



Article

Genome-Wide Identification of Nucleotide-Binding Site–Leucine-Rich Repeat Gene Family in *Cymbidium ensifolium* and Expression Profiles in Response to Fusarium Wilt Infection

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Abstract: Fusarium wilt in *Cymbidium ensifolium*, caused by *Fusarium oxysporum*, is highly contagious and poses a severe hazard. It significantly reduces the ornamental value of *C. ensifolium* and causes substantial economic losses in agricultural production. Nucleotide-binding site–leucine-rich repeat (NBS-LRR) genes are key regulatory factors in plant disease resistance responses, playing vital roles in defending against pathogen invasions. In our study, we conducted a comprehensive analysis of the NBS-LRR gene family in the genome of *Cymbidium ensifolium*. Phylogenetic analysis identified a total of 31 NBS-LRR genes encoding NB-ARC proteins, which were categorized into five classes (CNL, CN, NL, N, RNL) based on their protein structural domains. These genes were found to be unevenly distributed across eight chromosomes. Physicochemical analysis revealed significant variances in molecular weight and sequence length among the family members. Subcellular localization results indicated that most genes primarily reside in the cytoplasm and cell membrane, suggesting that the primary sites of disease resistance responses may be the cell membrane and cytoplasm. Furthermore, noticeable disparities were observed in gene structures and conserved motifs among different categories of family genes. Promoter analysis indicated that *cis*-regulatory elements are mainly associated with plant stress, jasmonic acid, gibberellin, and other development-related factors, suggesting that *CeNBS-LRR* genes mainly resist external stress through hormones such as abscisic acid and jasmonic acid. We characterized twenty-seven *CeNBS-LRR* gene expression patterns of healthy *C. ensifolium* at different periods after Fusarium wilt infection, and found that those genes exhibit a temporospatial expression pattern, and that their expression is also responsive to Fusarium wilt infection. By analyzing the expression pattern via transcriptome and qRT-PCR, we speculated that *JL006442* and *JL014305* may play key roles in resisting Fusarium wilt. This study lays the groundwork and holds considerable significance as a reference for identifying disease-resistant genes and facilitating genetic breeding in *C. ensifolium*.

Keywords: NBS-LRR; *Cymbidium ensifolium*; resistance expression; *R* gene; Fusarium wilt



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1. Introduction

Cymbidium ensifolium spp., a member of the Orchidaceae and genus *Cymbidium*, is an ornamental plant known for its vibrant colors, upright stature, and delightful fragrance, which holds high ornamental and economic value [1,2]. In recent years, the expansion of cultivation areas and various external factors, including biotic and abiotic stresses, have greatly impacted its cultivation. Among these factors, Fusarium wilt disease, caused by

Fusarium oxysporum, is particularly prevalent. This disease has broad infectivity and severe consequences, greatly diminishing the ornamental value of *C. ensifolium* and resulting in substantial economic losses to the industry [3,4]. Currently, disease prevention and control primarily rely on chemical agents and cultivation management measures, but their long-term effectiveness is questionable [5]. Therefore, the breeding of disease-resistant varieties is crucial for addressing wilt disease, with elucidating its resistance mechanisms being a primary focus.

Plants encounter various pathogens during different growth periods, including fungi, bacteria, nematodes, and viruses, among others, which significantly hinder their growth, development, metabolism, and absorption of water and nutrients, often resulting in severe diseases such as Fusarium wilt, Anthracnose, and powdery mildew [3,6–8]. To combat pathogen invasion, plants have evolved two distinct levels of defense responses over time: primary immune response and secondary immune response [9]. The primary immune response (PTI) constitutes the first level, where pattern recognition receptors (PRRs) on the surface of host cells recognize pathogen-associated molecular patterns (PAMPs) from pathogens and microorganisms, activating the PTI triggered by PAMPs (PAMP-triggered immunity). The second level is the secondary immune response (ETI). In general, PTI responses can block the invasion of most pathogens and microorganisms, but some pathogens or microorganisms may secrete effectors to inhibit PTI responses. To prevent further invasion by pathogens, plants produce effectors that trigger immunity (ETI). In the ETI response, *R* genes act as receptors, specifically recognizing pathogen effectors, thereby activating hypersensitive responses, cell death, and the accumulation of reactive oxygen species [10–12].

The NBS-LRR gene family represents the largest class of disease resistance genes, also known as the *R* gene family, named after the abbreviations of its two important domains: NBS (nucleotide-binding site) and LRR (leucine-rich repeat) [13]. The NBS-LRR genes play a vital role in plants by perceiving and responding to invasion by pathogens such as fungi, viruses, and activating defense responses, including the accumulation of antimicrobial substances and the synthesis of antimicrobial enzymes. [12]. The NBS domain can bind and hydrolyze ATP or GTP nucleotides, thereby facilitating signal transduction and response [14]. The LRR domain is composed of multiple repeats rich in leucine residues, which interact with exogenous signaling molecules, proteins, and other molecules, thereby exerting disease resistance effects [15]. Typically, the N-terminal domain of NBS-encoding proteins consists of TIR (Toll/interleukin-1 receptor-like), CC (coiled coil), or RPW8. Based on their domain composition, they are often classified into three major subfamilies: TNL (TIR-NBS-LRR), CNL (CC-NBS-LRR), and RNL (RPW8-NBS-LRR) [16,17]. Previous studies have shown that the TNL subfamily is predominantly present in dicotyledons, whereas in monocotyledons, the TN class may lack the LRR structural domain or even be completely absent, suggesting that the NBS-LRR family of genes underwent significant divergence during the evolution of monocotyledons and dicotyledons. [18]. Given its significance, the NBS-LRR gene family has been consistently at the forefront of research in plant disease resistance. Currently, identification analyses of the NBS-LRR gene family have been completed in various plant genomes such as *Arabidopsis thaliana*, *Oryza sativa*, *Triticum aestivum*, *Medicago sativa*, and *Actinidia chinensis* [19–23]. This study employs bioinformatics techniques to identify members of the NBS-LRR gene family in *C. ensifolium*, uncovering their chromosome localization, conserved domains, and evolutionary relationships. In addition, we analyzed the expression patterns of the *CeNBS-LRR* genes at different stages of infection and screened for potential genes responsive to Fusarium wilt. The genome-wide identification and expression profiling of the NBS-LRR gene family in *C. ensifolium* aims to lay a foundation for screening disease resistance genes in *C. ensifolium* and for genetic breeding research.

2. Materials and Methods

2.1. Identification of the *CeNBS-LRR* Genes

The complete genome data of *C. ensifolium* was obtained from the NGDC database (<https://ngdc.cncb.ac.cn/>, accessed on 27 December 2023), and the genomic data of

A. thaliana and the NBS-LRR protein sequences were obtained from the TAIR database (<http://www.arabidopsis.org/>, accessed on 27 December 2023) [24]. The NB-ARC domain Hidden Markov Model PF00931 was downloaded from the Pfam database (<http://pfam.xfam.org/>, accessed on 27 December 2023). The protein sequence files of *C. ensifolium* were searched and compared based on the PF00931 HMM model using the BLAST program of TBtools with a threshold E-value of $\leq 10^{-4}$ to initially screen candidate protein sequences. Subsequently, all potential candidate protein sequences were submitted to the CDD-SEARCH website (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>, accessed on 27 December 2023), the SMART website (<http://smart.embl.de>, accessed on 27 December 2023), and the PFAM website (<https://pfam.xfam.org/>, accessed on 29 December 2023) for further structural domain prediction, eliminating invalid genes lacking NB-ARC conserved structural domains and misannotated genes [25,26]. Finally, all candidate genes were validated using the INTERPRO website (<https://www.ebi.ac.uk/interpro/>, accessed on 29 December 2023). The final validated NBS-LRR genes were classified according to their domain arrangement.

