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Phylogeny, Expression Profiling, and Coexpression Networks Reveals the Critical Roles of Nucleotide-BindingLeucine-Rich Repeats on *Valsa* Canker Resistance

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Abstract: Rosaceae is one of the major families in the plant kingdom with important economic value. However, many of them are attacked by *Valsa* canker, resulting in serious loss of production and profits. Nucleotide-binding leucine-rich repeats (NLRs) play a key role in the plant immune response as the largest class of resistance genes. Currently, we performed a genome-wide identification of NLR genes in Rosaceae and revealed some NLR genes in response to *Valsa* canker using multispecies bioinformatics including co-expression network analysis and RNASeq data. A total of 3718 NLR genes were identified from genomes of 19 plant species (include 9 Rosaceae plants) and classified them into 15 clades. The NLRs display species- and group-specific expansions that are derived from both the whole genome duplication and the tandem duplication. Additionally, the expression of some NLR members was low under normal growth conditions in various plant tissues, while significantly enhanced after the infection of *Valsa* canker. Furthermore, co-expression network analysis shows that the 13 NLR members were distributed in key nodes of differentially expressed genes which could be considered as promosing key regulators for the resistance of *Valsa* canker. Therefore, our findings provide a reference for the evolution of NLR genes in Rosaceae and the key regulators of *Valsa* canker resistance.

Keywords: genome-wide analysis; rosaceae; expansion rate; gene duplication; gene expression

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1. Introduction

Plants have evolved complex and powerful immune mechanisms in the long-term to struggle against external threats. The receptors located on the cell membrane sense signals from external stimuli, and thus trigger the first layer of immunity, known as pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI) [1]. After that, the pathogenic bacteria employ effectors to inhibit the PTI responses, but the signals could be recongnized by resistance (R) proteins and activate the second layer of immunity, which is called effector-triggered immunity (ETI) [2]. Up to now, most of the characterized R genes are members in nucleotide-binding leucine-rich repeat (NLR) superfamily. In normal conditions, PTI is considered basic immunity and displays broad-spectrum resistance, whereas ETI derives from specific recognition of NLRs to effectors and exhibits species- or race-specific resistance [3]. Recent investigations revealed that several pattern recognition receptors (PRRs) complexes are essential for the occurrence of ETI, whereas

activation of Toll/interleukin-1 receptor (TIR) signaling is also required for PTI, indicating an interdependent relationship between PTI and ETI [4,5].

A typical NBS-LRR (Nucleotide-binding leucine-rich repeats) protein contains an N-terminal variable structural domain, a central NB-ARC (Nucleotide-Binding adaptor shared by Apaf1, certain R genes and CED4) domain, and a C-terminal Leucine-rich repeat (LRR) domain. The NB-ARC domain is mainly responsible for the binding and hydrolyzing ATP and GTP against pathogenic attack, whereas the LRR domain is responsible for the recognition of the effectors produced by pathogens [6]. Based on the type of N-terminal binding domain, NBS-LRRs could be classified into two categories, TIR-NBS-LRR (TNL) containing TOLL/interleukin-1 receptor (TIR), and CC-NBS-LRR (CNL) carrying coiled-coil (CC) motifs. Furthermore, CNLs could be further divided into CC-type NLRs (CNLs) and RPW8-type CC-NLRs (RNLs) according to the feature of the N-terminal RPW8 domain [7].

In general, TNLs and CNLs are capable of sensing specific pathogenic effectors, while RNLs are helpers in activating downstream defense signal transduction [8]. The flax (*Linum usitatissimum*) L locus encodes three TNL genes, which sense different variants of the flax fenugreek (*Melampsora lini*) effector protein AvrL [9]. In rice, *Pikm*, *Pikp*, and other NLR *Pik* genes recognize multiple AVR-Pik effector proteins from *Pseudomonas aeruginosa* and mediate host immunity against rice blast [10]. *Arabidopsis* RPM1-interacting protein 4 (RIN4), a receptor for multiple effector proteins, takes a vital role in ETI activation. The interaction of RIN4 with other R proteins, such as resistance to *P. syringae* 2 (RPS2) and resistance to *P. syringae* pv. *maculicola* 1 (RPM1), is also essential for effector perception of these R proteins [11]. In addition to recognition, some NLRs are also important for immune activation. For instance, RNL-helper genes are required for the activation of disease resistance 1 (ADR1) and N-requirement gene 1 (NRG1) during immunity process and cell death [12–15].

Compared with CNLs, the phylogeny of TNLs has a lower topological complexity and shorter branch length. During the early-stage evolution of angiosperm, the long-term contraction of TNLs and the gradual expansion of CNLs probably contribute to the above differences. The outbreak of fungus has enhanced the selection pressure on plant R genes, thereby accelerating the expansion of NBS genes before the differentiation in angiosperm families. That also is one of the major reasons for the different evolutional patterns of TNLs and CNLs [16]. RNLs have long been considered as a special lineage of CNLs, but have recently been differentiated classified due to their specific functions and unique domain composition. RNLs were conserved during the evolution of angiosperms and played a crucial role in pathogen detection or signal transduction [17].

Rosaceae family includes multiple fruit crops and ornamental plants with high economic value. In Rosaceae, apple (*Malus x domestica*), pear (*Pyrus bretschneideri* or *P. communis*), peach (*Prunus persica*), strawberry (*Fragaria vesca*), and apricot (*Prunus armeniaca*) are popular fruit crops worldwide [18]. Along with the development of the fruit industry, most fruit crops are exposed to attack by various pathogenic bacteria and fungi [19]. For instance, necrotrophic fungus belonging to *Valsa* species are pathogenic to apples and pears, and leads to *Valsa* canker, which is a destructive disease [20]. The pathogens invade the plants through the wounds caused by sunburn, frostbite, pruning, or other mechanical damage and result in the rot of phloem [21]. Little practices for effective prevention and control of the disease have been carried out, although some agricultural, biological, and chemical methods were extensively used in fields [22–24]. Breeding new varieties with tolerant resistance is high efficient, environment-friendly, and low input costs which has been becoming a widely accepted approach to ensure the healthy development of crop industry [25]. At present, the identification of resistance genes is valuable for accelerating the progress of breeding and carrying out comprehensive prevention and control measures.

