

Genome-wide identification and characterization of NBLRR genes in Finger millet (*Eleusine coracana* L.) and their expression in response to *Magnaporthe grisea* infection

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Abstract

Background

The nucleotide-binding site–leucine-rich repeat (*NBLRR*) genes significantly regulate defences against various phytopathogens. The genome-wide identification and analysis of *NBLRR* genes have been performed in several species. However, the evolution, structure, expression of *NBLRR*s and functional response to *Magnaporthe grisea* are not known in Finger millet (*Eleusine coracana* L.).

Results

The genome-wide scanning of the Finger millet genome resulted in 116 *NBLRR* genes named *EcNBLRR*s according to their chromosomal positions in ascending order. The phylogenetic analysis revealed divergence of *NBLRR*s as per the speciation of target species. The whole-genome duplications shaped the expansion of the *NBLRR* family in the finger millet genome, and purifying selection ($K_a/K_s < 1$) shaped expansions of *EcNBLRR* paralog and ortholog pairs. The maximum orthology and syntenic association of *NBLRR*s was observed between Finger millet and *Brachypodium distachyon* over Finger millet and *Arabidopsis thaliana*. The promoter sequence analysis showed various stress- and phytohormone-responsive *cis*-acting elements besides growth and development, indicating their role in disease defence and regulatory mechanisms. The functional analysis of 22 *EcNBLRR*s in the genotypes showing contrasting responses to *Magnaporthe grisea* infection revealed the role of target *EcNBLRR*s in finger millet- *Magnaporthe grisea* interactions at early (*EcNBLRR83*; *EcNBLRR55*; *EcNBLRR30* and *EcNBLRR32*) and late (*EcNBLRR76*; *EcNBLRR45*; *EcNBLRR26*; *EcNBLRR23* and *EcNBLRR21*) stage of infections.

Conclusion

The identification and functional analysis of *EcNBLRR*s showed the role of *EcNBLRR* genes in assigning blast resistance in finger millet. These results pave the foundation for in-depth and targeted functional analysis of *EcNBLRR*s through genome editing and transgenic approaches.

Background

Millet is one of the earliest domesticated small-seeded annual grass crops. Globally, ~130 countries are growing millets, which are the traditional food for more than 59 crore people in Asia and Africa. Among various millets used as food crops, viz. finger, foxtail, little, proso, barnyard, kodo millets etc., the *Eleusine coracana* L. Gaertn. (Finger millet) is considered one of the most important millets owing to wider cultivation in rainfed areas, its ability to sustain harsh dry environments and low soil fertility and its utility as a source of nutrition in poverty-stricken arid and semi-arid regions [1–3]. Since, Finger millet grains contain a significant portion of fibres, protein, vitamin B, minerals, essential amino acids, calcium and iron, thus it is considered nutritionally superior to wheat, rice and maize [1, 4, 5]. Apart from human consumption, the Finger millet straw is used as animal fodder, which possesses 60% of digestible

nutrients [6]. The current global Finger millet production of 4.5 million tons (<https://www.fao.org/faostat/en/>) is insufficient to meet the current and increasing demand of the population. On the other hand, Finger millet production and productivity are constrained by various stresses, viz. pests, diseases, drought, nutritional stresses *etc.* [7]. Among these production constraints, the Finger millet blast caused by an ascomycete filamentous fungus, *Magnaporthe grisea* (anamorphic stage, *Pyricularia grisea*), is the most devastating foliar pathogen that heavily affects the production and productivity of Finger millet [8] and causes substantial yield losses up to 50-100% [7]. Managing Finger millet blast by exploiting genetic resistance based on host-pathogen interactions is the most eco- and farmer-friendly sustainable approach.

The host has a genetically imprinted innate immune system that resists the pathogen attack mainly in the form of pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effectors-triggered immunity (ETI) [9]. Firstly, PTI will activate when the molecules from the pathogen interact with extracellular pattern-recognition receptors (PRR) lined up in the host plasma membrane [10, 11]. Nevertheless, to counteract the PTI, some pathogens evolve to secrete effector or avirulent (*Avr*) proteins (role in pathogenicity) into the plant cell and suppress the PTI, leading to cause infection. The secondary defence mechanism of ETI will be functioning where cytoplasmic immune receptors called R-proteins (resistance) recognize pathogen-derived *Avr* proteins, leading to a cascade of defence signalling pathways resulting in hypersensitive reaction (HR) or localized programmed cell death (PCD) [9, 12, 13]. Numerous R genes have been isolated in the last few years, especially from rice [14], cottonwood [15], papaya [16], *Arabidopsis thaliana* [17] and *Brassica rapa* [18] through genome-wide mining and characterization. The *NBLRR* (nucleotide-binding sites-leucine rich repeats), NBS domain with kinase1a, kinase2 and kinase3a motifs at the amino-terminal or central and LRR domain with leucine and hydrophobic amino acids towards the C terminal [13, 19, 20] constitute most of the existing R proteins in plants. The *NBLRR* genes encompass approximately 0.2 -1.6% of the genome of plant species [21]. The NBS domain is involved in signalling through interaction with bound ATP and GTP, whereas the C-terminal LRR domain help in the recognition of pathogen as well as protein-protein interactions in establishing disease resistance reactions [22, 23]. The role of *NBLRR* genes in disease resistance was reported in various crops, including powdery mildew in sunflower [24], various pathogens in cotton [25] and yam [26], powdery mildew in *Vitis vinifera* [13], downy mildew and black rot in Chinese cabbage [27] *etc.* The comparative genomic analysis showed homology between *NBLRR* and *EST* (expressed sequence tags) sequences of Finger millet and *Pi21* and *Pikh* of rice, which indicated conserved orthology of blast resistance among Finger millet and rice lineage [28, 29].

There were few efforts on the evolutionary and functional characterization of *NBLRR* genes in Finger millet [30]. However, these studies primarily relayed search-term-and PCR-based gene mining and could not capture the complete genome-wide snapshot of Finger millet *NBLRR* genes. Thus, we have framed our investigation to identify the Finger millet *NBLRR* genes in the latest Finger millet genome and comprehensive evolutionary and functional characterization and regulatory elements analyses.

Results

Mining of and physicochemical characterization of *EcNBLRR* genes and proteins

Genome-wide mining in Finger millet resulted in 116 *NBLRR* genes (**Table 1**). Further, we collected 159 and 79 orthologs of *Brachypodium distachyon* and *Arabidopsis thaliana*, respectively, for various evolutionary analyses. The physicochemical properties of *EcNBLRR* proteins showed significant variations in protein length ranging from 354 (*EcNBLRR50*) to 1452 (*EcNBLRR49*) amino-acids and molecular weight (MWs) from 40.07 (*EcNBLRR50*) to 1143.0 (*EcNBLRR21*) kDa. The maximum and minimum Isoelectric points (*pI*) were recorded as 9.30 (*EcNBLRR62*) and 4.8 (*EcNBLRR29*), respectively. The 50.86% of *EcNBLRR* proteins were acidic (*pI* < 7.0), and 49.14% were found basic (*pI* > 7.0). The majority of *EcNBLRR* proteins were localized in the cytoplasm (50), followed by the nucleus (30), chloroplast (29), whereas lowest *EcNBLRR* proteins found in the plasma membrane (4), endoplasmic reticulum (2; *EcNBLRR3*, *EcNBLRR23*) and peroxisomes (1, *EcNBLRR20*) (**Table 1**). Further, plotting of *EcNBLRRs* revealed uneven distribution across the Finger millet chromosomes. The maximum number (15) of *EcNBLRR* genes were found on chromosome 9A (15 genes), followed by 9B (13 genes) and 1B (12 genes). However, only one (*EcNBLRR21*) *EcNBLRR* gene was found on chromosome 2A (**Fig. 1; Table S1**).

Structural analysis of *EcNBLRR* genes and proteins in Finger millet

The term motif is a conserved amino acid or nucleotide sequence pattern that shows transcriptional or post-translational interactions of proteins or genes with assumed relation to a biological function of the macromolecule. Motifs identification server MEME identified ten conserved motifs structures distributed among 116 *EcNBLRR* proteins. Among ten motifs, motifs 3, 1 and 4 were predominantly present in most of the genes, followed by motifs 5, 2, 10, and 7. Motif 8 was present in 72 genes, followed by motif 9, recorded in 83 *EcNBLRR*. Except for *EcNBLRR4* and *EcNBLRR7*, motif 3 was found in all the *EcNBLRR* proteins in a repeated arrangement (**Fig. 2A**).

Structural analysis of *EcNBLRR* genes revealed the exons range from 1 to 10. The *EcNBLRR49* and *EcNBLRR37* showed highest number of exons (*n*=10), followed by *n*=6 (*EcNBLRR4*, *EcNBLRR103*, *EcNBLRR10*) and *n*=5 (*EcNBLRR35*, *EcNBLRR77*, *EcNBLRR109*, *EcNBLRR69*). The lowest number of exons (*N*=1) was observed in 52 (44.82%), followed by 2 in 28 (24.13%) and 3 in 18 (15.51%) *EcNBLRR* genes. Further, the majority of intronic sequences were found in phase-0 (70.47 %), followed by phase-1 (17.71 %) and phase-2 (11.81 %) (**Fig. 2B**).