2.2. Phylogenetic Analysis, Physicochemical Properties, and Chromosome Localization Analysis

The protein sequences of the NBS-LRR family were extracted from the protein sequence files of *C. ensifolium* and *A. thaliana* based on gene IDs. Multiple sequence alignment was performed using the CLustalW method in MEGA11.0.13 software with default parameters [27]. The ML (maximum likelihood) method was used to construct a phylogenetic tree of the CeNBS-LRR family protein sequences, using the p-distance model with bootstrap validation repeated 1000 times, partial deletion method, and a threshold of 50%. Ultimately, the web program Chiplot (<https://www.chiplot.online/tvbot.html>, accessed on 30 December 2023) was used to beautify the phylogenetic tree [28]. The subcellular localization of the NBS protein was predicted using the Plant-mPLoc website (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>, accessed on 30 December 2023) and physicochemical properties like isoelectric point and molecular weight were calculated using the EXPASY online tool (<https://web.expasy.org/protparam/>, accessed on 30 December 2023) [29,30]. Applying the chromosome information from the *C. ensifolium* annotation file, the TBtools 1.119 software was used to visualize the chromosomal positions of CeNBS-LRR genes [31].

2.3. Cis-Acting Elements and Genome Collinearity Analysis of CeNBS-LRR Genes

According to their gene position, the upstream 2000bp sequences were extracted by TBtools from the coding sequences (CDS) file of *C. ensifolium*. These sequence files were submitted to the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 3 January 2024) for investigating the putative *cis*-acting elements [32]. The resulting files were organized in Excel, irrelevant functional elements were removed, and the elements were categorized by function and visualized using a heatmap generated by Excel 2020 and TBtools. Genomic and annotation files for *A. thaliana* and *O. sativa* were downloaded from the Phytozome database (<https://phytozome-next.jgi.doe.gov/>, accessed on 5 January 2024). Genomic data analysis and visualization of the genomic collinearity between *C. ensifolium*, *A. thaliana*, and *O. sativa* were conducted using the quick-run MCScanX wrapper and multiple synteny plot programs in TBtools [31].

2.4. Analysis of Gene Structure and Conserved Domains

The CeNBS-LRR protein sequences were uploaded to the MEME website for motif analysis, with the number of motifs set to 10. The default parameters were used for other settings, resulting in an XML output file. Additionally, genetic structure and domain visualization were conducted via TBtools using the annotation file of *C. ensifolium* and the PFAM domain prediction result file. Conserved domain prediction was performed using the Jalview 2.11.3.2 software, and the logo of the conservative sequence was created using the Weblogo online tool (<https://weblogo.berkeley.edu/>, accessed on 12 January 2024). The final images were combined using Adobe Photoshop 2020 and Adobe Illustrator 2020 software.

2.5. Plant Material and Gene Expression Analysis to Fusarium Wilt Infection

The experimental plants were obtained from Fujian Agriculture and Forestry University (Forest Orchid Garden), and were cultivated in greenhouses. Healthy adult *C. ensifolium* ‘Da feng su’ plants were chosen as inoculation materials for the study. The pathogenic fungus *F. oxysporum* was isolated from diseased *C. ensifolium* roots, purified, and cultured on PDA medium. When the medium was colonized with mycelium, the mycelium was scraped and filtered to obtain a spore suspension with a concentration of 1×10^6 CFU/mL. The root-dipping method was used to treat the roots of plant materials. The roots were soaked in the spore suspension for 30 min before being transferred to an artificial culture chamber for incubation. Incubation conditions were kept at 25 °C with a 16 h light cycle and an 8 h dark cycle. Root samples were collected at 0 h (control), 6 h, 12 h, and 24 h post inoculation. All samples were subjected to three biological replicates. RNA extraction was conducted by an RNA extraction kit (OMEGA, Norcross, GA, USA) according to the instructions. cDNA was synthesized using a Hifair[®]III 1st Strand cDNA Synthesis Kit (gDNA digester plus), Yeasen Bio, Shanghai, China. Novozymes (Beijing, China) constructed the transcriptome data library using the Illumina Hiseq 6000 platform, quantifying gene expression levels in FPKM (fragments per kilobase of transcript per million mapped reads) (Table S1). The heatmap of gene expression was generated using TBtools.

2.6. Real-Time Fluorescence Quantitative Experiment

Based on the *CeNBS-LRR* genes expression values (FPKM > 10) and trends ($|\log_2\text{FoldChange}| \geq 1.0$), eight candidate genes were selected for expression validation. The primers for these genes were designed using Primer Premier 5 software and synthesized by Shanghai Sangon Biotech Company (Shanghai, China). The details of the primers are listed in Table S2. The *GAPDH* was used as the internal reference gene for qPCR experiments. Experiments were carried out on an ABI 7500 real-time system (Applied Biosystems, Foster City, CA, USA) using the Hieff[®] qPCR SYBR Green Master Mix (Low Rox Plus) kit according to the instructions given by the Shanghai Yisheng Biological Company (Shanghai, China). Three biological replicates were performed, and the 2^{-CT} formula was used to calculate the relative gene expression.

3. Results

3.1. Identification and Classification of NBS-LRR Genes in *C. ensifolium*

A total of 43 genes were obtained through retrieval and alignment using the HMM model (PF00931) in the *C. ensifolium* genome database. Gene structure prediction was further validated online using the Pfam, BatchCD search, Interprosearch, and SMART websites. Ultimately, 31 NBS-LRR family genes were identified, constituting approximately 0.1% of the entire genome. These genes were categorized into five classes based on different protein structural domains: CNL, CN, NL, N, and RNL. Specifically, there was 1 RNL gene, 9 CN genes, 11 CNL genes, 4 NL genes, and 6 N genes. All identified genes contained the NB-ARC domain, but the NBS-LRR gene carrying the TIR domain was not found (Table 1).

Table 1. Classification of the NBS-LRR genes in the *C. ensifolium*, *A. thaliana*, and *O. sativa* genomes.

Type	Domain	<i>C. ensifolium</i>	<i>A. thaliana</i>	<i>O. sativa</i>
CNL	CC NBS LRR	11	18	199
CN	CC NBS	9	3	61
NL	NBS LRR	4	32	123
N	NBS	6	6	109
TNL	TIR NBS LRR	0	101	0
TN	TIR NBS	0	2	3
RNL	RPW8 NBS LRR	1	5	0
RN	RPW8 NBS	0	1	0
Total		31	168	495

3.2. Phylogenetic Analysis, Physicochemical Properties, and Chromosomal Localization Analysis

A phylogenetic tree was constructed using the maximum likelihood (ML) method, based on 31 CeNBS-LRR and 166 AtNBS-LRR protein sequences (Figure 1). All NBS-LRR protein sequences were divided into three subfamilies: TNL, CNL, and RNL. Except for JL005200, which belonged to the RNL family, the remaining sequences were classified under the CNL subfamily. No clustering between CeNBS-LRR proteins and AtTNL subfamily members was observed. An analysis of the physicochemical properties of the 31 NBS-LRR protein sequences revealed that the average length of the CeNBS-LRR protein sequence was 2225.1 bp, with the longest being 3705 bp (JL006442) and the shortest being 384 bp (JL021707). A comparative analysis of the physicochemical properties of the 31 NBS-LRR family members revealed that most subfamily members had amino acid counts ranging from 127 to 1234. Specifically, JL021707 had the lowest amino acid count at 127, while JL006442 had the highest at 1234. The average theoretical isoelectric point was 6.68, and the molecular weight varied from 14,328.63 to 138,792.65 kDa. The average protein instability index was 48.38, indicating that the majority of the proteins were unstable. A high proportion of acidic amino acids were observed in the members, with an average aliphatic index ranging from 85.77 to 106.77. The average hydrophilicity ranged from -0.504 to 0.063; except for JL024806, the other proteins were hydrophilic (Table 2). Subcellular localization prediction using the Cell-PLoc 2.0 online website indicated that the majority were located in the cytoplasm and cell membrane, with a few found in the nucleus, suggesting that the primary sites of disease-resistance responses may be the cell membrane and cytoplasm. Chromosome localization analysis was performed based on the alignment of the *C. ensifolium* genome and annotation files. According to the analysis, CeNBS-LRR genes are distributed among eight chromosomes (Chr 2, Chr 4, Chr 5, Chr 6, Chr 8, Chr 12, Chr 13, Chr 17) out of the twenty chromosomes present in *C. ensifolium*. They form two gene clusters on chromosomes 8 and 17, containing up to 14 and 8 NBS-LRR genes, respectively (Figure 2). However, no genes from this family were found on the remaining 12 chromosomes, while the remaining chromosomes sporadically harbored one to two genes each.

