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GENOME-WIDE IDENTIFICATION, CHARACTERIZATION AND EXPRESSION ANALYSIS OF *NPR1*-LIKE IN *ORYZA SATIVA* GENOTYPES UNDER PATHOGEN STRESS

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Abstract *Rice (Oryza sativa* L.*) is the main food source in many countries and its production is continuously threatened by various pathogens and harsh environmental conditions. The family of NPR1 (Non-expressor of Pathogenesis-Related genes 1) proteins play a central role not only in signaling and plant immunity but also in tolerance to various types of stresses. In the present study, a systematic search and investigation of the functional relevance of OsNPR1 members, taking Arabidopsis NPR1 as the standard, were carried out. Five genes similar to NPR1 (OsNPR1-1 to OsNPR1-5) were identified in the rice genome by the use of Phytozome. Observations of gene structures showed variations in the arrangements of exon-intron, while chromosome mapping displayed that these genes were unevenly located on chromosomes 1 and 3; also some of these genes were absent on several other chromosomes. Physicochemical features revealed that proteins had lengths from 582 to 635 amino acids; they also displayed different isoelectric points. A phylogenetic tree was drawn and showed 5 distinct groups, revealing the evolutionary relationships established by conserved nodes. The search of conserved motifs through MEME substantiated the presence of NPR1-like motifs and this suggests functional conservation. Based on subcellular localization prediction, majority of OsNPR1 proteins may be present in the nucleus and this supports their involvement in transcriptional regulation. Ka/Ks analysis showed that while segmental duplications have led to a gene expansion, purifying selection has been keeping the genes stable throughout evolution. Analysis of synteny between OsNPR genes and other plants showed that OsNPR1-4 had strong collinearity mainly with Arabidopsis. Examination of the gene promoter regions revealed the presence of cis-elements related to hormone, stress, and light responses. Also, by predicting the miRNA-target, post-transcriptional regulation of OsNPR1-1 by several conserved miRNA families was suggested. As per GO annotation, OsNPR1-1, OsNPR1-2, and OsNPR1-5 participate in salicylic acid response which enhances defense. They have organic acid-binding capacity and get localized to the cytoplasmic INA complex, thereby elevating disease resistance of Oryza sativa. Expression profiling in response to nitrogen fertilizer and Pyricularia oryzae infection showed OsNPR1-1 as the gene having highest level of mRNA. That finding was statistically significant for biotic and abiotic stress conditions. Results indicate that OsNPR1-1 is essential in defense and nutrient signaling pathways in rice. This detailed characterization establishes a basis for upcoming functional studies and genetic enhancement initiatives focused on improving rice stress resilience via NPR1-mediated signaling.*

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Introduction

Rice, or *Oryza sativa*, is among the world's primary crops, providing more than twenty percent of the world's dietary energy and supporting over half the global population. In Asia, Africa, and some parts of Latin America, rice is in high demand, and improving its yield and resilience has now become a key agricultural focus (Mather et al., 2007). In some of the rice-producing regions, the use of inefficient field practices alongside water shortage, drought, salinity, and high temperatures greatly limits yield. Furthermore, production and yield are greatly affected by pests and pathogens, along with biotic pressures. Crops can be devastated and infected with *Xanthomonas oryzae pv oryzae* (Xoo), which is bacterial blight, greatly hindering crop yield. Even though the traditional breeding process incorporates resistance (R) genes like Xa21 and Xa4, these genes have become useless over time due to adaptive pathogen counter-responses (Gnanamanickam, 2009). The *NPR1* (Nonexpresser of Pathogenesis-Related genes 1) gene in *Arabidopsis thaliana* acts as a key molecular switch for the action of salicylic acid (SA)

in the systemic acquired resistance (SAR) pathway, managing the immune responses by binding to TGA transcription factors (Chern et al., 2001; Spoel et al., 2009; Srinivasan et al., 1999). NPR1's BTB/POZ and ankyrin-repeat domains perform oligomerization of the protein (by increase in concentration of SA), the protein monomerizes and then translocates to the nucleus, where PR genes are activated. Broad-spectrum resistance has been reported to be enhanced with NPR1 overexpression in transgenic crops, further substantiating its agronomic importance (Gadal et al., 2019).