In the present study, we performed a genome-wide investigation of NLRs in Rosaceae. Combined with bioinformatic analysis and expressional assay, the key members regulating *Valsa* canker resistance were screened in *Pyrus betulifolia*, a highly resistant germplasm.

2. Materials and Methods

2.1. Genetic Identification of NLRs of 19 Species

Genomic data of 19 species were downloaded from public genome databases (Supplementary Table S1). For the identification of NLRs, the NLR conserved domain NBS (Pfam: PF00931) of the HMM (Hidden Markov Model) was obtained from the Pfam database (http://pfam.sanger.ac.uk/, (accessed on 10 January 2022)). A genome-wide HMM model scan of 19 species was performed by using components hmmsearch in the HMMER v.3.3.2 (E-value < 1×10^{-10}), we used the default parameters of Steuernagel et al. [26]. For members with NBS structural domains, we further annotated other structural domains contained using hmmerscan. Members containing both NBS and LRR domains were identified as NLR candidates and manually validated in the Pfam databases and Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/, (accessed on 10 January 2022)). For classification, NLRs were classified into TNL, CNL, and NLR based on the type of their N-terminal structural domains. The TIR structural domains were judged based on the annotation results from the Pfam database. For the identification of coil-coiled motifs in these proteins, we used the PAIRCOIL2 program with a *P* score threshold of 0.025 [27,28].

2.2. Phylogenetic and Expansion Rate Analysis

In the process of constructing phylogenetic tree, we found that NLR was not suitable for constructing multi-species phylogenetic tree, and the tree of full-length amino acid sequence showed good guidance support. Therefore, we used Mafft v7.505 to perform multi-sequence alignment on the full-length amino acid sequences of NLR of 19 species. [29]. A phylogenetic tree was constructed using FastTree 2.1.11 with the maximum likelihood method, and estimated the genetic distances using the JTT model (1000 bootstrap replicates by default) [30]. The display, manipulation, and annotation of phylogenetic trees were performed using the Interactive Tree of Life (iTOL, http://itol.embl.de/, (accessed on 10 January 2022)). The subfamily expansion rate was defined as the number of all genes in a subfamily divided by the number of ancestral genes [31]. We selected two methods to estimate the number of ancestral genes based on whether this subgroup contains homologous group genes of 19 species. At the first method, the number of genetic ancestors in each subfamily can be counted only if at least one subfamily member exists in all comparative species. Considering that a subfamily will be missing in a species, we adopt the second method. There is an ancestral gene in the common ancestor, and this is because the loss of genes lead to the loss of subfamily members of some species, and the number of genetic ancestors of each subfamily are also calculated. [32] OrthoFinder v2.5.4 is able to identify orthologous groups formed during the evolutionary process between species by means of inter-sequence comparisons and tree building, thus inferring the number of ancestral genes [33].

2.3. Gene Duplication Events Analysis

Whole genome duplication and tandem duplication events were determined as previously described [34,35]. For gene duplication event analysis, we first performed an intra-species multiple sequence alignment of all protein sequences of 19 species using BLASTP (e-value < 1×10^{-5}, top five matches). Then, MCScanX was used to generate orthologous gene pairs of NLR genes within each species [36]. Whole genome duplication (WGD) and tandem duplication (TD) events were also determined using the MCScanX package [37].

2.4. Expression Analysis of NLR Gene in Response to Vp Metabolites

To determine the expression of NLRs in various tissues, the RNA-seq datas of NLRs in the apple (Accession: PRJNA183725) were used. For the expressional patterns of NLRs in response to *Valsa pyri* (*Vp*) signaling, raw RNA-seq data of 'Duli-G03' (Resistant varieties, *Pyrus betulifolia*) and 'Yuluxiang' (Susceptible varieties, *P. bretschneideri*) suspension cells treated with *Vp* metabolites (*Vp*M) at 20% concentration for 0, 1, 3, and 6 h were downloaded

from the NCBI website (https://www.ncbi.nlm.nih.gov/, (accessed on 10 January 2022), Accession: PRJNA745477) [38]. For each sample, three biological and technical replicates were included. STAR algorithm was used to map clean reads to the genome of *P. betulifolia* v1.0 and *P. bretschneideri* v1.0 [31]. Differentially expressed genes (DEGs) were identified by Deseq2, with a cutoff threshold $|\log_2\text{Fold-Change}| \geq 2$ and a p-value < 0.05 [39].

2.5. Co-Expression Network Analysis

Two previous 'Duli-G03'RNAseq datas (accession numbers PRJNA745477 and PRJNA829646) were used for co-expression network analysis. [40]. Among them, the samples in the PRJNA829646 project were processed directly using *Vp*. Specifically, suspension cells of 'Duli-G03'cultured under the same conditions were spread on uniformly growing *Vp* and sampled at 0, 1, 3, and 6 h, respectively. The modules with highly similar expression patterns were identified by using the R package WGCNA (https://CRAN.R-project.org/package=WGCNA, (accessed on 10 January 2022)). After fitting the parameters (soft threshold, 14; minimum module size, 30; merge cut height, 0.20), the modules were generated statistically and equitably using a one-step automatic construction method and distinguished by color codes [41]. All NLR genes with weights >0.5 were screened out for the construction of the network graph. Weight: Weighting and correlation of linkage between genes. Co-expression network relationship graphs were constructed using the software Cytoscape 3.8.0 [42].