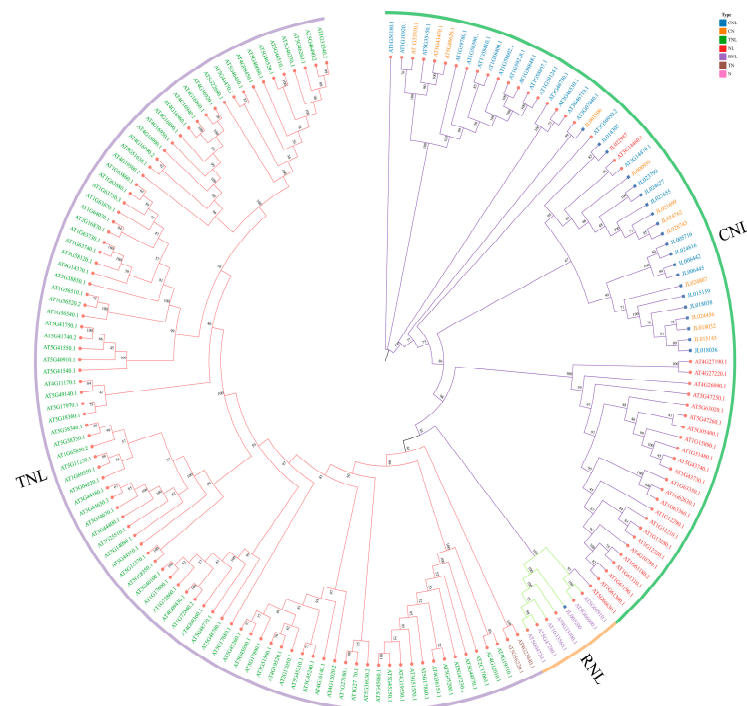


Figure 1. Phylogenetic tree of NBS-LRR proteins from *C. ensifolium* and *A. thaliana*. Different colors represent different NBS-LRR subfamilies. Solid squares and circles, respectively, represent *C. ensifolium* and *A. thaliana*. Percent bootstrap values (1000 iterations) are indicated in every branch.

Table 2. Physicochemical properties and subcellular localization of NBS-LRR family proteins in *Cymbidium ensifolium*.

Sequence ID	Length/bp	Size/aa	MW/Kda	pI	II	AI	GRAVY	Subcellular Location
JL000937	693	230	19,641.8	5.46	48.08	99.61	−0.381	Cytoplasm. Nucleus.
JL000939	3579	1192	136,675.62	6.08	46.76	102.42	−0.243	Cytoplasm.
JL003306	2655	884	100,476.43	9.23	57.12	103.82	−0.16	Cytoplasm.
JL005200	2457	818	93,067.12	6.32	47.07	104.82	−0.145	Cytoplasm.
JL005716	3192	1063	121,322.26	5.65	54.08	106.01	−0.114	Cell membrane. Cytoplasm.
JL006442	3705	1234	138,792.65	7.13	44.04	95.43	−0.252	Cell membrane. Cytoplasm.
JL006445	3156	1051	118,860.11	5.52	45.63	103.42	−0.153	Cell membrane. Cytoplasm.
JL014305	2865	954	109,640.25	6.27	42.86	104.25	−0.233	Cytoplasm.
JL015143	3279	1092	124,529.05	6.22	44.61	101.77	−0.187	Cytoplasm.
JL015159	2166	721	82,248.03	6.6	45.93	103.43	−0.21	Cytoplasm.
JL018032	3408	1135	128,937.11	6.45	43.25	101.26	−0.2	Cytoplasm.
JL018036	3417	1138	128,968.93	6.45	45.21	100.07	−0.177	Cytoplasm.
JL018038	2226	741	84,065.55	7.26	42.34	106.67	−0.119	Cytoplasm.
JL021699	1500	499	58,741.93	5.47	52.32	94.33	−0.323	Cytoplasm.
JL021705	600	199	22,529.59	4.67	46.89	94.47	−0.184	Cytoplasm. Nucleus.
JL021707	384	127	14,328.63	6.91	40.37	104.96	−0.102	Cytoplasm.
JL022987	1956	651	74,820.48	7.57	39.5	99.69	−0.28	Cell membrane. Cytoplasm.
JL023790	2424	807	93,218.67	5.43	56.47	99.42	−0.247	Cytoplasm.
JL024456	3150	1049	119,420.27	7.99	40.32	102.76	−0.202	Cytoplasm.
JL024627	3216	1071	121,917.88	5.67	51.05	97.4	−0.19	Cell membrane. Cytoplasm.
JL024762	2871	956	110,390.18	6.76	51.67	95.65	−0.292	Cytoplasm.
JL024805	1527	508	57,241.5	8.67	59.54	95.08	−0.267	Cell membrane. Cytoplasm.
JL024806	756	251	28,129.13	8.86	44.4	105.62	0.063	Cytoplasm.
JL024807	1980	659	74,718.97	7.19	49.51	99.59	−0.113	Cytoplasm.
JL024816	2322	773	87,897.62	6.71	52.46	105.27	−0.182	Cytoplasm.
JL025655	2943	980	113,173.42	6.47	50.31	95.3	−0.252	Cytoplasm.
JL025932	702	233	27,012.08	5.3	62.72	97.04	−0.141	Cytoplasm. Nucleus.
JL025933	972	323	37,748.46	5.37	61.94	96.56	−0.218	Cytoplasm.
JL027518	1524	507	57,848.73	8.9	44.18	106.77	−0.084	Cell membrane. Cytoplasm.
JL027520	1884	654	75,060.62	8.7	41.99	105.7	−0.1	Cell membrane. Cytoplasm.
JL028743	1470	489	56,510.41	5.7	47.08	85.75	−0.504	Chloroplast. Cytoplasm.

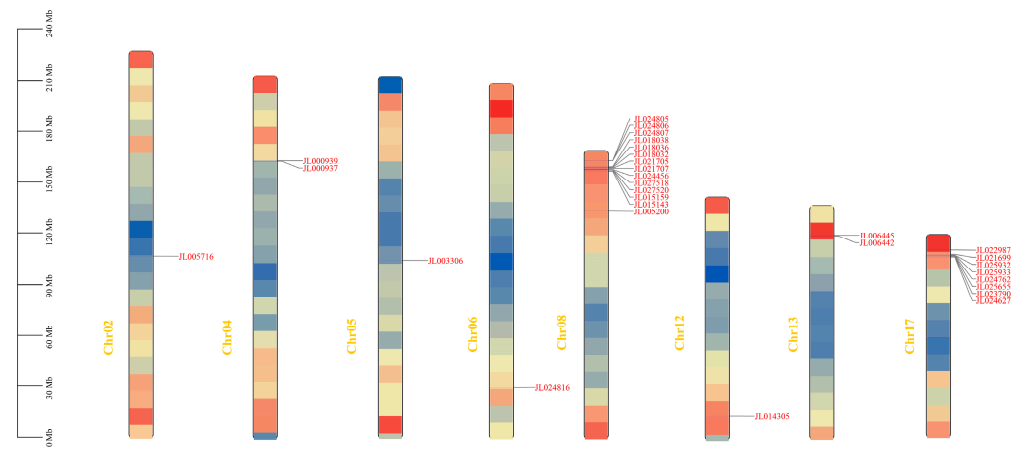


Figure 2. Localization and distribution of 31 NBS-LRR genes onto 8 *C. ensifolium* chromosomes. The scale represents the chromosomal distances (Mbp). The colors of chromosomes represent the gene distribution density on different chromosomes: red represents high gene density, while blue represents low gene density.