Evolutionary genetics of *NBLRRs* in Finger millet, *Arabidopsis* and *Brachypodium*

Phylogenetic analysis of *NBLRRs*

Phylogenetic analysis showed the clustering of 354 *NBLRR* proteins into four main clusters: I, II, III and IV. Cluster-IV emerged as the largest group with 155 *NBLRR* transporters, followed by cluster-I (98 *NBLRRs*), cluster-III (67 *NBLRRs*) and cluster-II (34 *NBLRRs*). Cluster-IV clustered the highest *EcNBLRRs* (*n*=64) with 83 *BdNBLRRs* and 8 *AtNBLRRs*. However, cluster-II emerged as the smallest group with 34 *NBLRRs*

transporters (27 from *Brachypodium*, six from Finger millet and one from *Arabidopsis*). Interestingly, most AtNBLRRs (68) were clustered in cluster-I (**Fig. 3**). Interestingly, clusters II, III and IV encompass only 2.94, 2.98 and 5.16% of AtNBLRRs in contrast to 69.38% in cluster-I. On the other hand, the subclusters IIb, IIIa, IVc and IVd are entirely devoid of AtNBLRRs otherwise clustered only EcNBLRRs and BdNBLRRs. Thus, in the current investigation, the overall topology of NBLRR phylogenetic tree showed the lineage-specific evolution and divergence of NBLRRs in the target species.

Duplication of *NBLRR* genes in Finger millet

The duplication analysis was performed to study the divergence of *NBLRR* genes in the Finger millet genome. The results revealed 473 duplication pairs among the 116 *EcNBLRRs* distributed across the 18 chromosomes. Further, the results showed that whole-genome duplication (WGD) is the primary evolutionary force in the divergence of *EcNBLRRs*. (**Fig. 4A**). The mean synonymous substitutions ($K_s=1.64$) were found predominately in *EcNBLRR* paralogs over the mean non-synonymous (0.75) substitutions suggesting the prominent role of purifying selection ($K_a/K_s = 0.46$) on the divergence of *NBLRRs* in Finger millet genome (**Fig. 4B; Table S2**).

Orthologs and synteny of *NBLRRs* of among Finger millet and model plants

Homology-based search by OrthoFinder identified 114 orthologs *NBLRR* pairs among model species *Arabidopsis thaliana* and *Brachypodium distachyon*, and Finger millet. As per evolutionary lineage, the maximum orthologs were identified between *Brachypodium distachyon* and Finger millet ($N=81$), followed by *Arabidopsis thaliana* and *Brachypodium distachyon* ($N=18$) and *Arabidopsis thaliana* and Finger millet ($N=15$) (**Fig. 5A**). The K_a/K_s ratio was obtained with statistical significance of $P > 0.001$ for *NBLRR* ortholog pairs from the three target species used in the current study. Selection pressure analyses were calculated and showed a mean of 0.29 K_a/K_s ratio, ranging between 0.09 (BdNBLRR90-EcNBLRR47) and 0.53 (BdNBLRR93-EcNBLRR42). Thus, the *NBLRR* orthologs evolved under strong purifying selection ($K_a/K_s < 1.0$) (**Fig. 5B; Table S3**).

To dissect the syntenic association of *NBLRR* genes among the target genomes, the pairwise-syntenic association analysis was performed among Finger millet, *Arabidopsis* and *Brachypodium*. Only four collinear blocks were observed between *Arabidopsis*-Finger millet with four *EcNBLRRs* and three *AtNBLRRs* (**Fig. 5C; Table S4**). However, the maximum number of collinear blocks of *NBLRR* genes observed between *Brachypodium*-Finger millet were $N = 29$, encompassing 32 *EcNBLRRs* and 23 *BdNBLRRs* (**Fig. 5D; Table S4**).

Functional analysis of *NBLRRs* in Finger millet

The *cis*-acting elements analysis of *NBLRR* genes in Finger millet

The *cis*-acting elements predicted in the 1.5kb upstream promoter sequences of *EcNBLRRs* were grouped into five different functional categories such as (i) core promoter, (ii) growth and development, (iii) hormonal-responsive, (iv) light-responsive and (v) stress-responsive elements (**Fig. 6**). The results

revealed that all the promoter sequences of *EcNBLRR* genes displayed a higher occurrence in core elements such as *CAAT-box* ($N=2571$), followed by *TATA-box* ($N=2186$) and *AT~TATA-box* ($N=231$), respectively (**Fig. S1**). Among the growth- and development-related elements, *CAT-box* found in higher occurrence ($N=75$) known to regulate meristem expression, followed by *O₂-site* ($N=56$) related to zein metabolism regulation, and circadian element control the circadian cycle, *GCN4-motif* regulate the endosperm growth and the element *RY* implicated in seed-specific regulation (**Fig. S2**).

Further, 162 copies of 6 hormonal-responsive *cis*-acting elements were categorized into auxin (*AuxRR*-core, *TGA*-element, *TGA*-box), gibberellins (*GARE*-motif, *TATC*-box, *P-box*) respectively. Among the hormonal-responsive *cis*-acting elements, the gibberellins regulating elements were found to be more ($N=91$), followed by auxin ($N=71$) (**Fig. S3**). A total of 860 light-responsive *cis*-acting elements falling under 34 types were identified. The maximum number of light-responsive elements were observed for *G-box* (279), followed by *Box-4* (90), *GT1-motif* (68), *TCT-motif* (62) and *GATA-motif* (56) (**Fig. S4**). The 34 stress-responsive *cis*-acting elements were present in 2,906 copies across the *EcNBLRRs* promoter sequences. The abscisic acid-responsive *ABRE* (251) elements were more predominant stress-responsive elements, followed by *CGTCA-motif* (210) related to exogenous methyl jasmonate (MeJA) responsive element and anaerobic responsive *ARE* (154) elements. Further, the *TCA* (39) and *SARE* (2) related to salicylic acid (SA) responsiveness were reported. Additionally, *TC-rich repeats* (44), *MBSI* (6) and *WUN-motif* (3) elements related to defence and stress-responsive, flavonoid gene synthesis and wound-responsiveness, respectively, were distributed among the promoter sequences of *EcNBLRRs* (**Fig. S5**).

Expression analysis of *EcNBLRR* genes against *M. grisea* infection

Upon *M. grisea* inoculation, the minute brown spots appeared in susceptible genotype Uduru Mallige at 3-4 dpi, thereafter, enlarged to develop a typical spindle-shaped blast lesion with grey or white centre characteristics at 7-8 dpi. Subsequently, these lesions spread to the entire leaf lamina and become dead leaves for which scale 8 and 9 were assigned. Conversely, the resistant genotype VL Mandua-352 developed a hypersensitive reaction (HR) with minute brown or little elongated spots randomly scattered at 4-6 dpi. However, these spots failed to expand further, for which scale 2 and 3 were assigned. The PDI of blast severity revealed 82.59% and 11.85% of blast incidence in Uduru Mallige (susceptible) and VL Mandua-352 (resistant), respectively. Control plants treated with sterile water had no symptoms (**Fig. 7; Table 2**).

The *EcNBLRRs* expression analysis was conducted to decipher the functional response of *EcNBLRRs* to blast in the genotypes Uduru Mallige (susceptible) and VL Mandua-352 (resistant) showing contrasting disease reactions (**Fig. 8**). A total of eight *EcNBLRR* genes showed significantly enhanced expression with the time of infection (*EcNBLRR2*, *EcNBLRR23*, *EcNBLRR26*, *EcNBLRR45*, *EcNBLRR53*, *EcNBLRR67*, *EcNBLRR68* and *EcNBLRR76*) in resistant genotype VL Mandua-352. *EcNBLRR21*, *EcNBLRR66* and *EcNBLRR71* showed significant expression at 72 hpi, however no significant difference was observed between 0 and 24 hpi, whereas in case of *EcNBLRR97* the fold change between 0 and 24 hpi was significant while 24 and 72 hpi remained unchanged. Also, *EcNBLRR97* showed enhanced

expression in both Uduru Mallige (susceptible) and VL Mandua-352 genotypes. The susceptible genotype Uduru Mallige has shown a significant inverse relationship between the time point of inoculation and expression of nine *EcNBLRRs* (*EcNBLRR23*, *EcNBLRR26*, *EcNBLRR45*, *EcNBLRR53*, *EcNBLRR55*, *EcNBLRR71*, *EcNBLRR76*, *EcNBLRR83*, and *EcNBLRR116*). Similarly, non-significant expression was observed between 24 and 72 hpi in the case of *EcNBLRR68* and 0 and 24 hpi for *EcNBLRR30*. On the contrary, the *EcNBLRR67* showed no significant expression in Uduru Mallige across the time points (0-72 hpi) (**Fig. 8**).