2.6. Quantitative Real-Time-PCR Assays

Total RNA was extracted from wild-type 'Duli-G03' suspension cells treated with *Vp*M using a column RNA extraction kit (160906-50, Tiandz, Beijing, China). cDNA was produced using the PrimeScript TM RT reagent Kit (TAKARA, CO., LTD., Dalian, China). qRT-PCR using SYBR Green Pro Taq HS pre-mixed qPCR Kit (ACCURATE BIOLOGY, CO., LTD., Changsha, China). The cycling program was as follows: polymerase activation at 95 °C for 30 s; and 40 cycles of PCR at 95 °C for 5 s and 60 °C for 30 s. The primers were designed on the online software primer3 input (http://bioinfo.ut.ee/primer3-0.4.0/, (accessed on 10 January 2022)) and the sequences of all primer pairs were listed in Supplementary Table S2. For each treatment, three technical repetitions are included. The mean fold was calculated using the $2^{-\Delta\Delta CT}$ method [43].

2.7. Statistical Analysis

Microsoft Excel 2010 was used for the statistical analysis. ANOVA and Tukey HSD tests were performed in R 4.2.1. The significance of the differences between means was statistically analyzed using t-test (* $p < 0.05$; ** $p < 0.01$).

3. Results

3.1. Genome-Wide Identification and Domain Composition of NBS-Encoding Genes

A total of 6330 NBS-encoding genes were identified from 19 species, including nine Rosaceae plants and several typical species in monocot, dicot, a bryophyte and fern (Supplementary Table S3). NBS-encoding members varied from 17 (*Selaginella moellendorffii*, (Smo) to 664 (*Populus trichocarpa*, Ptr) among the analyzed plant species, and from 151 (*Rubus occidentalis*, Roc) to 664 (*Rosa chinensis*, Rch) in Rosaceae plants. According to the division of domain constitution, the NLR genes could be divided into six subfamilies, NBS (consist of NB-ARC domain), NL (NBS-LRR), TNL (TIR-NBS-LRR), CNL (CC-NBS-LRR), CN (CC-NBS) and TN (TIR-NBS) (Figure 1). The subfamily members were largely varied among plant species and subfamilies. Compared with other subfamilies, NBS, NL, and TNL possess a large size of members. The TNL and TN subfamilies were absent in most monocots, whereas members of all six subfamilies were discovered in all detected dicots. In addition, member sizes of all six NLR subfamilies were obviously varied among Rosaceae species. For instance, only two TN genes were discovered in Roc, whereas 67 members in Pbr [44]. Furthermore, we also discovered distinct differences in NLR subfamily member

size in the same species. In the *Malus* species, Mde and Mba possess 121 and 85 NBS genes, respectively. The above results indicate that member sizes of NLR family and subfamilies were frequently changed among plant species.

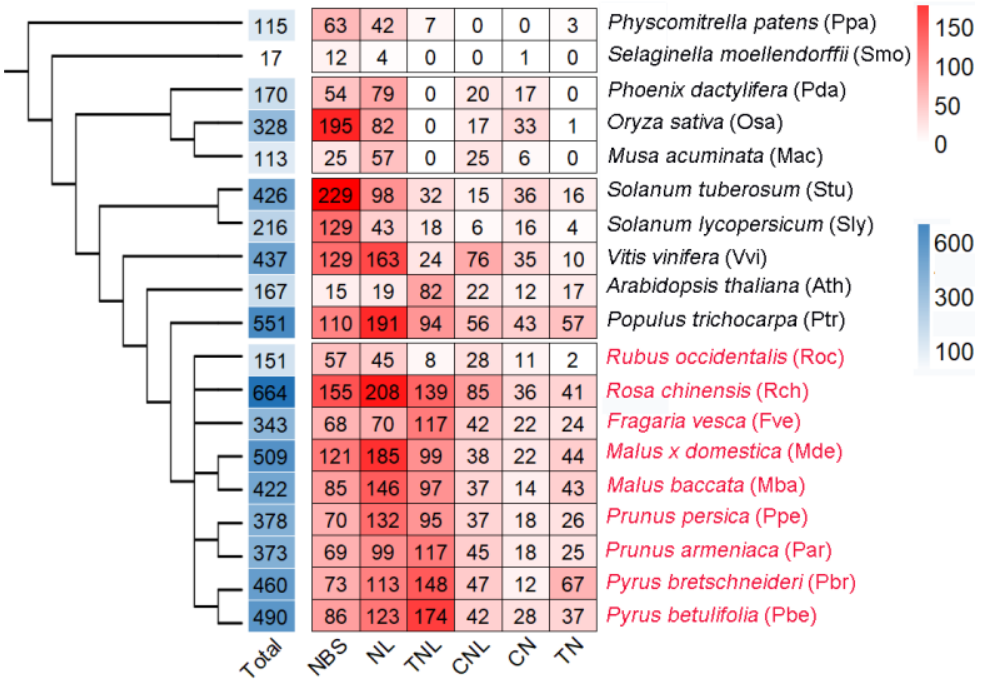

Figure 1. The number of Nucleotide-binding leucine-rich repeat (NLR) members varied between subfamilies and plant species.

3.2. Classification of NLRs Based on Phylogenetic Tree Construction

In normal conditions, typical resistance genes are distributed in NLRs which containing LRR domains [45]. We therefore selected NL, TNL, and CNL subfamilies for further analysis. Considering the numerous members in most NLR subfamilies, we further classified NLRs based on construction of the phylogenetic tree using the amino acid sequences (Supplementary Figure S1). Subsequently, we obtained a phylogenetic tree with high bootstrap support, which could be used for classification. Finally, 15 groups (phylogenetic branches), containing at least 12 NLRs, were determined as GroupI to XV (Figure 2A). For the other groups, each containing only 11 or fewer members and were integrated into GroupNX. Most groups contain two or more NLR subfamilies. Among these, GroupI, VIII and XV include NLR members from all three subfamilies, and 11 groups contain two subfamilies (Figure 2B,C). Some NLR groups include only NLR genes from several specific species. Two Groups (II and VI) contain only the members from monocots, but seven groups (IV, V, XI, XII, XIII, XIV, and XV) are existed in dicots. Four groups (I, III, VII, and VIII) include the members from both monocots and dicots, whereas GroupX was only existed in *Physcomitrella patens* (Ppa). NLR genes from Rosaceae plants are mainly distributed in 10 groups (GroupI, III, IV, V, VII, VIII, XII, XIII, XIV, and XV). The member sizes of the most groups changed evidently among species. For example, GroupXIII includes 23 NLRs from Pbe, but only nine from Pbr. These results demonstrated that the member size of NLR groups varies frequently among distinct plant species.