3.3. Gene Structure Analysis, Conserved Motif Analysis, and Conserved Domain Analysis in *C. ensifolium*

In the CeNBS-LRR family, it has been observed that the NB-ARC domain typically contains eight conserved motifs: P-loop, Kinase2, GLPL, MHDV, RNBS-A, RNBS-B, RNBS-C, and RNBS-D [33,34]. A total of 10 conserved motifs were identified through MEME analysis of the CeNBS-LRR proteins (Figure S1). Within the NB-ARC domain, six conserved motifs were identified (P-loop, Kinase2, GLPL, MHDV, RNBS-B, RNBS-D). Motif1 contains the sequence VFAIVGMGGIGKTTLAKLVFD, with the underlined part representing a

highly conserved amino acid sequence characteristic of the P-loop motif. Motif8 contains the sequence SVVSGKNLFLVLDDVWRADVW, characteristic of the Kinase2 motif [35]. The amino acid sequence NRLKGIGLQIAGNCGGLPLAIKAIAGVLAG of Motif6 can be inferred to represent the GLPL motif. Similarly, the sequence GSRVLVTTRNKE in Motif5 is indicative of a conserved feature of the RNBS-B motif. Notably, most *CeNBS-LRR* genes contain Motif1, Motif 5, Motif 6, and Motif 8, which collectively constitute highly conserved regions of the NB-ARC domain. The sequence LYLSFEDLP~~S~~HLKQCFLYFAL in Motif2 corresponds to a highly conserved motif of RNBS-D. The amino acid sequence FFTMHDLVYSLSR in Motif10 is a conserved region of the MHDV motif, and its presence or absence suggests the presence of the CC domain. Additionally, considering the different arrangements of the conserved motifs and the characteristics of the LRR domain, it can be inferred that the leucine-rich repeat sequence SFIPKGI~~G~~GNLQQLNH in Motif9 represents the LRR domain [36]. Gene structure analysis revealed that most *CeNBS-LRR* genes lack introns, constituting approximately 47% of the total. These genes are primarily found in the subfamilies of CNL and CN, with the number of exons ranging from one to six. NL-category genes typically consist of three exons and two introns. Variations in the numbers of introns and exons were observed among genes of different subfamilies (Figure 3).



Figure 3. Conserved motif and gene structure analysis of NBS-LRR genes in *C. ensifolium*. (a). Conserved motifs are shown by colored boxes. (b). Conserved domains are shown by colored columns. (c). Distribution of introns and exons.

3.4. Collinearity Analysis of *CeNBS-LRR* Genes

Furthermore, collinearity analysis between the *C. ensifolium* genome and the model plants *A. thaliana* and *O. sativa* revealed that *C. ensifolium* exhibits four collinear relationships with *O. sativa*, but only one with *A. thaliana* (Figure 4, Table S3). In the self-collinearity analysis of *C. ensifolium*, only one pair of segmentally duplicated genes (*JL014305* and *JL022987*) was identified (Figure 5).

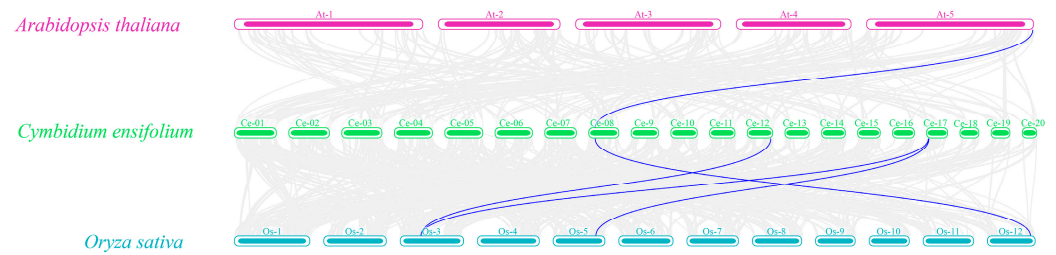


Figure 4. Collinearity analysis of NBS-LRR genes among *C. ensifolium*, *A. thaliana*, and *O. sativa*. Gray lines represent all syntenic areas between *C. ensifolium* and the other two genomes. The collinear pairs of NBS-LRR genes are highlighted by the blue lines.

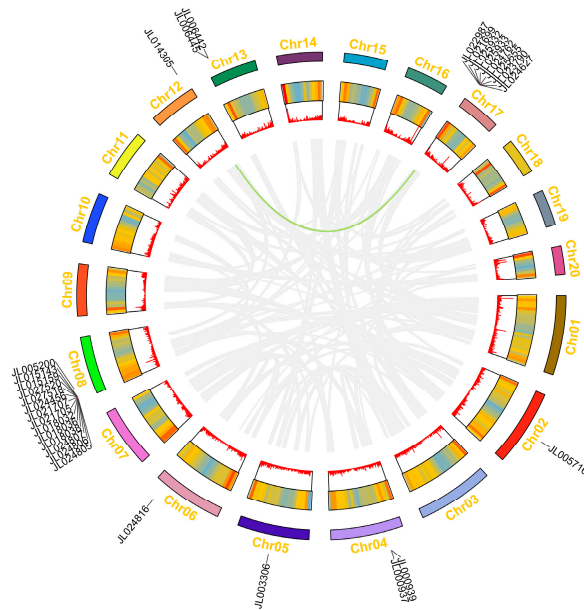


Figure 5. Collinearity analysis of *CeNBS-LRR* genes on chromosomes. Differently colored bars represent different chromosomes. The green line between chromosomes indicates segmentally duplicated genes. The heap map of bars represents the gene distribution density on different chromosomes.

3.5. Analysis of *Cis*-Acting Elements

To investigate the functions of NBS-LRR genes in *C. ensifolium*, the upstream 2000 bp gene sequences of the 31 *CeNBS-LRR* genes were analyzed to identify the related *cis*-regulatory elements. In the promoter region of the *CeNBS-LRR* genes, 19 *cis*-regulatory elements were identified after manual filtration to exclude those with low relevance (Table S4). These elements are involved in various plant life processes, including growth, development, stress response, hormone regulation, and signal transduction (Figure 6a). Each NBS-LRR gene was found to harbor *cis*-regulatory elements, with the gene *JL021705* containing the highest number (12) and the gene *JL000937* having the lowest (3). These *cis*-regulatory elements were classified into four categories: light-response-related, hormone-response-related, environmental-stress-related, and growth- and development-related. Among them, the most prevalent *cis*-regulatory elements were hormone-response-related, followed by environmental-stress-related elements (Figure 6b). Furthermore, *cis*-regulatory elements associated with abscisic acid and jasmonic acid were distributed among most genes, suggesting that *CeNBS-LRR* genes are mainly involved in the hormone induction process to resist external stress such as abscisic acid and jasmonic acid. The distribution of these *cis*-regulatory elements varies, with many clustering at the same locus or spanning limited lengths of the sequence.

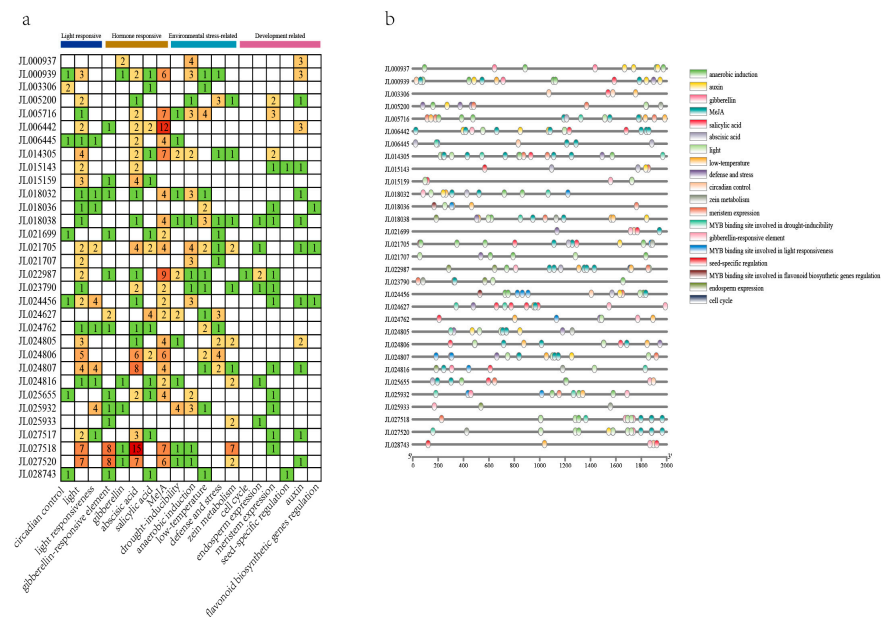


Figure 6. Analysis of the *cis*-elements of the promoter of the NBS-LRR gene of *C. ensifolium*. (a). The statistics of the *cis*-acting elements were visualized by a heat map and 19 *cis*-acting elements were classified into four categories represented by different colors. (b). Distribution of NBS-LRR gene *cis*-acting elements. Various elements are represented by the blocks of different colors.