The genes *EcNBLRR30*, *EcNBLRR32*, *EcNBLRR46*, *EcNBLRR55* and *EcNBLRR83* in VL Mandua-352 showed highest significant expression at 24 hpi than 72 hpi, whereas the vice-versa for *EcNBLRR1*, *EcNBLRR7*, *EcNBLRR19*, *EcNBLRR21*, *EcNBLRR23*, *EcNBLRR26*, *EcNBLRR33*, *EcNBLRR45*, *EcNBLRR53*, *EcNBLRR66*, *EcNBLRR67*, *EcNBLRR68*, *EcNBLRR76* and *EcNBLRR116* in same genotype. Furthermore, the susceptible Uduru Mallige showed peak significant expressions of *EcNBLRR1*, *EcNBLRR19*, *EcNBLRR21*, *EcNBLRR32*, *EcNBLRR33*, and *EcNBLRR46* at 24 hpi than 72 hpi, whereas vice-versa pattern for *EcNBLRR97* (**Fig. 8**).

The *EcNBLRR83* gene in VL Mandua-352 showed 3.07 FC expression compared to 0.77 FC in susceptible genotype Uduru Mallige at 24 hpi ($P = 0.0001$). The *EcNBLRR55* recorded higher FC (2.94) in VL Mandua-352 against 0.71 FC in susceptible genotype Uduru Mallige induced at 24 hpi ($P = 0.0001$). Similar patterns were followed by *EcNBLRR30* (FC: 2.42) and *EcNBLRR32* (FC: 2.42) in VL Mandua-352 compared with Uduru Mallige (*EcNBLRR30*: 0.9; *EcNBLRR32*: 1.38) at FC in 24 hpi ($P = 0.001$). However, all the above genes showed significantly decreased FC expression at 72 hpi ($P = 0.001$). The *EcNBLRR76* showed 2.45 fold expression in VL Mandua-352 compared to 0.87 FC in susceptible cultivar Uduru Mallige at 72 hpi ($P = 0.0001$). Similar trends of expressions were also followed by *EcNBLRR45* (FC: 2.31), *EcNBLRR26* (FC: 2.13), *EcNBLRR23* (2.11), and *EcNBLRR21* (2.06) recorded higher fold expression in VL Mandua-352 compared to FC expressions in susceptible line Uduru Mallige (*EcNBLRR45*: 0.46; *EcNBLRR26*: 0.60; *EcNBLRR23*: 0.26 and *EcNBLRR21*: 0.57) ($P > 0.0001$) (**Fig. 8**).

The statistically significant ($P > 0.001$) and more than 2-fold expression of, *EcNBLRR30*, *EcNBLRR32*, *EcNBLRR55* and *EcNBLRR83* were observed at 24 hpi and *EcNBLRR21*, *EcNBLRR23*, *EcNBLRR26*, *EcNBLRR45* and *EcNBLRR76* at 72 hpi compared to control as well as the expression levels in susceptible genotype Uduru Mallige indicating these could be putative target genes associated with blast resistance in Finger millet (**Fig. 8**).

Discussions

Finger millet is one of the most important millets, but the production is constrained by blast infection leading to severe yield losses. Thus, a better understanding of host-pathogen interactions is crucial in the genetic improvement of blast resistance in Finger millet. Several genomic regions carrying resistance (R) genes against potentially important pathogens have been reported in several plant species [31]. Among various R genes, the *NBLRR* genes are the immune receptors that recognize the pathogen effector

molecules (specific/non-specific) and trigger defence responses in host plants [32]. Studies on *NBLRRs* have already been extensively explored on various plant species, including *A. thaliana* [17], *O. sativa* [14], *Zea mays* [33], *P. trichocarpa* [15], *V. vinifera* [13], *D. rotundata* [26], Chinese cabbage [27], *Cucumis sativus* [34] etc in response to various disease resistance. However, identifying *NBLRRs* and detailed characterization and functional analysis in response to *M. grisea* infection in Finger millet is not yet explored. Hence, the current investigation aimed to characterize and validate the *NBLRR* genes against *M. grisea* infection in Finger millet.

Here, we identified 116 non-redundant NBS-encoding R genes with at least one leucine-rich repeat domain toward the carboxy-terminal end in the Finger millet genome (**Table 1; Table S1**) using both homology-based blast search and PFAM-based HMM-scan and -search. To study the evolutionary patterns of *NBLRRs* among three target species with the same approach, we have mined 79 and 159 *NBLRRs* homologs of the model plants *A. thaliana* and *B. distachyon* genomes, respectively. However, the mined *NBLRRs* homologs were part of previous reports in *A. thaliana* (167, [18]; 149, [35]) and *B. distachyon* (126, [36]), and the discrepancies in the number could be due to mining approaches employed, the E-values in BLAST search and HMM search and scan, and updated genome assemblies.

The physicochemical properties of EcNBLRR proteins showed high variation in protein length (354-1452 aa) and molecular weights (40.07-1143.00 kDa). The structural variation of EcNBLRRs could be associated with their functional diversification in dealing with various sorghum pathogens. The proper subcellular localization or targeting of R proteins is a prerequisite for assigning resistance to pathogens. The maximum EcNBLRRs showed subcellular localizations in the cytoplasm (50) and nucleus (30) (**Table S1**). Many reports showed the localization of the R proteins in nucleus and cytoplasm under pathogen infection, even without clear nuclear localization signals (NLS) in most of the R protein sequences [37, 38]. However, in some of the cases, the presence of NLS is shown to be essential for resistance reactions in barley, tobacco and Arabidopsis R proteins [37, 39–41]. Few studies showed differentiated immune responses from cell death signalling. For instance, the suppressed cell death and enhanced immune response activity of R protein MLA10 results when targeted to the nucleus, but cell death is enhanced when it is in the cytoplasm [42]. Further, the structural analysis of *EcNBLRRs* variation in the number of exons per EcNBLRR (1 to 10) and intronic phases. Most introns were found at phase-0 (70.47%), followed by phase-I (17.71%). The phase-0 intron does not disrupt a codon, whereas the phase-1 and phase-2 intron disrupt a codon between the first and second and third bases, respectively [43]. The distribution of intron phases is non-uniform: phase-0 introns occur most frequently, and phase-2 introns occur least frequently, suggesting that the proportion of phase-0 introns increased over evolution [43–46].

The topology of the *NBLRR* phylogenetic tree mostly showed clustering of *NBLRR* entities in lineage-specific patterns. The 86.07% AtNBLRRs falling in the dicot lineage are grouped in cluster-I with 12.93% and 9.43% of EcNBLRR and BdNBLRRs, respectively. Especially the subcluster Ib showed a prominent grouping of AtNBLRRs. The Finger millet and *B. distachyon* NBLRRs belonging to monocot lineages showed more mixed clustering than *A. thaliana*. The lineage-specific expansion of *NBLRRs*

could be associated with the lineage-specific evolution of host-pathogen interactions. Thus, the expansion of NBLRRs occurred along the evolutionary patterns of species lineages.

The whole genome duplications (WGDs) expanded *EcNBLRRs* under strong purifying selection in the Finger millet. The WGD, followed by functional diversification, played a prominent role in the expansion, evolution, and diversification of various polyploid gene families [47]. There are reports on both rapid and slow evolutionary patterns of NBLRRs in plants with frequent and rare gene conversions, respectively [48, 49]. This indicates that gene duplication and unequal crossing-over are followed by density-dependent purifying selection in the evolution of *NBLRRs* [49]. Thus, the functional diversification of duplicated *NBLRRs* supports adaptation and has likely been favoured by natural selection during evolution. Synteny gives the genomic framework for conserved homologs and their order between the different species' genomes. The syntenic association of *EcNBLRRs* with model plant species, *A. thaliana* and *B. distachyon*, showed maximum collinear blocks between Finger millet and *B. distachyon* than Finger millet and *A. thaliana* owing to close evolutionary association. The maximum shared synteny between Finger millet and *B. distachyon* genomic fragments from these species originated from a more immediate identical ancestor than Finger millet and *A. thaliana* in evolutionary lineage [50].

The scanning of *EcNBLRR* promoter sequences showed significant occurrences of stress-, hormonal- and light-responsive *cis*-elements besides core and growth & development related *cis*-elements (**Fig.6**). The results hint at the additional direct and indirect association of *EcNBLRRs* with various developmental and stress-responsive activities in addition to coding for disease resistance. The stress-responsive phytohormones, *viz.* salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), mediate the signal transduction pathways for defences against diseases and other stresses in plants [51]. For instance, enhanced expression of an *NBLRR* gene *ADR1* in the presence of salicylic acid resulted in drought tolerance [52]. Supportingly, 39 and 2 *EcNBLRRs* showed salicylic acid-responsive *SARE* and *TCA* elements, respectively. Similarly, salicylic acid-responsive TCA-element between -563 bp and -249 bp upstream of an R gene *OsPIANK1* regulates resistance mechanism to *M. oryzae* infection [53]. The *ABRE* elements were present in higher proportion among the *EcNBLRRs* promoters (291), which acts as a binding site for ABA-dependent transcription factors to regulate various stresses [54, 55]. The *G-box* is a ubiquitous light regulatory element in many of the promoters [56] and is highly represented light-responsive *cis*-element (279) among the *EcNBLRRs* promoters, followed by *Box-4* (90), *GT-1* (62) motif etc. Several studies have shown the essential roles of a particular spectrum of light in promoting plant defence against various pathogen infections [57]. For instance, the red-light spectrum enhances the plants' resistance to various pests and pathogens [58]; however, the detailed molecular basis still needs to be elucidated. Thus, the diverse *cis*-acting elements could modulate the expression of *EcNBLRRs* towards direct and indirect regulation of disease resistance in Finger millet.