Rice has an ortholog, *OsNPR1* (also known as NH1), which has the same function in regulating transcription of immune-related genes in *Oryza sativa* and in providing resistance regulation in plants. It is well known that *OsNPR1* is involved in BTH- and SA-induced resistance. also, suppression of *OsNPR1* results in a lack of resistance, while overexpression provides enhanced defense. In transcriptome profiling, 358 out of 1,228 BTH-upregulated genes and 724 of 1,069 BTH-downregulated genes were found to be *OsNPR1* dependent, particularly those associated with photosynthesis and defense metabolism. Moreover, promoter analysis has demonstrated *that OsNPR1 is inducible by SA, with key regulatory motifs, such as the W-box and ASF1*, contributing to its activation (Fahad et al., 2019).

Culling regulations under the phosphorylation modification of *OsNPR1* show that it is specifically targeted for degradation by CRL4^OsDWD1 ubiquitin E3 ligase complexes after being phosphorylated. This balancing act of immune enhancement alongside *OsNPR1*-dependent growth management finely tunes immune homeostasis. In addition, it is known that *OsNPR1* functions within the junction of hormone interactions, since loss-of-function mutants exhibit overexpression of genes responsive to jasmonic acid (JA) and ethylene (ET), suggesting *OsNPR1* negatively regulates the JA/ET crosstalk and simultaneously activates the SA pathway. Through dual RNA-seq, its participation both in host immunity and in the suppression of the pathogen has also been demonstrated, where Xoo virulence gene expression and rice PR and R genes are modified (Fageria et al., 2014).

Although the previously mentioned findings contribute greatly to our knowledge of *OsNPR1*, the molecular stress triggers and feedback processes that control *OsNPR1* activity remain elusive. This information gap motivates this study, which seeks to elucidate the expression, regulatory relationships, and signaling processes of immunity controlled by *OsNPR1*. The focus is on regulatory pathways such as expression and post-transcriptional modulation of target genes, which in turn shape immune functions in rice (Besler et al., 2001; Cha-um et al., 2007).

Materials and methods

Identification and retrieval of *NPR1* genes:

The UniProt database provided the protein sequence of the *NPR1* gene of *Arabidopsis thaliana* (Gene ID: NPR1_ARATH, At1g64280). The sequence was analyzed in the Pfam database (Gene ID: IPR021094) to verify the presence of conserved domains (Caldwell & Michelmore, 2009). A BLAST-P search was then conducted using the verified NPR1 sequence as a query against the *Oryza sativa* genome in the Phytozome database (https://phytozome-next.jgi.doe.gov). The Motif Finder tool (https://www.genome.jp/tools/motif/) was used to analyze homologous NPR1 sequences from *Oryza sativa*. The NCBI CDD (Conserved Domain Database) architectural viewer (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to analyze the resulting amino acid sequences using the default settings. We removed sequences that lacked real NPR1 conserved domains (IPR021094) (http://pfam.xfam.org/family/PF06943.12).

Table 1 Physicochemical characteristics and genomic information of identified OsNPR1 genes in Oryza sativa, including gene ID, chromosomal location, amino acid length, molecular weight, and isoelectric point.

