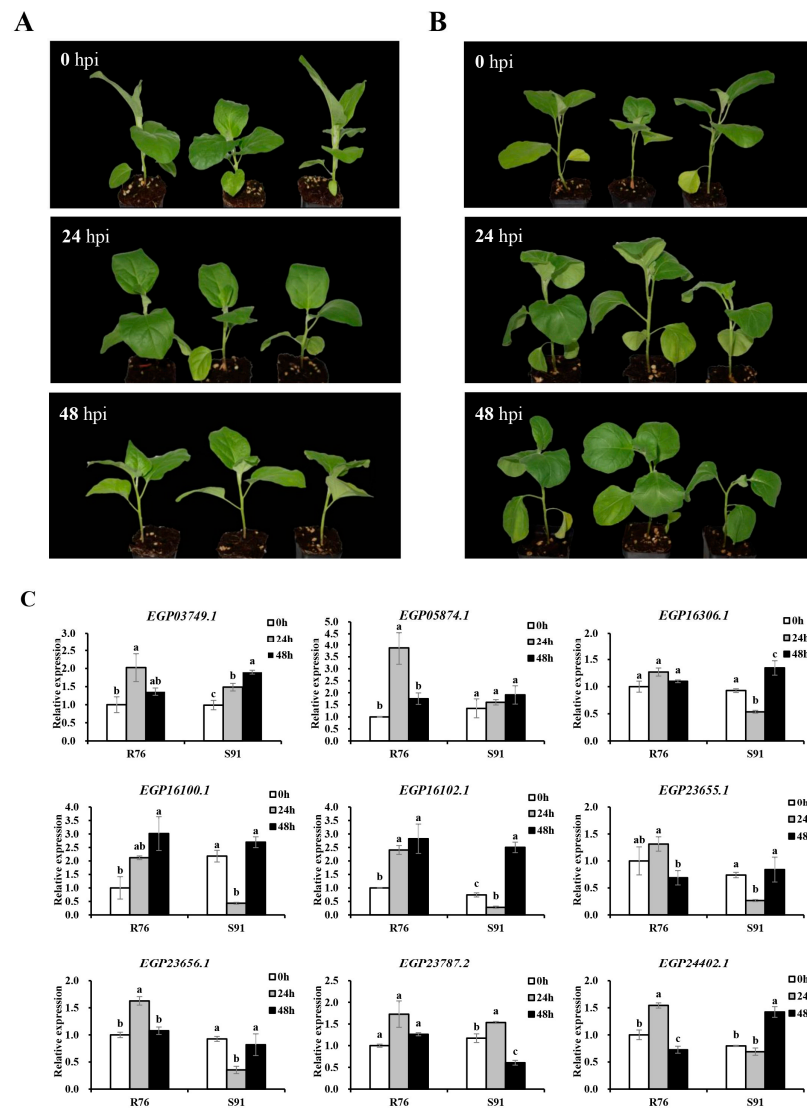

Figure 6. Phenotypes and gene expressions of R76 and S91 in response to *R. solanacearum* pathogen infection at different time points: 0, 24, and 48 hpi. (**A**) Phenotype of R76 in response to *R. solanacearum*

pathogen infection. (**B**) Phenotype of S91 in response to *R. solanacearum* pathogen infection; (**C**) The expression profiles of nine *SmNBSs* in R76 and S91 with *R. solanacearum* pathogen infection. Statistically significant differences are indicated by different lowercase letters using the multiple polarity test for variance (ANOVA) method ($p \leq 0.05$).

4. Discussion

NBS genes are the largest class in *R* genes and play crucial roles in plant pathogen defense [3,4]. The identification of the *NBS* genes not only contributes to our understanding of the structure and evolution of *NBS* genes in diverse plant species, but also it is of great significance in identifying and utilizing the functional *R* genes [57]. Currently, *NBS* genes have been identified and analyzed in a variety of species [17,18,21]. However, a comprehensive analysis of the *NBS* genes based on the chromosome-based eggplant genome has not been reported. As one of the important vegetable crops, it is of great significance to use bioinformatics analysis to explore the *NBS* genes in eggplant. In this study, we performed a comprehensive analysis of *NBS* genes based on eggplant whole-genome information.

Previous studies have reported significant variation in the number of *NBS* genes resulting from gene gain/loss events among plant species, even among closely related species [22,63]. Among *Solanaceae* species, this study has identified a total of 269 *SmNBSs* in eggplant, while 255, 306, and 447 genes were discovered in tomato, pepper, and potato, respectively [22]. According to domain classification and phylogenetic analysis, *SmNBSs* were categorized into three subgroups 231 CNLs (CC-NBS-LRR), 36 TNLs (TIR-NBS-LRR), and 2 RNLs (RPW8-NBS-LRR), which showed that the number of CNLs were significantly higher than the other subgroups, accounting for 85.87% of *SmNBSs*, which was consistent with the results of most angiosperms. The results may be caused by the earlier expansion of CNLs than TNLs during the evolution of angiosperms [7]. In addition, most functional *NBS* genes belong to CNLs, probably because it confers resistance to various pathogens, including fungi, bacteria, and viruses [28,64]. The discrepancy in the number of subgroups clearly indicates that *NBS* subgroups have undergone different evolutionary events, such as different types of genome duplications and varying degrees of natural selection [56].