Functional qRT-PCR analysis of 22 *EcNBLRRs* in response to *M. grisea* infection showed typical genotypic- and infection-specific expression patterns in Finger millet. The nine *EcNBLRR* genes were showed enhanced fold expressions at 24 hpi (*EcNBLRR83*: 3.07; *EcNBLRR55*: 2.94; *EcNBLRR30*: 2.42; *EcNBLRR32*: 2.02) and 72 hpi (*EcNBLRR76*: 2.45; *EcNBLRR45*: 2.31; *EcNBLRR26*: 2.13; *EcNBLRR23*: 2.11;

EcNBLRR21: 2.06) in the resistant genotype VL Mandua-352 compared to susceptible genotype Uduru Mallige. The compatible interactions between the susceptible host and the pathogen result in a substantial increase in the fungal growth on the host. On the other hand, incompatible interactions between resistant genotype and pathogen result in resistance reaction owing to arresting of fungal growth with increased plant defence reaction during early colonization stages [59]. Further, the expression of host-specific resistance genes temporally differs significantly with the point of infection, which could add to the resistance in stepwise mode, *i.e.*, at pathogen penetration and multiplication modes. The defence response in the resistant genotypes is associated with enhanced expression of *R* genes (*EcNBLRR83*: 3.07; *EcNBLRR55*: 2.94; *EcNBLRR30*: 2.42; *EcNBLRR32*: 2.02) during an early stage of pathogen infection leading to a hypersensitive reaction. On the other hand, the *R* genes differentially expressed in the contrasting genotypes VL Mandua-352 and Uduru Mallige in the late infection stage (*EcNBLRR76*: 2.45; *EcNBLRR45*: 2.31; *EcNBLRR26*: 2.13; *EcNBLRR23*: 2.11; *EcNBLRR21*: 2.06) are probably involved in the post-penetration multiplication of pathogen [60].

Conclusion

The current study identified 116 *EcNBLRR* genes through genome-wide mining with homology-based BLAST with query NBLRR sequences, and HMM search and scan with PFAM database. We also explored various structural characteristics features like motif analysis, subcellular prediction, and functional analysis for 116 identified *EcNBLRR* genes. The *EcNBLRR* genes have a broad range of variations in length, number of exons and intronic phases, molecular weight, isoelectric point and subcellular localization. *Cis*-acting analysis revealed various hormonal and light signalling and stress-responsive elements. The syntenic association indicated that *EcNBLRR* genes displayed higher homology in the *Brachypodium*-Finger millet pair than in the *Arabidopsis*-Finger millet. The qRT-PCR gene expression analysis showed 9 *EcNBLRR* candidate genes associated with resistance reaction *M. grisea* in resistant genotype VL Mandua-352. The present study provides the fundamental resource in molecular breeding to enhance blast resistance in Finger millet.

Materials and Methods

Genome-wide mining, physiochemical characterization and chromosomal localization of *EcNBLRR* genes and proteins

In the first step, 88 and 126 *NBLRR* proteins of *Arabidopsis thaliana* and rice, respectively, were retrieved from TAIR (<http://www.arabidopsis.org/>) and the rice genome annotation project (<http://rice.uga.edu>) using search term 'NBLRR' were BLAST aligned with latest Finger millet proteome (Ecorocona_560>v1.1.protein.fa) retrieved from EnsemblPlants (<https://plants.ensembl.org>) with an e-value cut-off of $1e-5$. Secondly, the HMM models were built for *Arabidopsis thaliana* and rice *NBLRR* query protein sequences and the NB-ARC Pfam domain (PF00931; <http://pfam.xfam.org>) was scanned against Finger millet proteome (Ecorocona_560.v1.1.protein.fa) with an e-value cut-off of 0.01. The non-redundant hits from the first and second steps were subjected to HMM search with the Pfam database

(<http://pfam.xfam.org>). The sequences showing the NBS domain towards N (amino) terminal and LRR domain towards C-terminal were considered for subsequent analysis (**Fig. S6A**). Based on chromosomal positions, the gene model hits were sequentially named based on chromosomal positions, and physiochemical properties were predicted using the ProtParam tool (<https://web.expasy.org/protparam>). The subcellular localization was determined using the WoLFPSORT: Protein Subcellular Localization Prediction(<https://wolfpsort.hgc.jp>).

Domain, motifs, and gene structure analysis of *NBLRR* proteins in Finger millet

The domain features of *EcNBLRR* proteins were examined using Pfam and SMART databases with default parameters. The top ten conserved motifs of *EcNBLRR* proteins were predicted through MEME suit (<https://meme-suite.org/meme>) with default parameters [61]. The gene structure and intronic phases of *EcNBLRR* genes were visualized in TBtool [62] using the GFF3 file of *Eleusine coracana* downloaded from the EnsemblPlants database (<https://plants.ensembl.org/>) [63].

***Cis*-acting element analysis**

The 1.5kb upstream promoter region of each *EcNBLRR* gene from the start codon was examined for *cis*-acting elements with the PlantCare server (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) [64]. The predicted *cis*-acting elements were classified into subcategories, and the distribution was visualized within the 1.5 kb upstream regions of *EcNBLRR* genes.

Sequence alignment and phylogenetic analysis

For evolutionary analysis, the *EcNBLRR* homologs from two model plant species *Arabidopsis thaliana* and *Brachypodium distachyon* were mined (**Fig. S6B**). The phylogenetic analysis was used to determine the evolutionary association among *NBLRR* sequences of Finger millet with model plant species *Arabidopsis thaliana* and *Brachypodium distachyon*. The multiple sequence alignment of *NBLRRs* was carried out using the MUSCLE [65]. The multiple sequence alignment was trimmed using the trimAL tool (<http://trimal.cgenomics.org/>) [66]. Then, the aligned and trimmed sequences were used for phylogenetic tree construction using IQTREE.v.16.12 (<http://www.iqtree.org/>) with Maximum Likelihood Method and 1000 bootstrap replicates. The phylogenetic tree was visualized with iTOL v6 (<https://itol.embl.de>) server.

Duplication, orthology, collinearity, syntenic and selection pressure analyses

The self-BLASTp search (e-value < $1e-5$) within the Finger millet proteome was performed using BLAST+ 2.12.0. Further, the DupGen_finder was used to analyse various duplications [67]. The orthologs of Finger millet *NBLRR* genes with model plants *Arabidopsis thaliana* and *Brachypodium distachyon* was deciphered with OrthoFinder (<https://github.com/davidemms/OrthoFinder>) [68] and visualized through Cytoscape software (Shannon et al., 2003). The sequences from the whole proteome of Finger millet, *Arabidopsis thaliana* and *Brachypodium distachyon* were aligned with the BLASTp program (e-value < $1e-5$). The MCS-X program was employed to identify the collinear blocks [69]. The ParaAT2.0 (<https://ngdc.cncb.ac.cn/tools/paraat>) software was used to align the *NBLRR* ortholog pairs among

Finger millet, *Arabidopsis* and *Brachypodium* [70] and aligned orthologues were used to calculate the non-synonymous (Ka), and synonymous rate (Ks). Subsequently, the evolutionary constraint of Ka and Ks among each *NBLRR* ortholog pair was calculated using KaKs_calculator 3.0 with the GY-HKY method [71].

Plant material, pathogen inoculation, blast phenotyping and sample collection

The virulent Finger millet *M. grisea* isolate Ragi Almora was used in this study to inoculate on resistant (VL Mandua-352) and susceptible (Uduru Mallige) genotypes under artificial conditions [72]. The fungal isolate was retrieved from preservation stock, multiplied on oatmeal agar medium (HiMedia Laboratory Pvt. Limited Bombay, India) and incubated at 26 °C with 12 hrs light and dark alternation periods [73]. The plants were grown under a controlled environment at a blast phenotyping facility of ICAR-IARI, New Delhi in a randomized complete block design (RCBD) with three replications. Each replicating plastic pot (9.5 × 10 cm) carried approximately fifty plants. After ten days of fungus incubation, the mycelium was gently scrapped using a sterile glass slide containing 10 ml of sterile water and filtered through a double-layered sterilized muslin cloth. Finally, culture suspension of *M. grisea* was adjusted to 1×10^6 conidia/ml and added with 0.05% Tween 20 (HiMedia Laboratory Pvt. Limited Bombay, India) before inoculation on 20 days old plants. The inoculated plants were maintained at $25 \pm 1^\circ\text{C}$ and 95% RH with 12 hrs alternate photoperiods for blast development [72, 73]. The similar inoculation with sterile water alone served as a control. Leaf blast symptoms were calculated at 7-8 dpi (days post inoculation) using a 0-9 disease scale grade suggested by Babu and co-workers [73] (Table S5). The blast disease severity was assessed and expressed as per cent disease incidence (PDI) [72].