Gene ID	**Source Assession**	**Chromosome**	**Chromosome No.**		**Direction**	**Amino acid(AA)**	**pI**	**Mw**
Name	No.	No.	Start	End		No.	Volume	Dalton
Os_NPR1-1	**LOC_Os01g09800**	1	5060415	5065236	Reverse	582	5.44	63765.57
Os_NPR1-2	**LOC_Os01g56200**	1	32367682	32371816	Forward	635	5.81	68495.03
Os_NPR1-3	**LOC_Os03g46440**	3	26259736	26267273	Reverse	589	6.04	65047.00
Os_NPR1-4	**LOC_Os03g46440**	3	26259736	26267273	Reverse	583	6.04	64471.26
Os_NPR-5	**LOC_Os03g46440**	3	26259736	26267273	Reverse	583	6.04	64471.26

Physicochemical characterization of identified NPR1

Protein length (number of amino acid residues), molecular weight, and theoretical isoelectric point (pI) of *NPR1* proteins were measured using the ExPasy ProtParam tool (http://web.expasy.org/protparam/)(Tantasawat et al., 2015; Tarang et al., 2013). Phytozome and Ensemble plants provided the gene IDs, chromosomal locations, gene sequences, and protein sequences. In order to facilitate better data organization and comprehension, these NPR1 genes were renamed according to their physical locations and ease of understanding. The subcellular localization of *OsNPR1* genes was ascertained using the online application WoLFPSORT (https://wolfpsort.hgc.jp).

Multiple sequence alignment and phylogenetic Analysis:

The MEGA 11 v11.0.10 program was used to align the amino acid sequences for the *NPR1* gene and conduct the phylogenetic analysis via neighbor joining (NJ), with bootstrapping set at 1000 replications using pairwise deletion. For phylogenetic analysis, five rice *NPR1* protein sequences, six Arabidopsis *NPR1* protein sequences, and four additional crops were used. The phylogenetic tree was elaborated and annotated using Itol (https://itol.embl.de/)(Nelson et al., 1998).

Cis-regulatory elements and conserved motifs recognition:

Cis-regulatory elements were identified from promoter site sequences up to 600 bases upstream of the start of the ORF codon area using the Plant Care website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The final peptide sequences of the NPR1 (*Oryza sativa*) were examined using the Multiple EM for Motif Elicitation (MEME) tool (http://meme.nbcr.net/meme/), with a maximum number of motifs at 20. A minimum width of 5 and a maximum width of 50 for the motif were among the default settings(Nawaz et al., 2010). We also utilized NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to generate domains conserved against Pfam v33.1 with a maximum number of 500 hits using the default parameters. Data from MEME, NCBI CDD, and a phylogenetic tree created with Tbtools were combined to provide a final graphical result (Myśliwa-Kurdziel and Strzałka, 2002).

Chromosome mapping and gene structure analysis

To forecast the arrangement of exons and introns, the CDS and genomic sequences of *OsNPR1* genes were obtained from the Phytozome online database. Additionally, other complete genomic, gff, and protein files of the rice genome were also retrieved from Phytozome. Gene sequences were placed into the Gene Structure Display Server (GSDS v2.0) (http://gsds.cbi.pku.edu.cn) to illustrate sequences that were subsequently employed to represent the gene structure using Gene Structure Display Server (GSDS v2.0) (accessible at http://gsds.cbi.pku.edu.cn).

Gene Duplication and Synteny Analysis

Using Ka and Ks values, the divergence time (t) of the rice NPR1 gene family was theoretically computed. Phytozome databases provided the *Oryza sativa* peptide and CDS sequences. To determine the rate of synonymous and nonsynonymous substitution, the TBtool Ka/Ks calculator was utilized. By entering the Ks value into the $T = Ks /2\lambda$ equation, where λ represents a value of $7.85\times10\text{-}9$, the period of divergence (million years ago, Mya) of *Oryza sativa* was calculated. The gene duplication occurrences were predicted using MCScanX (Multiple Collinearity Scan Toolkit) using the default settings. The MCScanX software, which is integrated into TBtool, was used to undertake a synthesis study of the NPR1 gene family. The Dual Synteny plotter was used to build the synteny connection diagram of the NPR1 gene family in both wild and farmed rice(Mullan and Pietragalla, 2012; Munns et al., 2010).