3.3. Expansion of NLRs among Species and Groups

To gain a more comprehensive acknowledgment of evolutional characteristics, we detected the expansion rates of NLRs in each group from all 19 species (Figure 3, Supplementary Table S4). The global expansion rates varied from 0 to 4.07 among species, and from 0.32 to 6.68 among groups. GroupII and VI largely expanded only in monocots, GroupI, IV, VIII, IX, XII, XIII, XIV, and XV expanded only in dicots, whereas GroupIII, V,

and VII expanded in both monocots and dicots. Many groups exhibit a typical species-specific expansion. For instance, GroupXII was enlarged in Ptr, Mde, Mba, Pbe, and Pbr, while GroupXV was expanded specifically in Ath. In Rosaceae, the expansion rates of most groups are largely changed in different species. The value of GroupV was recorded as 19.33 in Mde, whereas 0.33 in Par and Pbr. Above all, the diversity of global and detailed expansion rates implies that NLR genes were specifically expanded among species and groups.

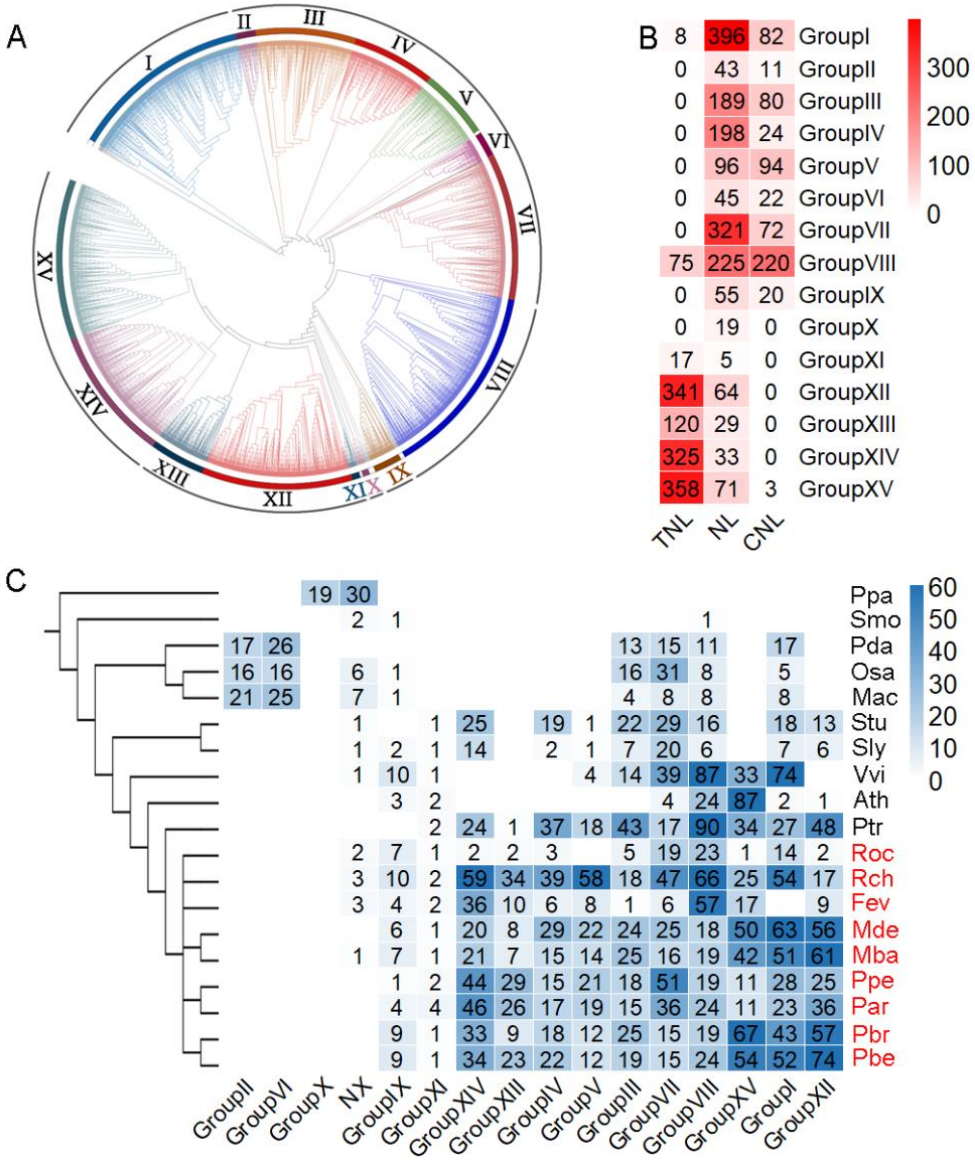

Figure 2. The classification of NLRs. (**A**) A phylogenetic tree was constructed using the full-length amino acid sequences of 3718 NLRs from 19 species. (**B**) The member size of NLR groups in each subfamily. (**C**) The exact member size of NLR groups in each species.