3.6. Expression Patterns of CeNBS-LRR Genes in Response to *Fusarium* Wilt Infection

Plant materials of *C. ensifolium* were sampled at 0 h, 6 h, 12 h, and 24 h after inoculation with *F. oxysporum*, and the root samples were used for transcriptome analysis. We used transcriptome data to validate the expression patterns of *CeNBS-LRR* genes at 0 h, 6 h, 12 h, and 24 h post inoculation. After inoculation, different *CeNBS-LRR* genes showed diverse expression patterns (Figure 7). Among the *CeNBS-LRR* gene family members, a total of 27 genes showed differential expression patterns, with 13 *CeNBS-LRR* genes displaying their lowest expression levels at 0 hpi and 9 genes reaching their peak expression levels at 6 hpi. While 17 genes showed a decline in expression levels after 6 hpi, their expression trends varied at 12 hpi and 24 hpi. Five genes reached their peak expression levels at 24 hpi (Figure 7). Overall, the expression levels of most NBS-LRR gene family members exhibited an initial increase followed by a decrease after pathogen infection.

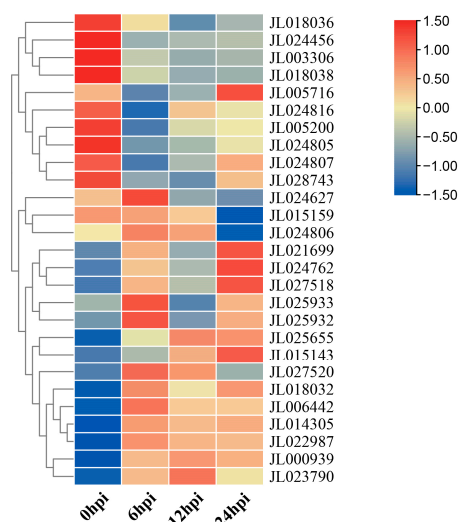


Figure 7. The expression of *CeNBS-LRR* genes in response to *Fusarium* wilt infection post-inoculation at 0 h, 6 h, 12 h, and 24 h.

Based on the transcriptome data and expression profiles, eight *NBS-LRR* genes were selected for real-time fluorescence quantitative experiments to validate the reliability of the transcriptome data. The fluorescence quantification expression trends of most *CeNBS-LRR* genes were consistent with the transcriptome data (Figure 8). The expression levels of *JL00642* and *JL014305* showed a significant increasing trend from 0 hpi to 6 hpi, followed by a slight decline at 12 hpi. The expression of *JL005200* and *JL003306* exhibited a gradual decrease at 6 hpi with mild changes at 12 hpi; *JL021699* was upregulated at 6 hpi, followed by a decline at 12 hpi, and a peak at 24 hpi. Additionally, it was noteworthy that the expression of *JL006442* and *JL014305* increased by more than 3-fold compared to 0 hpi, suggesting their primary involvement in the early disease response.

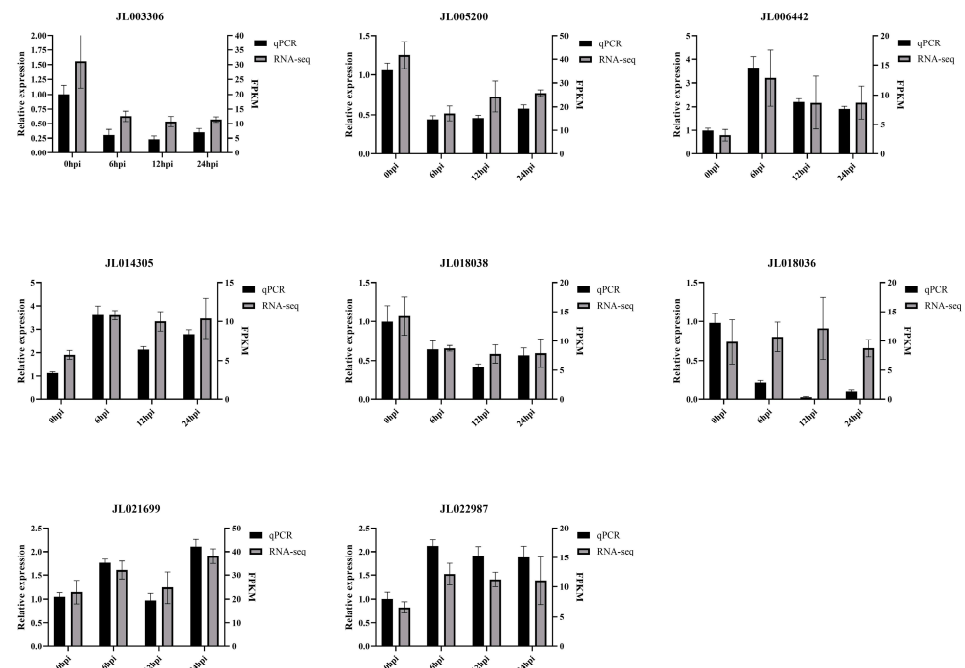


Figure 8. qRT-PCR analysis of eight *NBS-LRR* genes in *C. ensifolium* in response to *Fusarium* wilt infection. Error bars indicate the SD of three biological replicates.

4. Discussion

C. ensifolium boasts a rich history and legacy in China, is renowned for its unique flower shape and diverse colors, and holds a significant position in the market [37]. However, despite its prominence, the continuous advancement and intensification of the *C. ensifolium* industry have led to an escalating incidence of diseases. Various pathogens are increasingly developing resistance to traditional chemical agents [38]. This phenomenon profoundly impacts the growth and prosperity of the *C. ensifolium* industry. Notably, *Fusarium* wilt, caused by *F. oxysporum* and often referred to as the ‘cancer of orchids’, stands out as a formidable threat to the health and vitality of *C. ensifolium*. Given these challenges, breeding *C. ensifolium* varieties resistant to diseases emerges as a pivotal solution. The completion of the whole-genome sequencing of *C. ensifolium* has provided a robust foundation for the exploration and identification of resistance genes.

NBS-LRR genes represent one of the most important classes of plant disease resistance genes, with the majority of the currently identified disease resistance genes falling within this class. Examples include the *AvrRPS4* and *RLM3* genes in *A. thaliana*, which confer resistance to *Pseudomonas syringae*, the *Pto* gene in *Lycopersicon esculentum*, providing resistance to spotted wilt, the *Xa21* gene in *O. sativa*, imparting resistance to bacterial blight, and the *Pm41* gene in *T. aestivum*, offering resistance against powdery mildew [39–43]. Researchers have found that overexpression of the *NLR* gene *Rps11* in *Glycine max* can effectively enhance resistance to *Phytophthora* root rot. In *T. aestivum*, the *CNL* class *TaRCR1*

has been found to regulate downstream disease resistance gene expression by maintaining the balance of reactive oxygen species in plants, thereby reducing the damage caused by the wheat stripe rust pathogen to wheat. Despite these notable findings, there is a paucity of reports on disease resistance-related research in *C. ensifolium*, and the molecular mechanisms underlying its response to pathogens remain unclear. Therefore, by leveraging the identification of *CeNBS-LRR* genes through *C. ensifolium* genome data, the exploration of disease resistance-related genes can provide valuable insights for breeding disease-resistant *C. ensifolium* varieties. Based on the bioinformatic analysis of the *C. ensifolium* genome, this study identified a total of 31 *CeNBS-LRR* genes, including zero members of the TNL family and one member of the RNL family, while the remaining genes belonged to the CNL family. Similar situations have been found in various monocots such as rice, wheat, grapes, sugarcane, and sorghum, indicating a gradual loss of the TNL family members in the evolutionary history of monocot plants [44–46]. Although it has been suggested that the number of NBS-LRR genes is positively correlated with genome size, the number of *CeNBS-LRR* genes is extremely low compared to common agricultural and horticultural crops, and it is speculated that this may be related to the growing environment and the small number of ancestors [13]. In the phylogenetic tree, the *CeNBS-LRR* genes were clustered into CNL and RNL, similar to *A. thaliana*, *O. sativa*, and *Phalaenopsis equestris*. TNL genes form a distinct cluster separate from CNL genes, indicating a distant relationship between the TNL and CNL classes. None of the genes clustered with TNL; presumably, the TNL category was lost in early evolution, which is consistent with the fact that monocots contain very few or no TNL genes. In addition, noticeable clustering phenomena between the RNL subfamily and the CNL subfamily are observed, suggesting a high degree of homology between RNL and CNL proteins. Some researchers have reported that RNL genes and CNL genes share a common ancestor [17]. Only one RNL gene was identified in *C. ensifolium*, a pattern consistent with observations in other monocots, where only a very limited number of RNL genes are present [47,48]. It is speculated that RNL genes evolved from CNL genes and may play a specific role in disease resistance, akin to the *RPW8* gene in *Arabidopsis*, which exhibits significant resistance to powdery mildew [49]. Additionally, CNL genes are considered to be an older category with a higher abundance in plants [50]. Furthermore, the number of NBS-LRR genes in *C. ensifolium* is extremely low, a phenomenon observed in other orchids such as *Apostasia shenzhenica*, *Gastrodia elata*, and *P. equestris*, where only a small number of NBS-LRR genes have been identified [51]. These orchids also lack TNL genes in their genomes. In contrast to common crops like *Arabidopsis*, rice, wheat, and tomato, orchids generally possess a significantly lower number of NBS-LRR genes. This discrepancy could be attributed to gene loss events in the ancestral genome of orchids, which experienced fewer expansions of gene families due to their relatively uniform habitat environments.