Transcriptome analysis

Expression profiling of NPR1 genes in rice under controlled developmental conditions and dual RNA-seq-based analysis of nitrogen-influenced host-pathogen dynamics. We retrieved previously acquired rice RNA-seq data from the Expression Atlas website: (https://www.ebi.ac.uk/gxa/home), also available on NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/). Gene IDs were then uploaded to the Ensemble Plant website (https://plants.ensembl.org) using GEO accession numbers Os01g0194300, Os03g0767900, Os02g0667100, Os03g0667100, and Os04g0667100, respectively. To determine fold change values for expression profiling, the Reads Per Kilo bases per Million mapped reads (RPKM) values from the RNA-seq data were log2 transformed. The p-value < 0.05 was used to distinguish between significant and insignificant differences in gene expression. A table was made with five different treatment combinations. This table was also sent to the TBTool to generate heatmap data(Maathuis et al., 1996).

Analysis of microRNA Target Sites

The PmiREN database (https://www.pmiren.com) provided the rice microRNA (miRNA) datasets. To predict potential miRNAs targeting rice NPR1 genes, the coding sequences (CDS) of OsNPR1 genes were uploaded to the psRNATarget website (https://www.zhaolab.org) along with the designated miRNA and target input fields. Complementary sequences for the identified miRNAs were acquired using the same platform(Isayenkov and Maathuis, 2019; Liu et al., 2017).

Gene ontology, and Sub-cellular localization Analysis

Gene ontology (GO) enrichment information of each *NPR1* gene was retrieved from the web tool STING database https://string-db.org/. To study the particular

involvement of the *NPR1* genes of *O sativa* in terms of biological function (BP), molecular function (MF), and cellular component (CC). Subcellular localization of NPR1 genes in *O. sativa* was identified using the online tool WoLF PSORT (https://wolfpsort.hgc.jp/)(Dixon and Massey Jr, 1951).

Results

Identification of the NPR1 genes

NPR1 genes were identified in *Arabidopsis thaliana, Dacus carota, Gossypium herbaceum, oryza sativa* and *Solanum tuberosum* when *NPR1* domain sequences were blasted against the whole genome of *Arabidopsis thaliana* which were extracted from phytozome database. Initially, the no. of protein sequences obtained were 4, 4,10, 5 and 2 respectively in *Arabidopsis thaliana, Dacus carota, Gossypium herbaceum, oryza sativa and Solanum tuberosum (*Chaabene et al., 2017*)*.

The protein size of *Oryza sativa* ranged from 582 to 635 amino acids, the molecular weight (MW) ranged from 63765.57 to 68495.03 Da, and their Isoelectric point varied from 5.41 to 6.04 in *Oryza sativa. The* detailed information is shown in Table 1.

Gene structures and recognition of conserved motifs and domains

Exons and introns are basic components of genes and play an important role in investigating evolutionary evidence among organismal genes. The number and distribution of exons and introns within a gene family provide information about its evolutionary properties. A thorough description of the exon-intron structures of *NPR1* genes in *Oryza sativa*, together with phylogenetic analysis, revealed that the gene structural pattern was similar to the phylogenetic study. All genes have only exons and no introns, as illustrated in Figure 1 and Table S1 (Antolovich et al., 2002).