3.4. TD Events Are More Important for the Rapid Expansion of NLRs

To better explore the source resulting in rapid expansion, the gene duplication events were further investigated. A total of 1031 duplicate gene pairs were found in 19 species, including 374 gene pairs involving WGD and 657 gene pairs involving TD. The number of WGD and TD gene pairs changed frequently among plant species or NLR groups (Figure 4A,B). In Rosaceae, a large number of TD gene pairs were distributed in GroupI, VII, VIII, XII, XIV, and XV, whereas WGD was distributed in Group I, V, XII, XIII, XIV, and XV

(Figure 4C,D). These results demonstrated that TD events were more important than the WGD for the rapid expansion of NLRs in most species or groups, although some expansion were caused by both TD and WGD events.

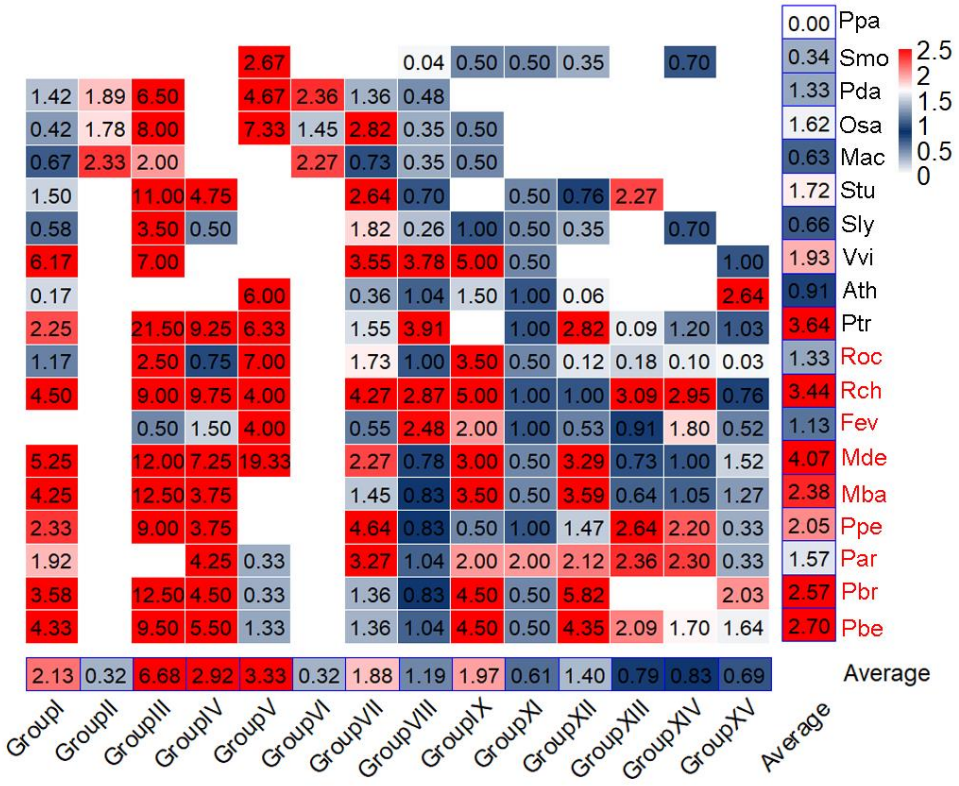

Figure 3. Global and exact expansion rates for each subfamily from 19 species. The value of each group was calculated by the member size divided by the number of ancestral genes.

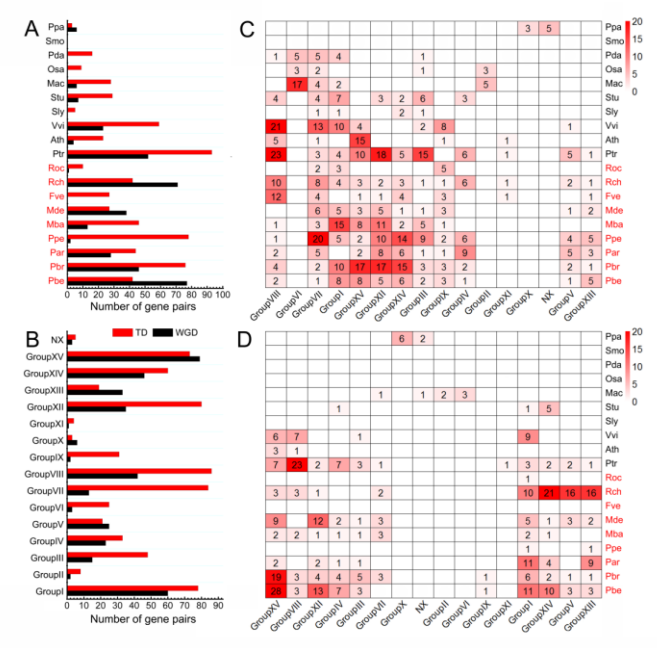

Figure 4. The total and exact duplication events of NLR family in 19 species. (**A**) and (**B**) Duplicated gene pairs in each species (**A**) and group (**B**), respectively. (**C**,**D**) Exact WGD (**C**) and TD (**D**) events in each NLR group from all 19 species.

3.5. NLRs Are Low Expressed in Various Tissues under Normal Growth Conditions

To investigate the tissue-specific expressions of NLRs, we extracted the expressional data of NLRs in apple from previously published transcriptomic data (PRJNA183725) [46]. These included expressional patterns in seven tissue types, leaf, root, fruit flesh, apex, stem, whole seedling, and flower full bloom (Figure 5, Supplementary Table S5). The FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) mean value of all genes was recorded as 15.49, but the mean value of NLRs was only 2.38. Furthermore, the mean values of NLR groups varied from 1.48 to 3.60. Among these, GroupXII exhibited higher expression levels than other groups. In addition, GroupXII and GroupXV displayed higher expression levels in the root. We also confirmed the low expression of NLRs in suspension cells of 'Yuluxiang' (Pbr) and 'Duli-G03' (Pbe) calli during normal growth conditions (Supplement Table S6). These results probably indicate that NLRs were rarely involved in the growth and development of apple.