RNA isolation, cDNA synthesis and qRT-PCR expression study

The total RNA was isolated from the replicated biological and technical samples from 0, 24 and 72 hrs intervals using Trizol reagent, Invitrogen, USA [72]. RNA quality and integrity were quantified in NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and visualized in 1.5 % agarose gel electrophoresis. After that, RNA was converted into cDNA (complementary-DNA) using a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, USA). Twenty-two PCR primers were designed for selected *EcNBLRR* genes using primer3blast software (<https://primer3plus.com/cgi-bin/dev/primer3plus.cgi>) (Table S6). For qRT-PCR analysis, the first-strand cDNA was used (Roche Life Science, Penzberg, Germany) with Finger millet endogenous coding gene *Actin* as internal control with the following conditions: denaturation at $95^\circ\text{C}/2$ min (initial), annealing of 35 cycles at $58^\circ\text{C}/1.0$ min and extension at $72^\circ\text{C}/10$ s followed by three-step melting at $95^\circ\text{C}/10$ s, $63^\circ\text{C}/60$ s, and $97^\circ\text{C}/10$ s, and cooling at $37^\circ\text{C}/30$ s. For the calculation of $2^{-\Delta\Delta\text{CT}}$ [74] the Ct mean values were considered, and fold change expression changes were estimated and interpreted [72, 75]. Two-way ANOVA was performed using the Bonferroni post-hoc test to determine the statistical significance.

Declarations

Ethics approval and consent to participate.

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All the raw data sets used in this study were downloaded from publicly available databases. The necessary supporting data is provided as a supplementary file.

Competing interests

The authors declare no competing interests.

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Authors' contributions

GP and MGM conceived and designed the experiment; AB, MGM, SB, SCN and HR performed the experiments and analysis.; AB and MGM wrote the original draft; GP, MGM and TSC supervised and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Tables

Tables 1 to 2 are available in the Supplementary Files section

Figures

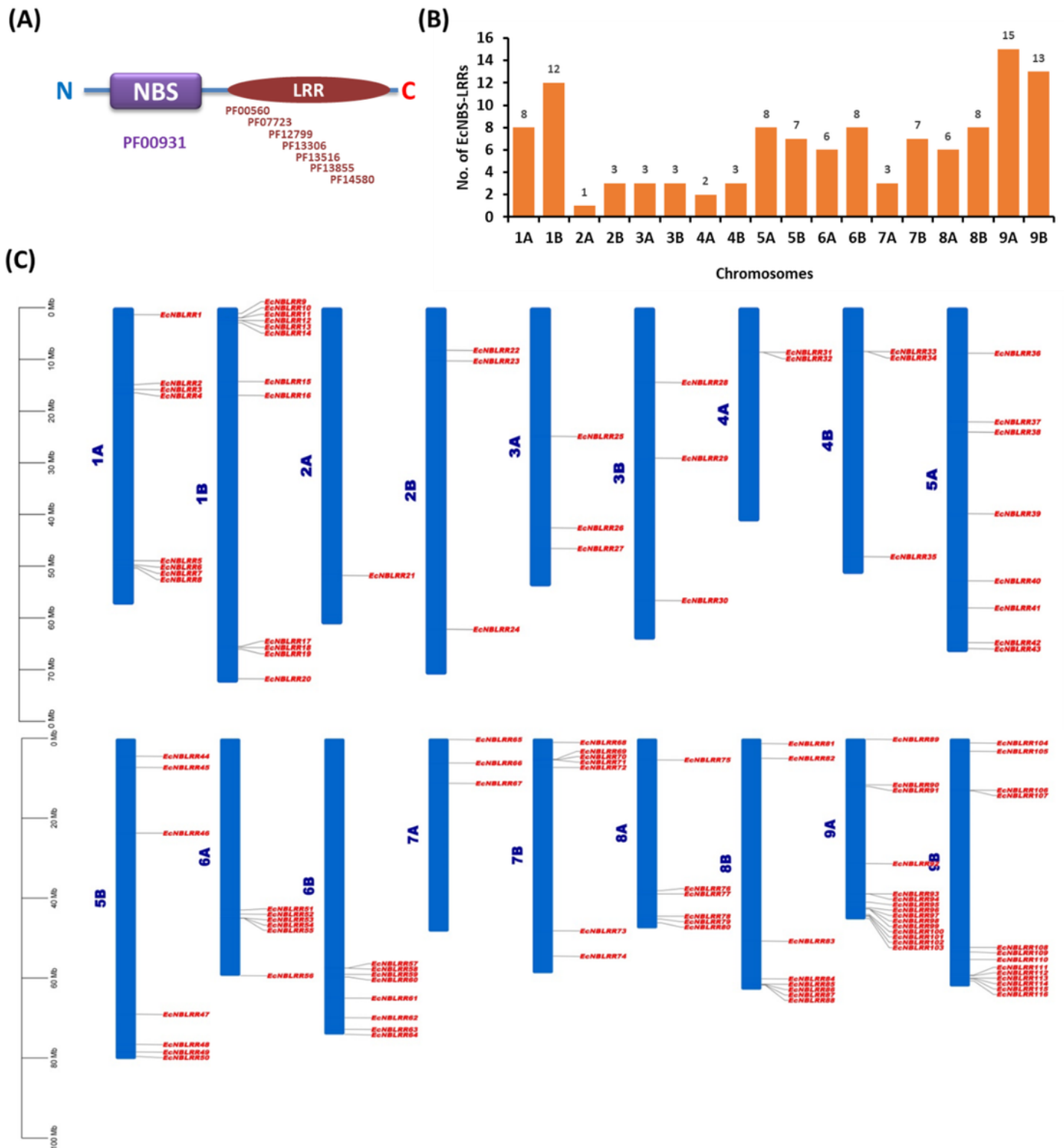


Figure 1

(A) The structural features considered for mining of NBLRRs in Finger millet; (B) The chromosome wise allocation of *EcNBLRRs* and (C) The distribution of *EcNBLRR* gene family in Finger millet genome.

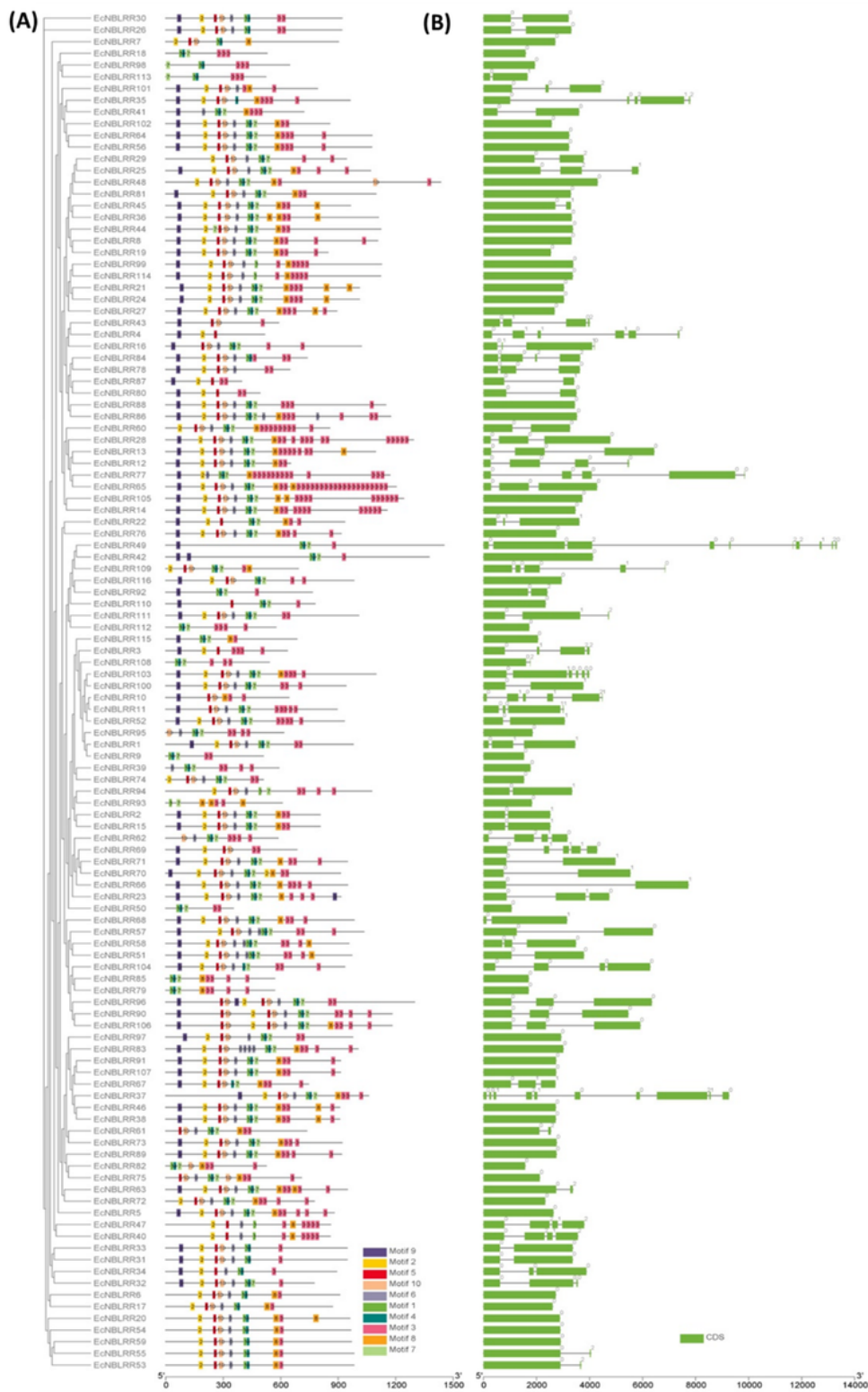


Figure 2

The phylogenetic association, distribution of conserved motif and gene structure analyses of EcNBLRR proteins and genes. (A) Distribution of conserved motifs in EcNBLRR proteins. The best ten conserved motifs in EcNBLRRs are displayed in different coloured and numbered boxes. (B) EcNBLRRs gene structures. The description of exons and introns adopts image tools. The green boxes coding exons and the line represents the intron sequences.