In the conserved domain and motif analysis of the *CeNBS-LRR* genes, representative motifs of the NBS-LRR gene family such as P-loop, Kinse2, GLPL, RNBS-B, and RNBS-D are all found in *CeNBS-LRR* genes. Motif positions are arranged in the physical structure order of motif10-motif1-motif4-motif3-motif2-motif5-motif9-motif8, which is typically consistent with the typical structure of NBS-LRR genes in other plants [52]. Within the same subfamily, different genes maintained a similar motif arrangement sequence, with genes exhibiting closer phylogenetic relationships showing higher sequence similarity and functional similarity. Except for motif7 and motif10, the other motifs are highly conserved. P-loop, GLPL, and RNBS-B constitute the most conserved part of the NB-ARC domain in *C. ensifolium*, suggesting their important role in disease resistance response. Gene structure analysis revealed significant differences in the number of introns and exons among the *CeNBS-LRR* genes, with exon counts ranging from 1 to 6. The number of domains is proportional to the number of exons, and overall, the number is relatively small. Approximately half of the genes have zero introns, suggesting that exon loss events may have occurred during early evolution.

Cis-acting element analysis enables the visual depiction of gene expression levels across different elements, facilitating the prediction of potential gene functions and the biological processes they participate in [53]. In our analysis, jasmonic acid, hypoxia-specific induction, and abscisic acid were the top three elements in terms of proportion. Jasmonic acid can induce the production of stress-resistant genes when plants are subjected to biotic stress, enabling them to defend themselves from damage. Exogenous treatment with jasmonic acid has been shown to significantly enhance plant disease resistance [54]. Abscisic acid is another important regulatory factor in plants. When subjected to external environmental, biotic, and abiotic stresses, it helps alleviate sustained damage to plants by initiating stress responses through signal transduction pathways [55]. Therefore, it is plausible to suggest that most *CeNBS-LRR* genes may be involved in various stress responses through hormonal regulation, a phenomenon similar to that in *Dendrobium officinale* [56].

Homologous analysis can reveal the correlation between homologous genes across different species or the occurrence of gene fragment duplication within the same species. The collinearity results show that the only RNL gene (*JL005200*) in *C. ensifolium* is homologous to those in *A. thaliana* and *O. sativa*, suggesting that the *CeNBS-LRR* gene family is highly conservative in evolution. There are more collinear regions between *C. ensifolium* and *O. sativa* compared to those between *C. ensifolium* and *A. thaliana*, indicating that the closer the relationship between species, the more unified the evolutionary trend in gene families. This homologous gene can be used to predict functions based on reports in other species. It is well established that plants trigger their own immune response upon pathogen infection, which leads to the upregulation of specific disease-resistant genes. Therefore, we conducted further analysis on the expression changes of 27 *CeNBS-LRR* genes at different time points after pathogen inoculation, and subsequently validated the findings using qRT-PCR. Remarkably, the results were consistent with the transcriptome data. Interestingly, different genes exhibited distinct expression patterns, with each reaching its maximum peaks at different time points post-inoculation. Specifically, the expression of *JL006442* and *JL014305* displayed a substantial increase at 6 hpi, and reaching their maximum expression levels. Conversely, *JL003306* and *JL005200* showed linear downregulation at 6 hpi. *JL021699* showed an initial increase followed by a decrease in expression levels at different time points post-inoculation, ultimately reaching a peak at 24 hpi. These findings suggested that *JL006442* and *JL014305* may play key roles in resisting pathogen-induced stress. Further functional validation and protein interaction analysis are warranted for these candidate genes to provide a theoretical basis for disease resistance breeding in *C. ensifolium*.

5. Conclusions

In conclusion, this study identified a total of 31 *CeNBS-LRR* family genes in the whole genome of *C. ensifolium* and analyzed their physicochemical properties. Based on their diverse structures, 31 *CeNBS-LRR* family members were classified into two major categories. Different categories of *CeNBS-LRR* genes show distinct differences in their domains, with RNL genes featuring a unique RPW8 domain. The conserved motifs exhibited variations among genes of different subfamilies, while the motifs of NB-ARC domains were similar among different subfamilies. Only one pair of duplicated genes was identified in the *CeNBS-LRR* gene family, suggesting that the *CeNBS-LRR* gene family exhibits conservative evolution. Promoter analysis revealed that the most *cis*-acting elements are associated with plant stress response, growth, and development. Furthermore, the gene expression profiling and qRT-PCR results demonstrate that *CeNBS-LRR* has different trends in response to Fusarium wilt infection with varying degrees of expression. In summary, this study provides a solid theoretical foundation for the exploration of disease-resistant genes in *C. ensifolium* and for genetic breeding aimed at enhancing disease resistance in *C. ensifolium* varieties.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10060634/s1>, Figure S1: The conserved motif analysis of the *CeNBS-LRR* gene; Table S1: Average FPKM value of *CeNBS-LRR* genes transcriptome data before and after Fusarium wilt infection; Table S2: Primer information used in this study; Table S3: Synteny

analysis of *CeNBS-LRR* gene IDs among *C. ensifolium*, *A. thaliana*, and *O. sativa* genomes; Table S4: Details of *cis*-acting elements in the promoter region of 31 *CeNBS-LRR* genes (2000 bp upstream of the initiation codon).

Author Contributions: Q.-H.Z. and Y.A. conceived and designed the experiments. L.Y. performed the experiments and wrote the manuscript. B.-X.S., J.-J.L., Y.-Y.L. and S.-Y.C. conducted the bioinformatics analysis. C.-Y.F. and Y.T. contributed materials. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The sequences of *C. ensifolium* used in this study are available at the National Genomics Data Center (NGDC). The RNA-Seq data have been deposited in NCBI under SRA accession codes: PRJNA1096825.