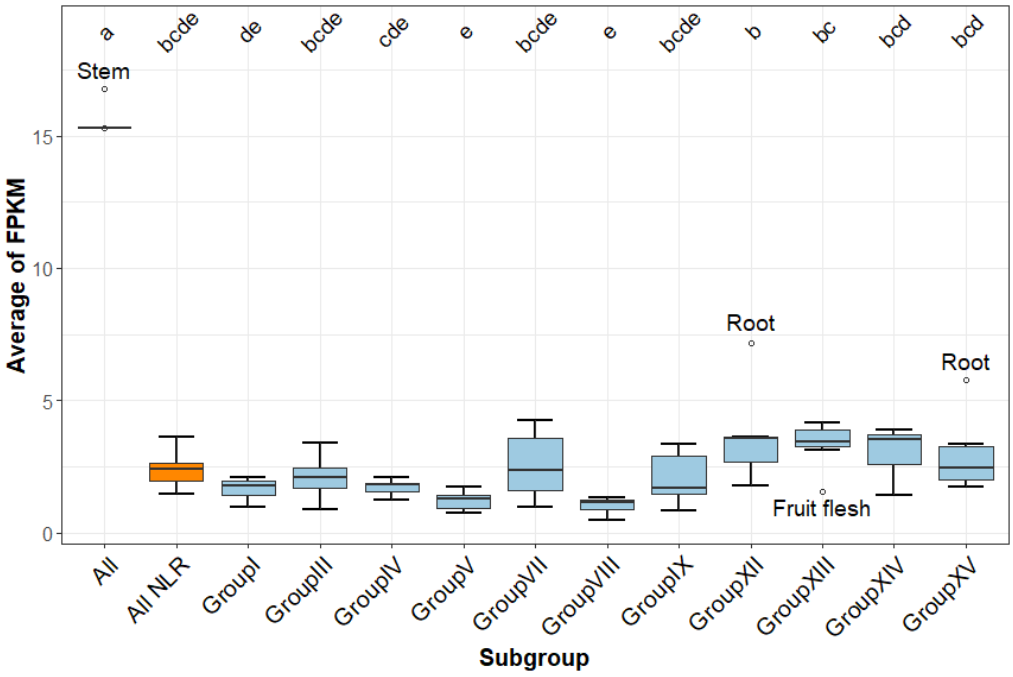

Figure 5. The NLRs were little expressed in various tissue types under normal growth condition. The mean FPKM (Fragments Per Kilobase of exon model per Million mapped fragments) value of each group. An ANOVA showed significant differences of the mean FPKM values among the tissues. The TukeyHSD test was used to determine differences between groups and marked by level of significance (a–e).

3.6. Large Numbers of NLRs Respond to Signals of Valsa Canker

To select the NLRs in response to signals of *Valsa* canker, the expression datas were extracted from transcriptomic data of 'Yuluxiang' (Pbr, susceptible to *Valsa* canker) and 'Duli-G03' (Pbe, resistant to *Valsa* canker) calli cells treated or untreated with *Vp*M. Subsequently, a total of 127 differentially expressed NLRs were determined, including 68 genes from 'Duli-G03' and 59 from 'Yuluxiang'. Based on the phylogenetic analysis and expressional patterns, these genes could be divided into 50 gene subgroups. For the seven subgroups with the tree branches colored red, the members were expressed distinctly in 'Duli-G03' and 'Yuluxiang'. For instance, in the first branch of GroupI, all members are up-regulated in 'Duli-G03', but down-regulated in 'Yuluxiang'. Among them, 25 subgroups branches were labeled pink, including only DEGs from 'Duli-G03' or 'Yuluxiang'. Besides, 18 subgroups, the branches colored blue, exhibited similar expressional patterns between 'Duli-G03' and 'Yuluxiang' (Figure 6). The expressional patterns of seven genes were confirmed by qRT-PCR assay, indicating the reliability of RNA-seq data (Supplementary

Figure S2). These results displaied that a large number of NLRs respond to the signals caused by *Valsa* canker.