The diversity of genetic structure could be one of the mechanisms that promote the evolution of multigene families [65]. The genetic structure analysis of *SmNBSs* revealed that the gain or loss of exons occurred in different *NBS* subgroups, and TNLs comprise a higher number of exons compared to CNLs, which is similar to other species [17,18,56]. The results suggest that the higher number of exons in the TNLs may be attributed to their earlier evolutionary history compared with CNLs. The chromosomal distribution map showed that *SmNBSs* were unevenly distributed across all the chromosomes (52.04% on chromosomes 10, 11, and 12) and were mainly clustered in the two terminal regions of those chromosomes, a pattern observed in Brassicaceae and legume plants [27,66]. This phenomenon may be explained by the fact that genes located in the terminal regions of chromosomes are more susceptible to mutations during the course of evolution [67,68].

The NBS domains consisted of functionally conserved and strictly ordered motifs (P-loop, GLPL, Kinase-2, RNBS-A, RNBS-B, RNBS-C, RNBS-D, and MHDL) [14]. Conserved motif analysis revealed that the *SmNBSs* also contained eight common conserved motifs, with the P-loop, GLGL, and Kinase-2 being the most common and conserved. Both the P-loop and Kinase-2 motifs play a role in ATP/GTP binding, and their presence in R proteins is critical for protein function [14,69]. The amino acid sequences of the P-loop, Kinase-2, and GLPL in the NBS domain of eggplant were conserved. Furthermore, the strict order of these motifs indicates that conserved order is just as important as conserved sequence.

Gene duplication plays a key role in the evolution of plant gene families, and segmental and tandem duplications are considered as the two primary evolutionary patterns [70]. Studies have shown that segmental and tandem duplication events may be the primary drivers of *NBS* gene amplification [22]. To be more specific, a total of twenty-two tandem duplication gene pairs and nine segmental duplication gene pairs were identified and

predominately distributed on chromosomes 10, 11, and 12. Tandem duplicate genes commonly exist in the form of clusters, which is the primary factor contributing to the formation of *SmNBSs* clusters on the three chromosomes. Similar to other species, the expansion of *SmNBSs* was mainly achieved through tandem duplication events, which play a key role in the evolution of *NBS* genes [26,27]. The Ka/Ks values in *SmNBS* gene pairs were all less than one, suggesting that *SmNBSs* mainly experienced purifying selection (i.e., removal of harmful mutations and the maintenance of protein conservation) during the evolution process. Gene pairs within chromosome syntenic blocks represent highly conserved orthologous genes among species [60]. The synteny analysis of *NBS* genes in different plant species (eggplant, *Arabidopsis*, and tomato) revealed that *SmNBSs* exhibit strong homology with *SlNBSs* in tomato while displaying low homology with *AtNBSs* in *Arabidopsis*. The results implied that *NBS* genes in eggplant and tomato may have undergone similar evolutionary patterns and share some duplication events. Furthermore, nine gene pairs were identified in eggplant, *Arabidopsis*, and tomato, indicating that these genes play a crucial role in the expansion and evolution of the eggplant *NBS* genes.

The expression patterns of *SmNBSs* were analyzed to study their transcriptional response to pathogen infection. The expression levels of 13 *SmNBSs* were significantly changed after *R. solanacearum* infection, indicating their response to pathogen infection. To further investigate the potential resistance mechanism of *SmNBSs*, RNA was extracted at different time points after pathogen treatment and analyzed using qRT-PCR. The results demonstrated that nine up-regulated genes are probably involved in the resistance response. These nine genes could be divided into two types based on their expression trends, of which one type was up-regulated at 24 hpi and down-regulated at 48 hpi, and the other type was constantly up-regulated at both 24 and 48 hpi. Seven genes were significantly up-regulated at 24 hpi followed by down-regulation at 48 hpi in R76, but they were significantly down-regulated at 24 hpi in S91, which indicated these genes could likely respond to the disease at the initial stage of inoculation. There were two genes whose expressions were constantly up-regulated, suggesting that they may be involved in more/longer stages (not only 48 hpi) of the disease response. Additionally, *EGP05874.1* showed a positive regulatory effect and may be the main gene involved in the response to *R. solanacearum.* Overall, the results showed these genes responded more quickly to *R. solanacearum* in R76 compared with S91.

5. Conclusions

In this study, 269 *SmNBS* genes were identified in the eggplant genome and categorized into three subgroups (231 CNLs, 36 TNLs, and 2 RNLs) based on domain classification and phylogenetic analysis. *SmNBSs* were unevenly located on all chromosomes with a majority (52.04%) located on chromosomes 10, 11, and 12 in the form of clusters. Eight conserved motifs were identified in *SmNBSs*, and their amino acid sequences and orders were highly conserved. Tandem duplication was the main driver of *NBS* gene amplification. qRT-PCR analysis showed that *SmNBSs* responded to pathogen stresses, and *EGP05874.1* may be involved in the resistance response. Most importantly, this study provides a comprehensive insight into *SmNBS* genes in eggplant and lays a significant foundation for further studies on the functional characteristics of *SmNBSs*.

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