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References

1. Ai, Y.; Li, Z.; Sun, W.-H.; Chen, J.; Zhang, D.; Ma, L.; Zhang, Q.-H.; Chen, M.-K.; Zheng, Q.-D.; Liu, J.-F.; et al. The *Cymbidium* genome reveals the evolution of unique morphological traits. *Hortic. Res.* **2021**, *8*, 255–264. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Li, X.; Liu, L.; Sun, S.; Li, Y.; Jia, L.; Ye, S.; Yu, Y.; Dossa, K.; Luan, Y. Transcriptome analysis reveals the key pathways and candidate genes involved in salt stress responses in *Cymbidium ensifolium* leaves. *BMC Plant Biol.* **2023**, *23*, 64. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Paul, S.K.; Mahmud, N.U.; Gupta, D.R.; Alam, M.N.; Chakraborty, M.; Islam, M.T. First Report of *Fusarium sacchari* Causing Sugarcane Wilt in Bangladesh. *Plant Dis.* **2022**, *106*, 319. [\[CrossRef\]](#) [\[PubMed\]](#)
4. Jin-Ai, Y.; Peng, H.; Cheng-Zhong, L.; De-Yi, Y. Stem rot on *Cymbidium ensifolium* (Orchidaceae) caused by *Fusarium oxysporum* in China. *Can. J. Plant. Pathol.* **2017**, *40*, 105–108. [\[CrossRef\]](#)
5. Chung, W.C.; Chen, L.W.; Huang, J.H.; Huang, H.C.; Chung, W.H. A new ‘forma specialis’ of *Fusarium solani* causing leaf yellowing of *Phalaenopsis*. *Plant Pathol.* **2010**, *60*, 244–252. [\[CrossRef\]](#)
6. Staskawicz, B.J.; Ausubel, F.M.; Baker, B.J.; Ellis, J.G.; Jones, J.D.G. Molecular Genetics of Plant Disease Resistance. *Science* **1995**, *268*, 661–667. [\[CrossRef\]](#)
7. Wei, W.R.; Yue, H.L.; Nie, Y.X.; Chen, T.X. First Report of *Colletotrichum siamense* Causing Daylily (*Emerocallis citrina*) Anthracnose in China. *Plant Dis.* **2022**, *106*, 1061. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Ajithkumar, K.; Savitha, A.S.; Mahadevakumar, S.; Sujatha, M.; Maharachchikumbura, S.S.N.; Sreenivasa, M.Y.; Renuka, M.; Yenjerappa, S.T. First Report of Powdery Mildew Caused by *Erysiphe diffusa* on Cluster Bean in India. *Plant. Dis.* **2022**, *106*, 3203. [\[CrossRef\]](#)
9. Ji, H.M.; Mao, H.Y.; Li, S.J.; Feng, T.; Zhang, Z.Y.; Cheng, L.; Luo, S.J.; Borkovich, K.A.; Ouyang, S.Q. *Fol-milR1*, a pathogenicity factor of *Fusarium oxysporum*, confers tomato wilt disease resistance by impairing host immune responses. *New Phytol.* **2021**, *232*, 705–718. [\[CrossRef\]](#)
10. Kourelis, J.; van der Hoorn, R.A.L. Defended to the Nines: 25 Years of Resistance Gene Cloning Identifies Nine Mechanisms for R Protein Function. *Plant Cell* **2018**, *30*, 285–299. [\[CrossRef\]](#)
11. Ellis, J.G.; Lawrence, G.J.; Dodds, P.N. Further analysis of gene-for-gene disease resistance specificity in flax. *Mol. Plant Pathol.* **2007**, *8*, 103–109. [\[CrossRef\]](#) [\[PubMed\]](#)
12. DeYoung, B.J.; Innes, R.W. Plant NBS-LRR proteins in pathogen sensing and host defense. *Nat. Immunol.* **2006**, *7*, 1243–1249. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Shao, Z.Q.; Xue, J.Y.; Wang, Q.; Wang, B.; Chen, J.Q. Revisiting the Origin of Plant NBS-LRR Genes. *Trends Plant Sci.* **2019**, *24*, 9–12. [\[CrossRef\]](#) [\[PubMed\]](#)
14. McDowell, J.M.; Woffenden, B.J. Plant disease resistance genes: Recent insights and potential applications. *Trends Biotechnol.* **2003**, *21*, 178–183. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Kobe, B.; Kajava, A.V. The leucine-rich repeat as a protein recognition motif. *Curr. Opin. Struct. Biol.* **2001**, *11*, 725–732. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Yu, X.; Zhong, S.; Yang, H.; Chen, C.; Chen, W.; Yang, H.; Guan, J.; Fu, P.; Tan, F.; Ren, T.; et al. Identification and Characterization of NBS Resistance Genes in *Akebia trifoliata*. *Front. Plant Sci.* **2021**, *12*, 758559. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Meyers, B.C.; Kaushik, S.; Nandety, R.S. Evolving disease resistance genes. *Curr. Opin. Plant Biol.* **2005**, *8*, 129–134. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Bai, J.; Pennill, L.A.; Ning, J.; Lee, S.W.; Ramalingam, J.; Webb, C.A.; Zhao, B.; Sun, Q.; Nelson, J.C.; Leach, J.E.; et al. Diversity in Nucleotide Binding Site–Leucine-Rich Repeat Genes in Cereals. *Genome Res.* **2002**, *12*, 1871–1884. [\[CrossRef\]](#) [\[PubMed\]](#)

19. Meyers, B.C.; Kozik, A.; Griego, A.; Kuang, H.; Michelmore, R.W. Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. *Plant Cell* **2003**, *15*, 809–834. [[CrossRef](#)] [[PubMed](#)]
20. Liu, W.; Ghouri, F.; Yu, H.; Li, X.; Yu, S.; Shahid, M.Q.; Liu, X. Genome wide re-sequencing of newly developed Rice Lines from common wild rice (*Oryza rufipogon* Griff.) for the identification of NBS-LRR genes. *PLoS ONE* **2017**, *12*, e0180662. [[CrossRef](#)]
21. Liu, J.; Qiao, L.Y.; Zhang, X.J.; Li, X.; Zhan, H.X.; Guo, H.J.; Zheng, J.; Chang, Z.J. Genome-wide identification and resistance expression analysis of the NBS gene family in *Triticum urartu*. *Genes Genom.* **2017**, *39*, 611–621. [[CrossRef](#)]
22. Ameline-Torregrosa, C.; Wang, B.B.; O'Bleness, M.S.; Deshpande, S.; Zhu, H.; Roe, B.; Young, N.D.; Cannon, S.B. Identification and characterization of nucleotide-binding site-leucine-rich repeat genes in the model plant *Medicago truncatula*. *Plant Physiol.* **2008**, *146*, 5–21. [[CrossRef](#)] [[PubMed](#)]
23. Wang, T.; Jia, Z.H.; Zhang, J.Y.; Liu, M.; Guo, Z.R.; Wang, G. Identification and Analysis of NBS-LRR Genes in *Actinidia chinensis* Genome. *Plants* **2020**, *9*, 1350. [[CrossRef](#)] [[PubMed](#)]
24. Garcia-Hernandez, M.; Berardini, T.Z.; Chen, G.; Crist, D.; Doyle, A.; Huala, E.; Knee, E.; Lambrecht, M.; Miller, N.; Mueller, L.A.; et al. TAIR: A resource for integrated *Arabidopsis* data. *Funct. Integr. Genomics* **2002**, *2*, 239–253. [[CrossRef](#)] [[PubMed](#)]
25. Lu, S.; Wang, J.; Chitsaz, F.; Derbyshire, M.K.; Geer, R.C.; Gonzales, N.R.; Gwadz, M.; Hurwitz, D.I.; Marchler, G.H.; Song, J.S.; et al. CDD/SPARCLE: The conserved domain database in 2020. *Nucleic Acids Res.* **2020**, *48*, D265–D268. [[CrossRef](#)] [[PubMed](#)]
26. Letunic, I.; Khedkar, S.; Bork, P. SMART: Recent updates, new developments and status in 2020. *Nucleic Acids Res.* **2021**, *49*, D458–D460. [[CrossRef](#)] [[PubMed](#)]
27. Tamura, K.; Stecher, G.; Kumar, S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Mol. Biol. Evol.* **2021**, *38*, 3022–3027. [[CrossRef](#)]
28. Xie, J.; Chen, Y.; Cai, G.; Cai, R.; Hu, Z.; Wang, H. Tree Visualization By One Table (tvBOT): A web application for visualizing, modifying and annotating phylogenetic trees. *Nucleic Acids Res.* **2023**, *51*, W587–W592. [[CrossRef](#)] [[PubMed](#)]
29. Chou, K.C.; Shen, H.B. Cell-PLOC: A package of Web servers for predicting subcellular localization of proteins in various organisms. *Nat. Protoc.* **2008**, *3*, 153–162. [[CrossRef](#)]
30. Gasteiger, E.; Gattiker, A.; Hoogland, C.; Ivanyi, I.; Appel, R.D.; Bairoch, A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* **2003**, *31*, 3784–3788. [[CrossRef](#)]
31. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant* **2020**, *13*, 1194–1202. [[CrossRef](#)] [[PubMed](#)]
32. Lescot, M.; Dehais, P.; Thijs, G.; Marchal, K.; Moreau, Y.; Van de Peer, Y.; Rouze, P.; Rombauts, S. PlantCARE, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **2002**, *30*, 325–327. [[CrossRef](#)] [[PubMed](#)]
33. Lupas, A.; Van Dyke, M.; Stock, J. Predicting coiled coils from protein sequences. *Science* **1991**, *252*, 1162–1164. [[CrossRef](#)] [[PubMed](#)]
34. Shao, Z.Q.; Xue, J.Y.; Wu, P.; Zhang, Y.M.; Wu, Y.; Hang, Y.Y.; Wang, B.; Chen, J.Q. Large-Scale Analyses of Angiosperm Nucleotide-Binding Site-Leucine-Rich Repeat Genes Reveal Three Anciently Diverged Classes with Distinct Evolutionary Patterns. *Plant Physiol.* **2016**, *170*, 2095–2109. [[CrossRef](#)] [[PubMed](#)]
35. Wang, J.; Song, W.; Chai, J. Structure, biochemical function, and signaling mechanism of plant NLRs. *Mol. Plant* **2023**, *16*, 75–95. [[CrossRef](#)] [[PubMed](#)]
36. Zhang, H.; Chen, C.; Li, L.; Tan, X.; Wei, Z.; Li, Y.; Li, J.; Yan, F.; Chen, J.; Sun, Z. A rice LRR receptor-like protein associates with its adaptor kinase OsSOBIR1 to mediate plant immunity against viral infection. *Plant Biotechnol. J.* **2021**, *19*, 2319–2332. [[CrossRef](#)]
37. Ke, Y.-J.; Zheng, Q.-D.; Yao, Y.-H.; Ou, Y.; Chen, J.-Y.; Wang, M.-J.; Lai, H.-P.; Yan, L.; Liu, Z.-J.; Ai, Y. Genome-Wide Identification of the MYB Gene Family in *Cymbidium ensifolium* and Its Expression Analysis in Different Flower Colors. *Int. J. Mol. Sci.* **2021**, *22*, 13245. [[CrossRef](#)] [[PubMed](#)]
38. Ahmad, S.; Chen, G.; Huang, J.; Yang, K.; Hao, Y.; Zhou, Y.; Zhao, K.; Lan, S.; Liu, Z.; Peng, D. Beauty and the pathogens: A leaf-less control presents a better image of *Cymbidium* orchids defense strategy. *Front. Plant Sci.* **2022**, *13*, 1001427. [[CrossRef](#)]
39. Saucet, S.B.; Ma, Y.; Sarris, P.F.; Furzer, O.J.; Sohn, K.H.; Jones, J.D.G. Two linked pairs of *Arabidopsis* TNL resistance genes independently confer recognition of bacterial effector *AvrRps4*. *Nat. Commun.* **2015**, *6*, 6338. [[CrossRef](#)]
40. Pedley, K.F.; Martin, G.B. Molecular Basis of Pto-Mediated Resistance to Bacterial Speck Disease in Tomato. *Annu. Rev. Phytopathol.* **2003**, *41*, 215–243. [[CrossRef](#)]
41. Ercoli, M.F.; Luu, D.D.; Rim, E.Y.; Shigenaga, A.; Teixeira de Araujo, A.; Chern, M.; Jain, R.; Ruan, R.; Joe, A.; Stewart, V.; et al. Plant immunity: Rice *XA21*-mediated resistance to bacterial infection. *Proc. Natl. Acad. Sci. USA* **2022**, *119*, e2121568119. [[CrossRef](#)] [[PubMed](#)]
42. Li, M.; Dong, L.; Li, B.; Wang, Z.; Xie, J.; Qiu, D.; Li, Y.; Shi, W.; Yang, L.; Wu, Q.; et al. A CNL protein in wild emmer wheat confers powdery mildew resistance. *New Phytol.* **2020**, *228*, 1027–1037. [[CrossRef](#)] [[PubMed](#)]
43. Staal, J.; Kaliff, M.; Dewaele, E.; Persson, M.; Dixelius, C. RLM3, a TIR domain encoding gene involved in broad-range immunity of *Arabidopsis* to necrotrophic fungal pathogens. *Plant J.* **2008**, *55*, 188–200. [[CrossRef](#)] [[PubMed](#)]
44. Meyers, B.C.; Dickerman, A.W.; Michelmore, R.W.; Sivaramakrishnan, S.; Sobral, B.W.; Young, N.D. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant J.* **1999**, *20*, 317–332. [[CrossRef](#)] [[PubMed](#)]