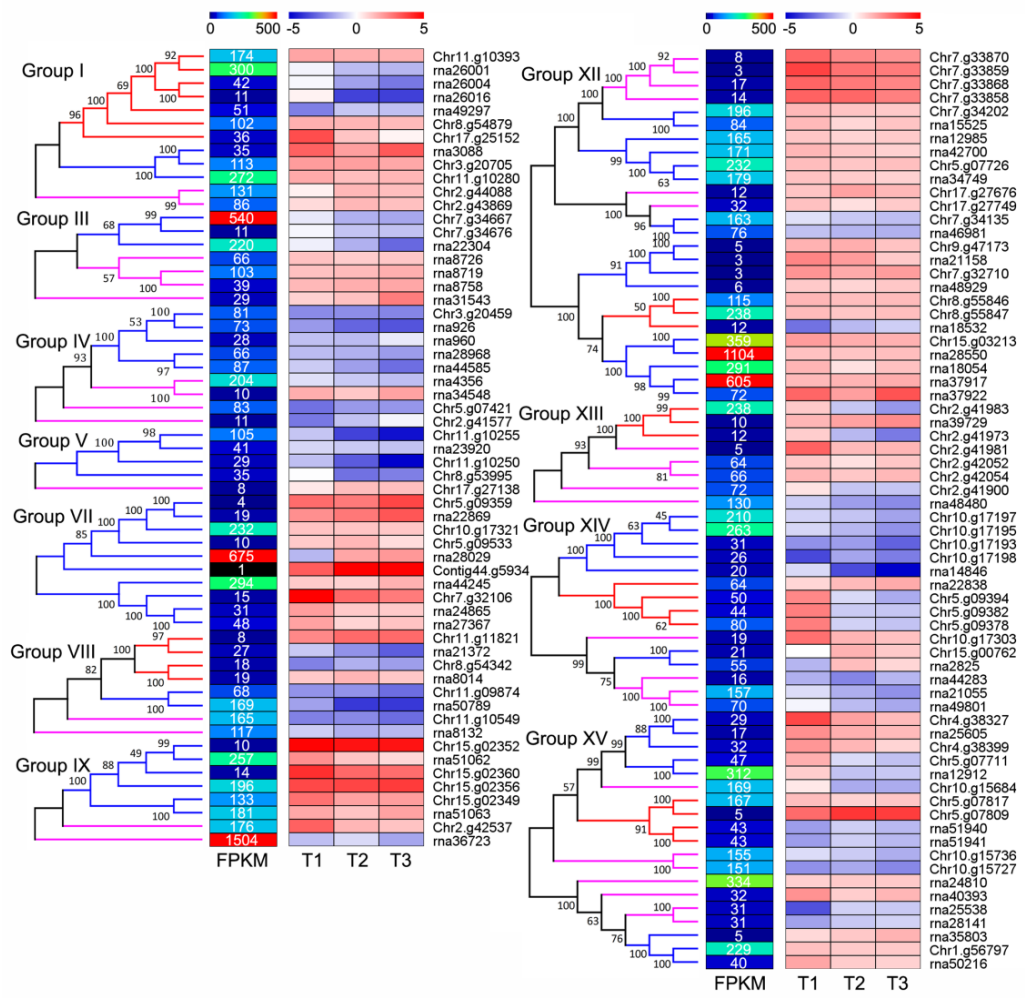

Figure 6. The amount of NLR was differentially expressed during 'Yuluxiang' (*P. bretschneideri*, susceptible pear variety) and 'Duli−G03' (*P. betulifolia*, resistant pear rootstock) respond to signals from *Valsa pyri* (*Vp*). The trees were contructed based on amoino acid sequences.

3.7. NLRs Take a Crucial Role in 'Duli-G03' Response to Vm Signals

We screened a total of 18,931 DEGs from two 'Duli-G03' RNA-seq datas under *Vp* or *Vp*M treatment and performed co-expression network analysis. All related genes were screened according to a reliability threshold (weight > 0.5) and annotated for NLR genes among them. Further, we constructed a co-expression network for NLR genes. Notably, 13 genes are distributed on the key nodes of the network (Figure 7). Among these, four members are located at the most central position and are likely to play an important role in the regulation of the *Valsa* canker, including two genes from GroupVII (*Chr3.g19943* and *Chr7.g32106*), one gene from GroupIX (*Chr2.g42537*) and one from GroupXII (*Chr5.g07640*). Besides, we also discovered nine genes from other NLR groups, including GroupI (*Chr7.g34568* and *Chr7.g34388*), GroupXIII (*Chr2.g41974* and *Chr2.g41981*), GroupXIV (*Chr10.g17303*, *Chr5.g09388*, and *Chr5.g09378*) and GroupXV (*Chr10.g15727* and *Chr4.g38327*), which are co-expressed with multiple genes. The above genes probably take a crucial part in 'Duli-G03' in response to *Vp* signals and could be considered as candidates for further functional investigation.

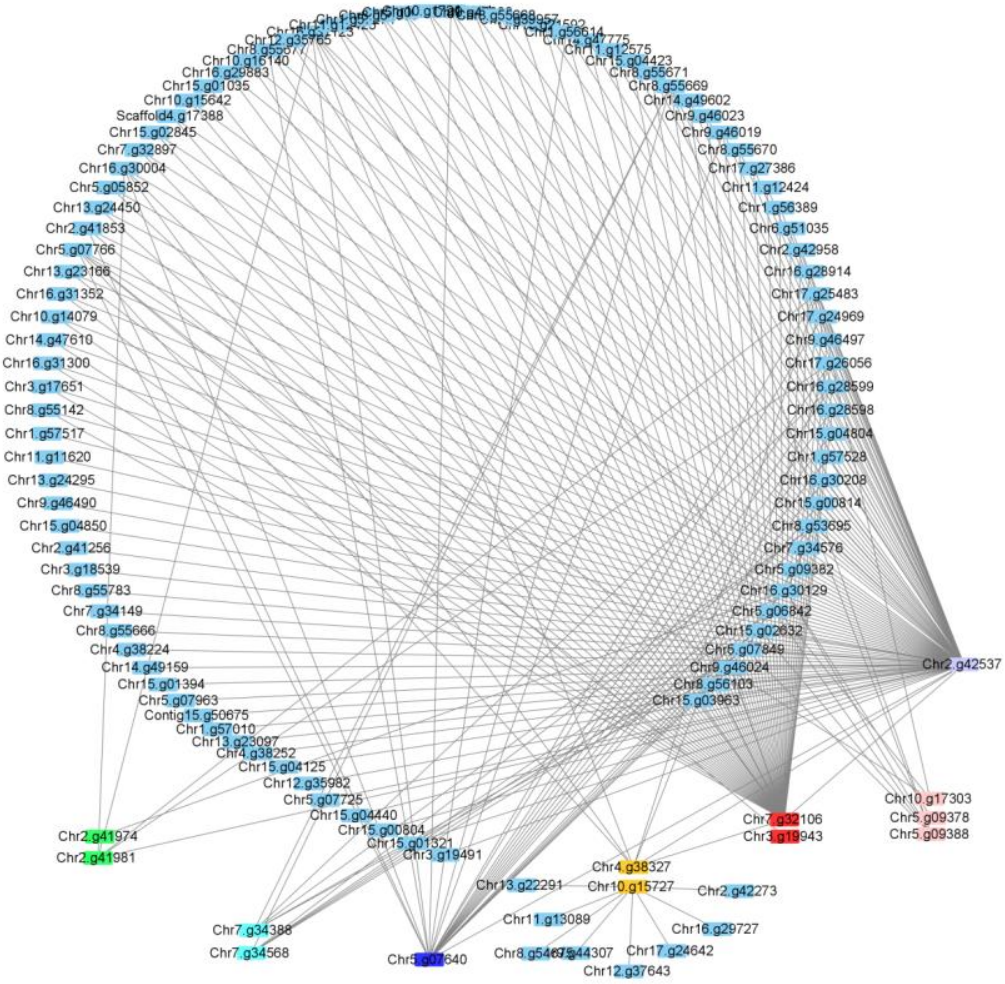

Figure 7. Co-expression network of NLR genes. All NLR genes were annotated and network relationships were constructed (Cut-off weight > 0.5).