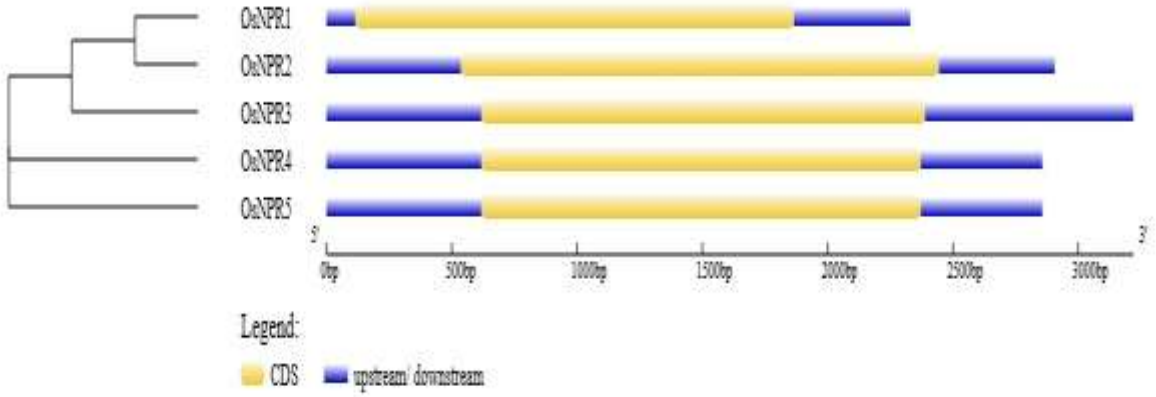

Figure 1 Gene structure analysis of OsNPR1 gene family in Oryza sativa showing exon–intron organization. All genes exhibit intron-less structure, indicating conserved genomic architecture

For the prediction of 20 conserved motifs in amino acid sequences of all 5 *OsNPR1* genes, the MEME program was employed. From the conserved motif analysis, phylogenetically closely related *OsNPR1* protein amino acids shared nearly identical motif composition, and also possessed the same domain (NPR1_like_C_super family). Therefore, these findings suggest that among all the 5 members of *OsNPR1* of they may possess functional similarities.

The *OsNPR1-1* gene had motif number 1,2,3,5,6,8, 9, 13, 14, 15, and 19; the *OsNPR1-2* gene had motif number 1,2,3,5, 6,7 8, 9, 10, 11, 13, 14, 15, and 20; the OsNPR1-3 gene had motif number 1,2,3,5,6, 7, 8, 9,10,11,12, 13, 15, 17, 18 and 19; whereas gene *OsNPR1-4* and gene *OsNPR1-5* contained motif number 1, 2, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,16, and 17 (Fig 2 and Table S2). Comparable motifs were dispersed across different *NPR1* genes, suggesting that such genes may have come into existence due to divergent evolution.