45. Jiang, Z.; Zhao, M.; Qin, H.; Li, S.; Yang, X. Genome-wide analysis of NBS-LRR genes revealed contribution of disease resistance from *Saccharum spontaneum* to modern sugarcane cultivar. *Front. Plant Sci.* **2023**, *14*, 1091567. [[CrossRef](#)]
46. Tetreault, H.M.; Grover, S.; Scully, E.D.; Gries, T.; Palmer, N.A.; Sarath, G.; Louis, J.; Sattler, S.E. Global Responses of Resistant and Susceptible *Sorghum* (*Sorghum bicolor*) to Sugarcane Aphid (*Melanaphis sacchari*). *Front. Plant Sci.* **2019**, *10*, 145. [[CrossRef](#)] [[PubMed](#)]
47. Li, Q.; Jiang, X.-M.; Shao, Z.-Q. Genome-Wide Analysis of NLR Disease Resistance Genes in an Updated Reference Genome of Barley. *Front. Genet.* **2021**, *12*, 694682. [[CrossRef](#)] [[PubMed](#)]
48. Qian, L.-H.; Wang, Y.; Chen, M.; Liu, J.; Lu, R.-S.; Zou, X.; Sun, X.-Q.; Zhang, Y.-M. Genome-wide Identification and Evolutionary Analysis of NBS-LRR Genes From *Secale cereale*. *Front. Genet.* **2021**, *12*, 771814. [[CrossRef](#)] [[PubMed](#)]
49. Xiao, S.; Ellwood, S.; Calis, O.; Patrick, E.; Li, T.; Coleman, M.; Turner, J.G. Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by RPW8. *Science* **2001**, *291*, 118–120. [[CrossRef](#)]
50. Cannon, S.B.; May, G.D.; Jackson, S.A. Three Sequenced Legume Genomes and Many Crop Species: Rich Opportunities for Translational Genomics. *Plant Physiol.* **2009**, *151*, 970–977. [[CrossRef](#)]
51. Xue, J.Y.; Zhao, T.; Liu, Y.; Liu, Y.; Zhang, Y.X.; Zhang, G.Q.; Chen, H.; Zhou, G.C.; Zhang, S.Z.; Shao, Z.Q. Genome- Wide Analysis of the Nucleotide Binding Site Leucine-Rich Repeat Genes of Four Orchids Revealed Extremely Low Numbers of Disease Resistance Genes. *Front. Genet.* **2019**, *10*, 1286. [[CrossRef](#)]
52. Loutre, C.; Wicker, T.; Travella, S.; Galli, P.; Scofield, S.; Fahima, T.; Feuillet, C.; Keller, B. Two different CC-NBS-LRR genes are required for Lr10-mediated leaf rust resistance in tetraploid and hexaploid wheat. *Plant J.* **2009**, *60*, 1043–1054. [[CrossRef](#)] [[PubMed](#)]
53. Rushton, P.J.; Reinstädler, A.; Lipka, V.; Lippok, B.; Somssich, I.E. Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. *Plant Cell* **2002**, *14*, 749–762. [[CrossRef](#)] [[PubMed](#)]
54. Spoel, S.H.; Koornneef, A.; Claessens, S.M.C.; Korzelius, J.P.; Van Pelt, J.A.; Mueller, M.J.; Buchala, A.J.; Métraux, J.-P.; Brown, R.; Kazan, K.; et al. NPR1 Modulates Cross-Talk between Salicylate- and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. *Plant Cell* **2003**, *15*, 760–770. [[CrossRef](#)] [[PubMed](#)]
55. Yang, W.; Zhang, W.; Wang, X. Post-translational control of ABA signalling: The roles of protein phosphorylation and ubiquitination. *Plant Biotechnol. J.* **2016**, *15*, 4–14. [[CrossRef](#)]
56. Yang, J.; Xiong, C.; Li, S.; Zhou, C.; Li, L.; Xue, Q.; Liu, W.; Niu, Z.; Ding, X. Evolution patterns of NBS genes in the genus *Dendrobium* and NBS-LRR gene expression in *D. officinale* by salicylic acid treatment. *BMC Plant Biol.* **2022**, *22*, 529.

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