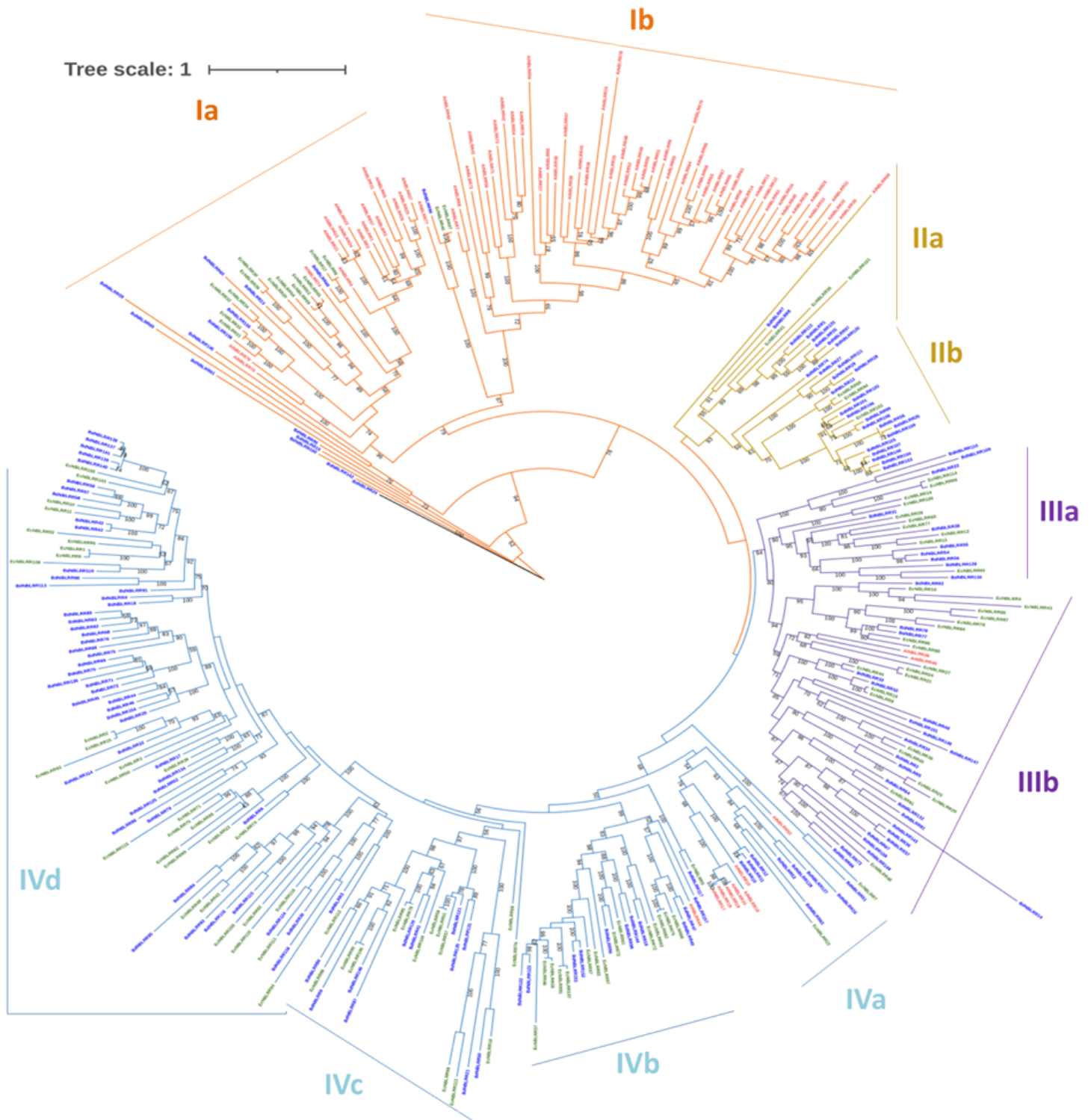


Figure 3

The phylogenetic tree of NBLRRs from Finger millet and model plant species *Arabidopsis thaliana* and *Brachypodium distachyon*. The clades I, II, II and IV are represented by orange, dark yellow, indigo and blue colours, respectively. The NBLRRs from Finger millet, *Arabidopsis thaliana* and *Brachypodium distachyon* proteins are represented with green, red and blue colours, respectively. Phylogenetic trees were constructed with IQTREE.v.16.12 using Maximum Likelihood method and 1000 bootstrap replicates.

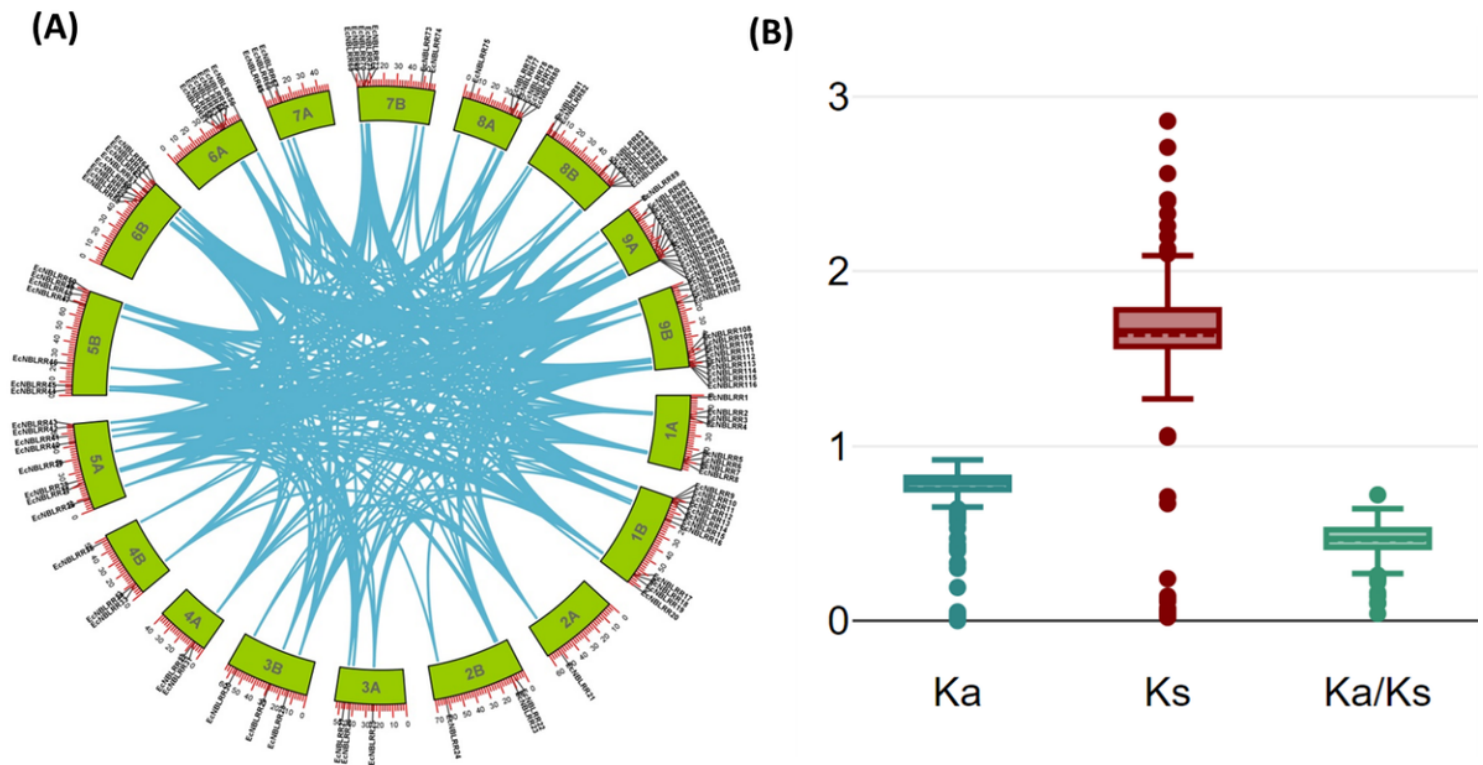


Figure 4

(A) Schematic representations of the chromosomal distribution and duplication relationships of Finger millet *NBLRR* genes and (B) The box and whisker plots for non-synonymous substitutions (K_a), synonymous substitutions (K_s) and ratio of non-synonymous and synonymous substitutions (K_a/K_s) among the *NBLRR* paralogs of Finger millet.