4. Discussion

NLR activates the second layer of the plant immune system against pathogenic bacteria by directly or indirectly recognizing the effectors secreted by the pathogenic bacteria [47]. The genome-wide identification of NLR genes have been detailed and analyzed in various plant species, such as *Arabidopsis*, Rice, Maize, *Haynaldia villosa*, barley, *Solanaceae* and Legume Plants, etc. [48–55]. The genome-wide identification and evolutionary pattern of NLRs were also investigated in Rosaceae species [56,57]. However, there is less in the comparative analysis and discussion of NLRs between different species of Rosaceae. In this paper, we systematically studied NLR genes in nine species of Rosaceae. Moreover, candidate NLRs which might play important roles in response to *Valsa* canker were also examined for further investigation.

The NLR gene appeared earlier than in terrestrial plants, and it expanded rapidly after the plants landed [17]. That also resulted in exaggerated changes of NLR family sizes in various plants. In monocots, 139 NLRs were determined in *Zea mays*, but 498 in *Oryza sativa*, and 424 in *Setaria italic* [16]. In Rosaceae, only 81 members were identified from Roc, while 432 in Rch. Besides, NLR resistance genes were largely reduced in aquatic, parasitic, and insectivorous plants in angiosperms [58]. Above all, we suggest that the member size of NLRs was rapidly changed in landing plants associated with the difference in the environment.

To better investigate the potential function, several methods had been taken to ideally classify the NLR super-family. Based on the presence of a Toll/IL-1 Receptor-like (TIR)

domain, the NLRs have been traditionally divided into TIR-NBS-LRR (TNL) and the non-TIR-NBS-LRR (nTNL) [59–61]. Combining exon-intron structures and DNA motif sequences, three NLR classes were determined, named TNLs, CC-NBS-LRR (CNLs), and RPW8-NBS-LRR (RNLs) [62]. By comparative analysis, the distribution of NLRs in our study was almost identical to that previously reported [63]. Nevertheless, it is difficult to determine the detailed evolutional characteristics of NLRs. At present, we classified NLRs into 15 groups by phylogenetic analysis using full-length amino acid sequences. To our acknowledgment, each group included small-size members and was beneficial for evolutional and functional investigations.

In most cases, the enlargement of the member size of a gene family was derived from duplication events, such as WGD and TD [64]. In normal conditions, the enlargement of a gene family could be assessed by calculating the expansion rate [65]. For instance, several subfamilies of Leucine-Rich Repeat Receptor-Like kinases (LRR-RLKs), such as XIIa, and XIV, were promptly expanded in plants [31]. From our results, an investigation of duplication events between species and groups suggests that WGD mainly leads to the overall NLR gene expansion, while WGD and TD together lead to individual group-specific expansion.

The potential functions of various genes could be preliminarily predicted by tissue-specific expression. To exactly investigate the function of LRR-RLKs in Arabidopsis, the expressional patterns in various tissue types were tracked by using generation promoter: *GUS* transgenic plants [66]. In Rosaceae, the tissue-specific pattern of all genes in *Malus* species was systemically detected by using transcriptomic sequencing [46]. Our previous studies confirmed the reliability of the data [67,68]. Interestingly, most NLRs were little expressed in various tissues from *Malus* plants under normal environmental conditions. Indeed, most stress-related genes were low expressed in tissues but could be enhanced by biotic and/or abiotic stresses [69]. Therefore, we suggest that NLRs are mainly associated with stress responses but are less involved in cell growth and development.

As a destructive fungal disease, the identification of genes against the infection of *Valsa* canker pathogens becomes increasingly important for efficient control of wide-spreading pathogens. To date, we have screened several genes that regulate *Valsa* canker resistance, including some members in RLK, Receptor-like protein (RLP), and the Cyclic nucleotide-gated channel (CNGC) gene family [70,71]. As important candidates in plant immunity, the roles of NLRs against *Valsa* canker had not yet been comprehensively investigated. Currently, based on the transcriptomic data, 68 NLR genes were screened from highly resistant rootstock 'Duli-G03'. Furthermore, seven subgroups were distinctly expressed between susceptible and resistant varieties. In addition, 13 genes located at the key nodes of the co-expressional network, which might take a crucial part in *Valsa* canker response could be considered as candidates for further functional analysis.

In conclusion, a total of 3718 NLRs were systematically identified in the whole genome of 19 species. Based on phylogenetic tree construction, all NLRs could be divided into 15 groups. The different group displays distinct expansion rates caused by both WGD and TD events. Among the 50 subgroups, 127 NLRs were differentially expressed under *Vp* induction. There are 13 key NLR genes located at key nodes of the differentially expressed gene co-expression network that could be candidates for key regulators of *Valsa* canker resistance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9030345/s1, Table S1: 19 species-related information and online data sites, Table S2: All primers are required for Real-Time PCR, Table S3: NBS structural domains of 19 species were identified, Table S4: Expansion rates of each group of NLR for 19 species, Table S5: Expression data of total genes and expression data of NLR genes in apple, Table S6: NLR gene expression data of suspension cells of Pyrus betulifoliar and Pyrus bretschneideri under normal growth conditions, Figure S1: Real-Time PCR of seven genes in 20% *Vp*M-treated wild-type 'Duli-G03' suspension cells; Figure S2: From 127 genes, seven genes that responded strongly to the *Valsa* canker were selected for validation. Expression of seven NLR genes in wild-type suspension

cells treated with 20% VpM. Asterisk means significantly different at $p < 0.05$ (*) and $p < 0.01$(**). Vertical bars represent ± SD.

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