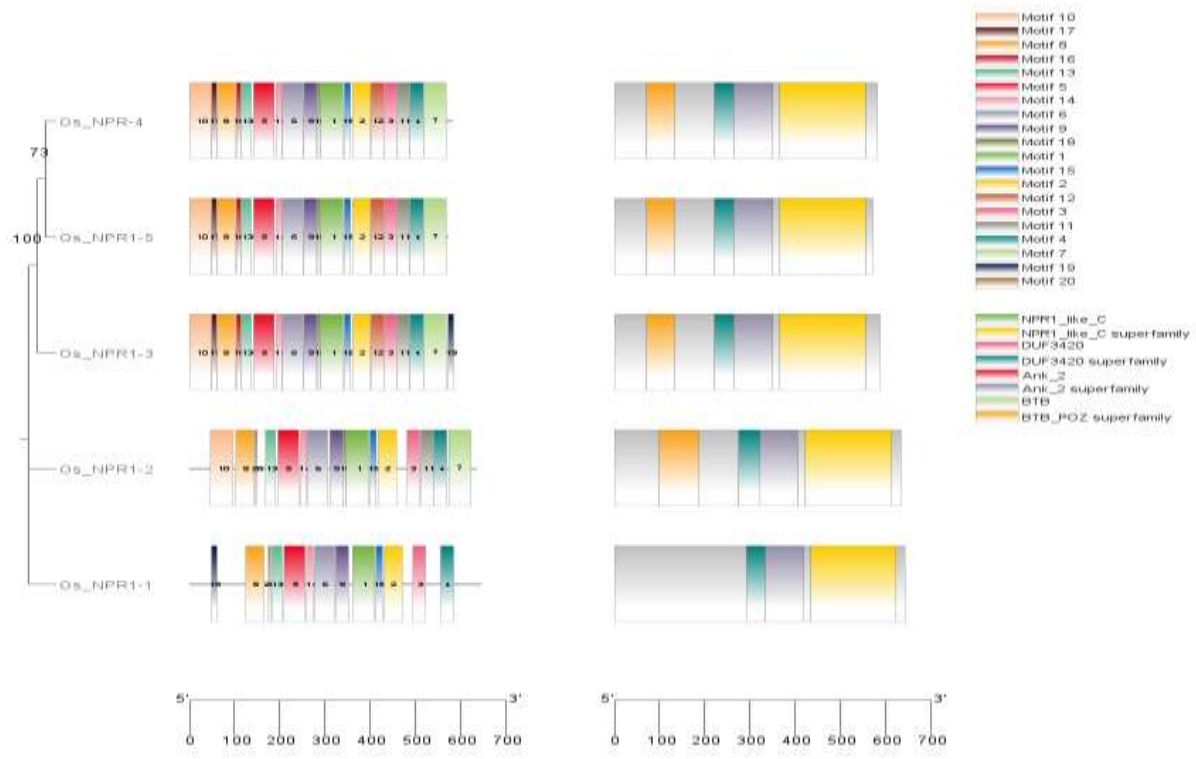

Figure 2 Conserved motif composition of OsNPR1 proteins identified using MEME tool. Different colored boxes represent distinct motifs distributed across protein sequences.

Comparative phylogenetic relatedness of Rice *NPR1* subfamily with Arabidopsis, Potato, Cotton, and Carrot

The evolutionary relationships between *Arabidopsis thaliana*, *Dacus carota*, *Gossypium herbaceum*, *oryza sativa* and *Solanum tuberosum* was studied by a Neighbor-Joining (NJ) phylogenetic tree, in accordance of *NPR1* family, was constructed through MEGA X and Itol tools by aligning their full-length peptide sequences. The results depicted that 23 *NPR1* proteins were distributed among 5 groups based on the number of NPR1 domain present, named as *NPR1 (*Non-expressor of Pathogenesis-Related genes 1) (Table S3, Fig 3). Group 1 contain 7 genes, group 2 contain 2 genes Arabidopsis *(At-NPR4 & AtNPR1-2)*, group 3 contain 2 genes of rice *(OsNPR1-2 & OsNPR1-3)*, group 4 contain 11 genes and group 5 contain 1 gene carrot (*DcNPR1-4*). Therefore, it can be inferred that *NPR1* proteins belonging to similar clades may possess identical structure and preform similar function (Table S3, Fig 3).