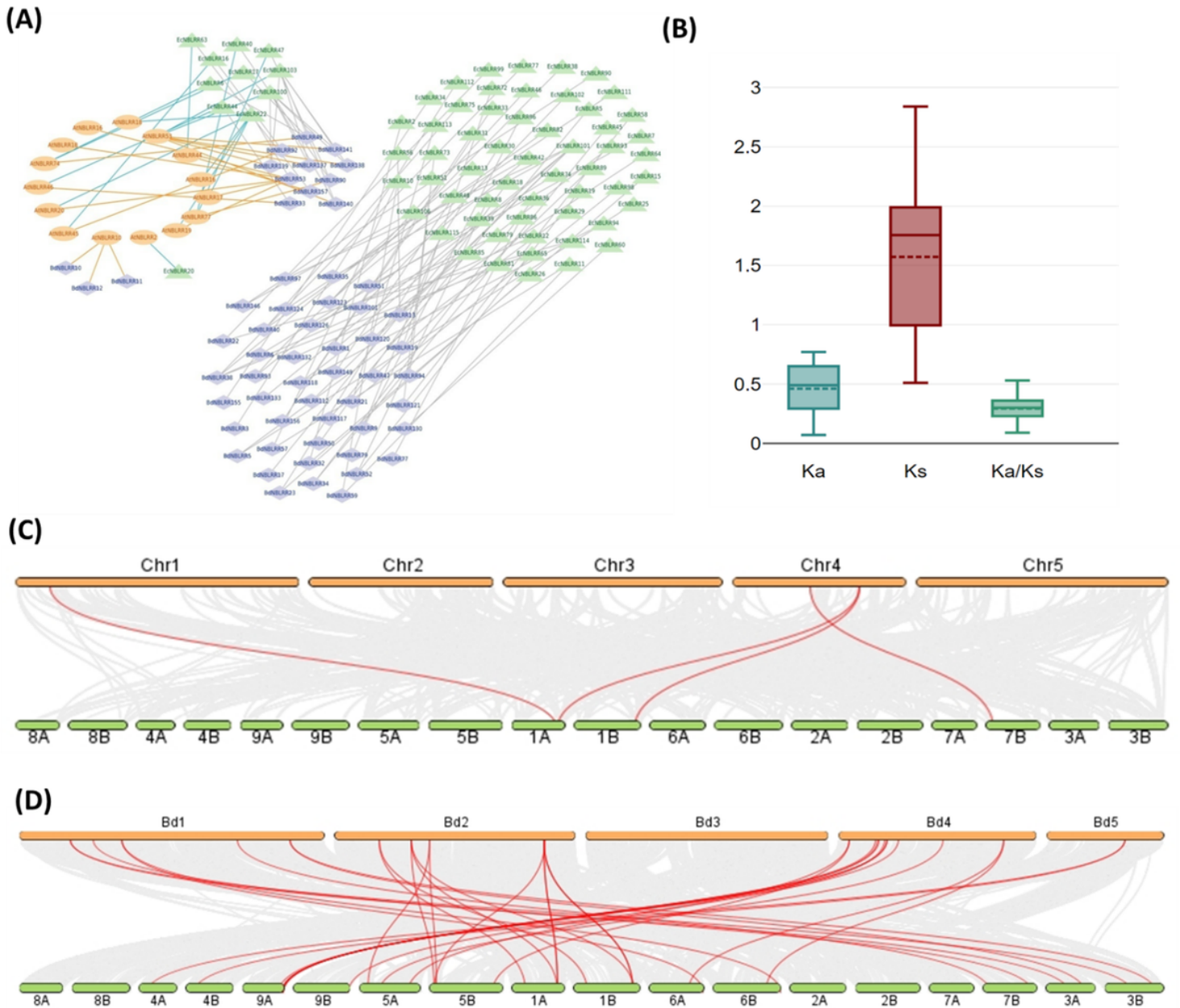


Figure 5

(A) The orthology among the NBLRR genes of Finger millet (green triangle), *Arabidopsis thaliana* (orange ellipse) and *Brachypodium distachyon* (indigo diamond); (B) The box and whisker plots for non-synonymous substitutions (Ka), synonymous substitutions (Ks) and ratio of non-synonymous and synonymous substitutions (Ka/Ks) among the NBLRR ortholog of target species; (C) The collinearity relationships between NBLRR genes of *Arabidopsis thaliana* and Finger millet. The orange and green blocks represent the chromosome of *Arabidopsis thaliana* and Finger millet, respectively and (D) The collinearity relationships between NBLRR genes of *Brachypodium distachyon* and Finger millet. The orange and green blocks represent the chromosome of *Brachypodium distachyon* and Finger millet, respectively.

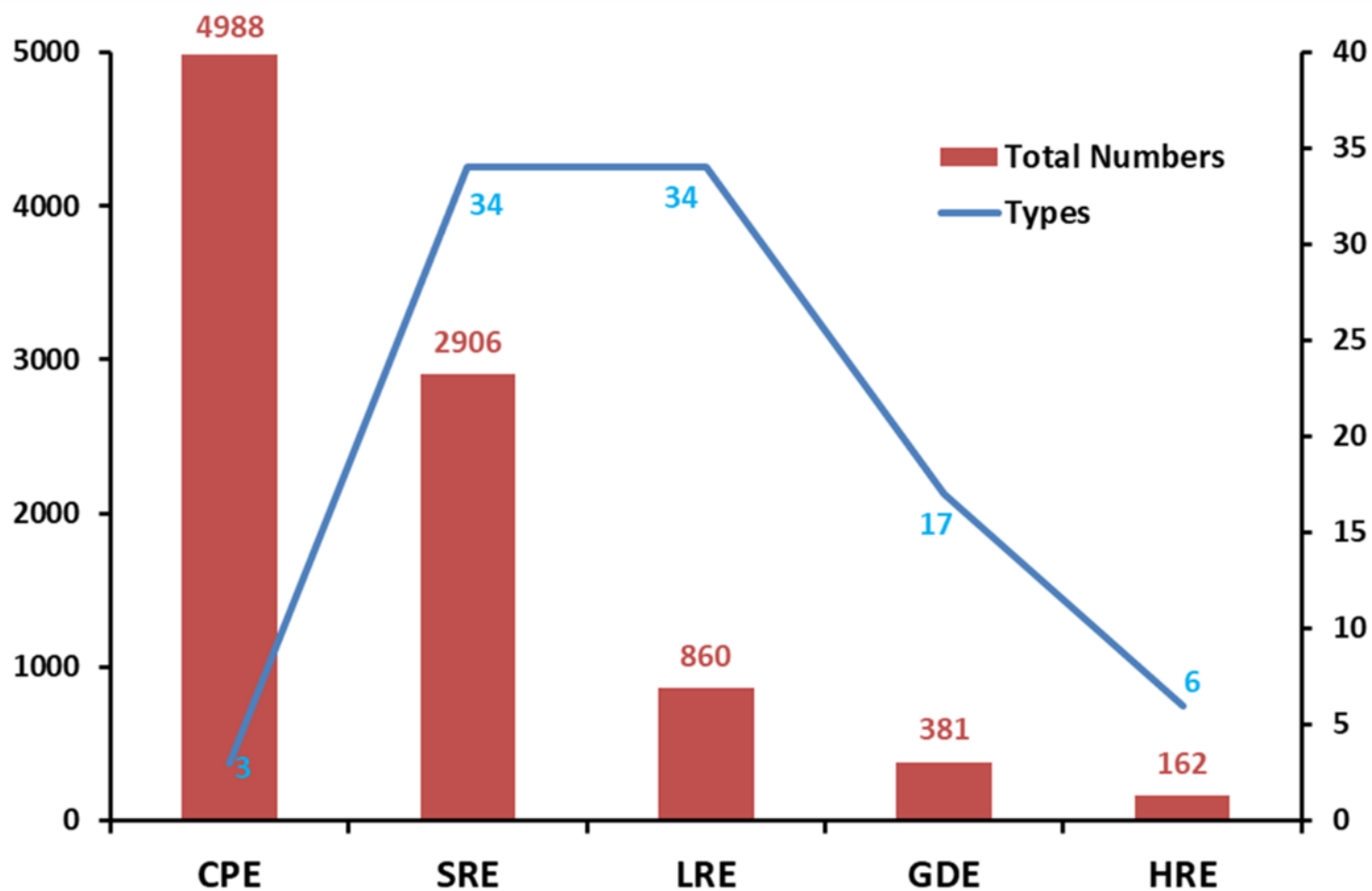


Figure 6

The different kinds and numbers of cis-acting elements identified in the 1.5 kb upstream promoter sequences of *EcNBLRRs*.

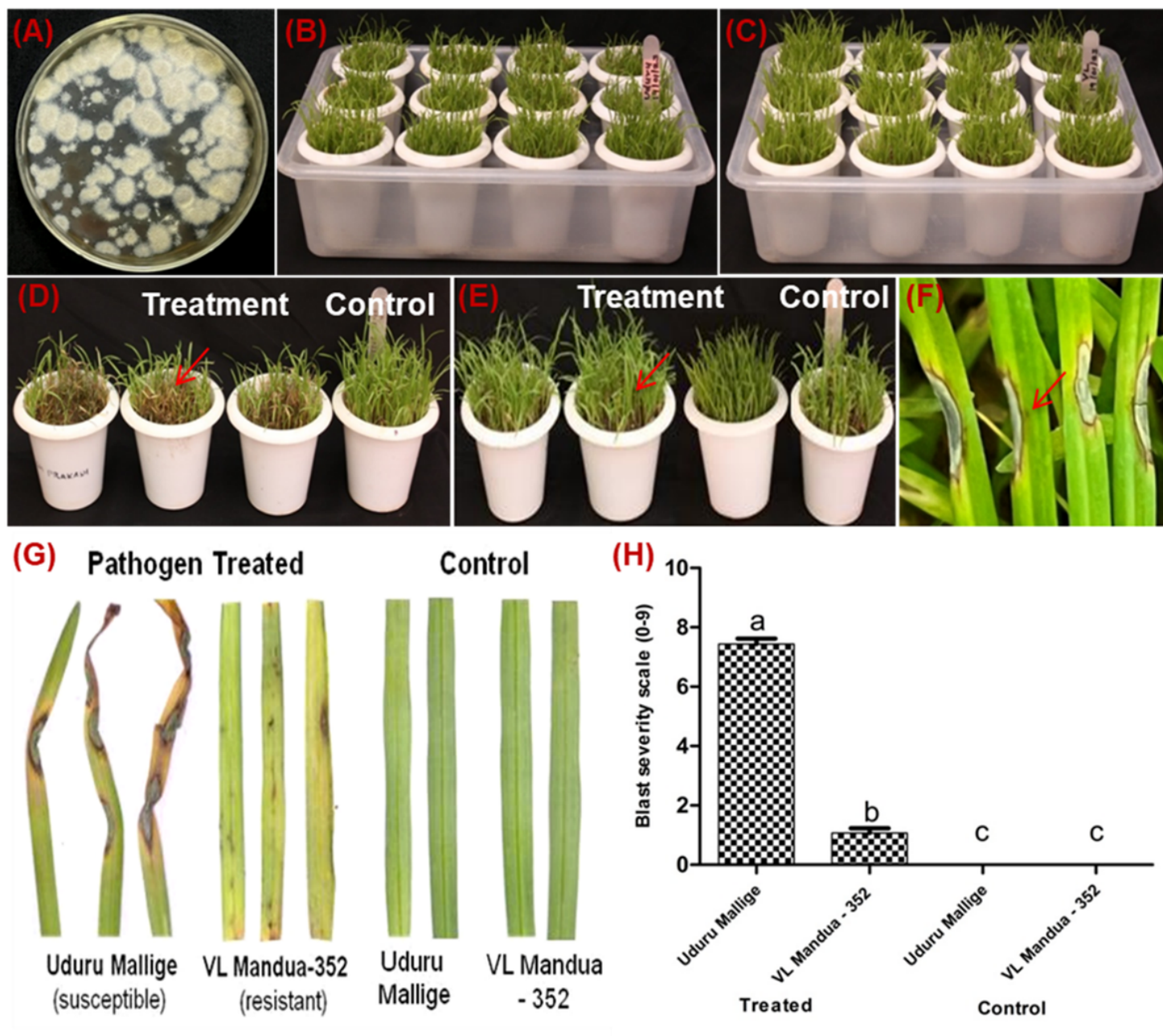


Figure 7

(A) *M. grisea* isolate Ragi Almora multiplied on Oatmeal agar; (B) Finger millet cultivars Uduru Mallige (susceptible) before inoculation of *M. grisea* isolate Ragi Almora; (C) Finger millet cultivar VL Mandua-352 (resistant) before inoculation of *M. grisea* isolate Ragi Almora; (D) Typical blast symptoms with higher blast on Uduru Mallige (PDI 82.59 %) cultivar at 8 dpi; (E) Minute brown spots (hypersensitive reaction) on resistant cultivar VL Mandua-352 at 8 dpi (PDI 11.85 %); (F) The close-up view of severe blast symptom observed on susceptible cultivar Uduru Mallige; (G) Scanned images representing the both susceptible and resistant disease reactions on the contrasting Finger millet cultivars Uduru Mallige and VL Mandua-352; (H) The bar graph plotted using severity of blast lesion as per 0-9 scale grade in the contrasting Finger millet cultivars Uduru Mallige and VL Mandua-352. Note: dpi - days post inoculation, PDI – per cent disease incidence.

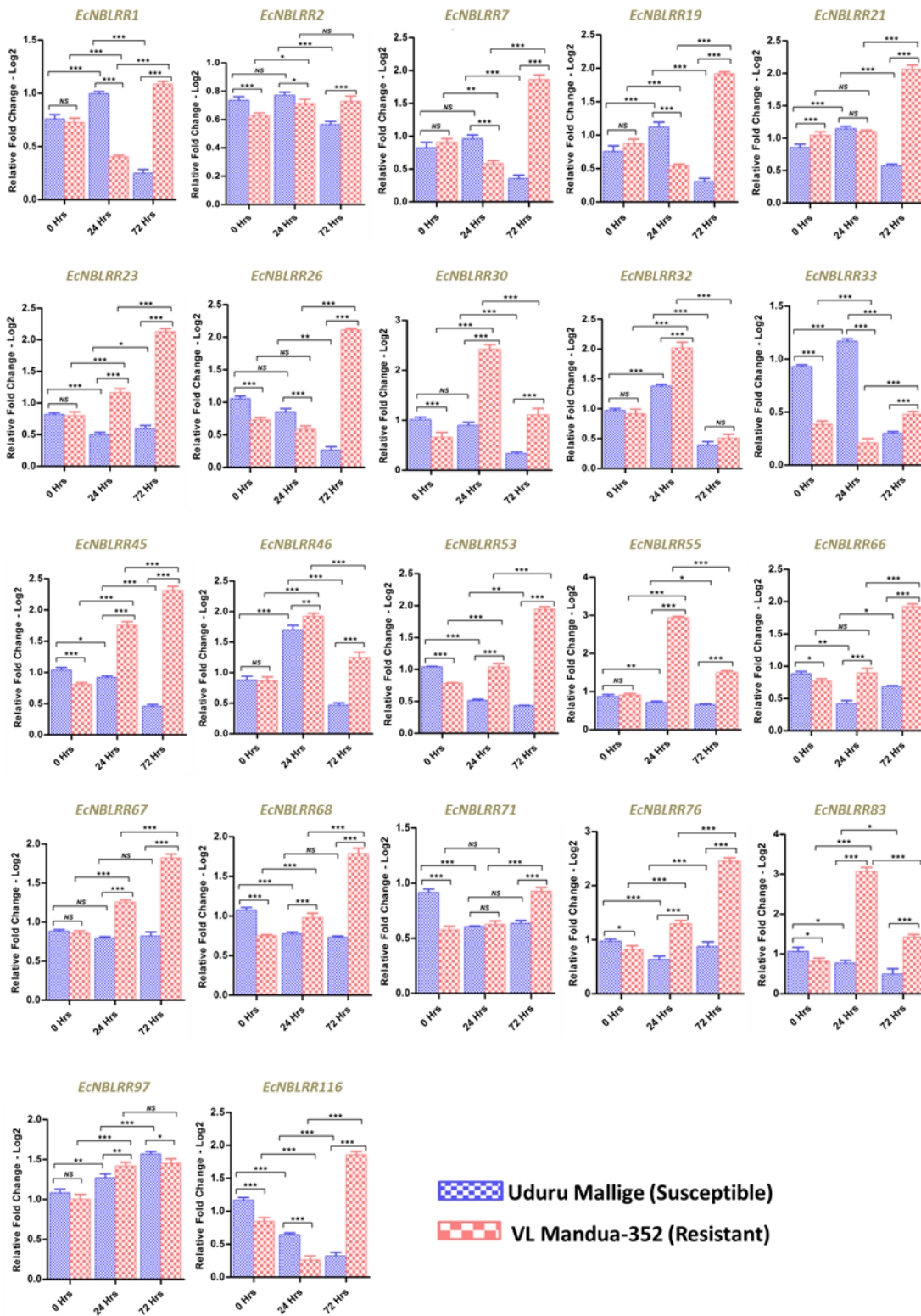


Figure 8

The relative expression level of 22 *EcNBLRR* genes in the Uduru Mallige and VL Mandua-352 genotype showing contrasting results in response to *M. grisea* isolate Ragi Almora infection at 24 and 72 hpi during RT-qPCR analysis. The error bars represent the deviations from three biological replicates, the asterisk indicated significant differences at $P < 0.01$ (*), $P < 0.001$ (**) and $P < 0.0001$ (***) and NS refers to no significant differences.

Supplementary Files

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