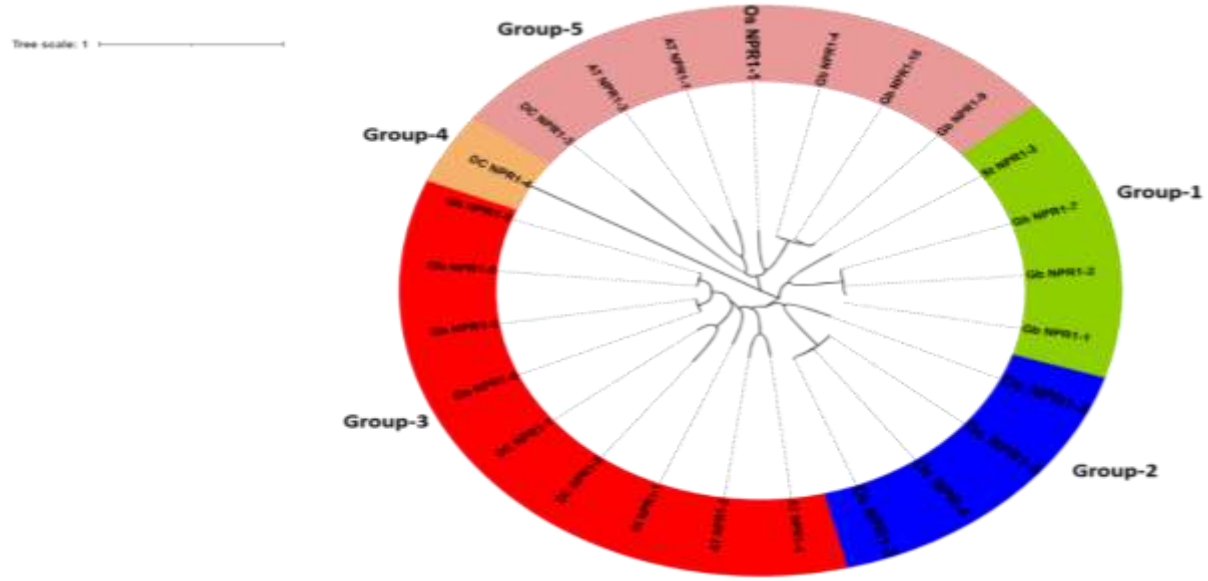

Figure 3 Phylogenetic analysis of NPR1 proteins from Oryza sativa, Arabidopsis thaliana, and other plant species constructed using the Neighbor-Joining method

Location of chromosomes and assessment of gene duplication of Rice NPR1 genes

Chromosome mapping was utilized to determine the distribution of NPR1 gene members in the rice genome (Figure 4). The NPR1 subfamily members were found only on chromosomes 1 and 3. There were two NPR1 genes on Chr1 (*OsNPR1-1* and *OsNPR1-2*). The Chr2 had only one NPR1 gene, *OsNPR1-3*. All of the genes had segmental duplications, and no tandem duplications were found (Figure 4). A single syntenic study was undertaken to determine the duplication of the *OsNPR1* gene family on chromosomes. In rice, *OsNPR1-1* was found to be paralogous with *OsNPR1-2* and *OsNPR1-3*. In contrast, *OsNPR1-2* was paralogous with *OsNPR1-3* (Fig 5). To learn more about orthologous, a dual syntenic analysis *of Oryza sativa* and *A. thaliana* was performed, as highlighted by the threads. In this graph, seven genes were duplicated (Figure 6).

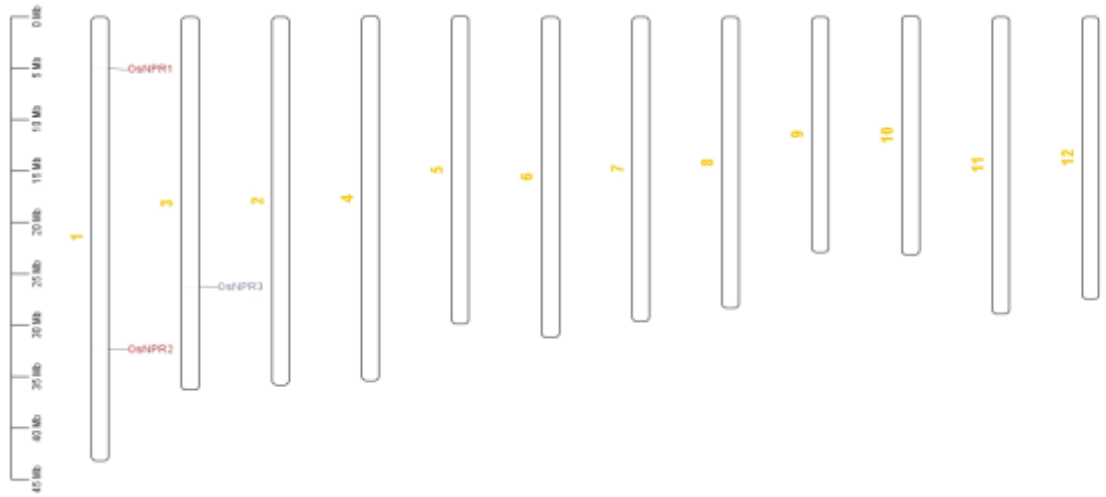

Figure 4 Chromosomal distribution of OsNPR1 genes in the rice genome showing their localization on chromosomes 1 and 3.

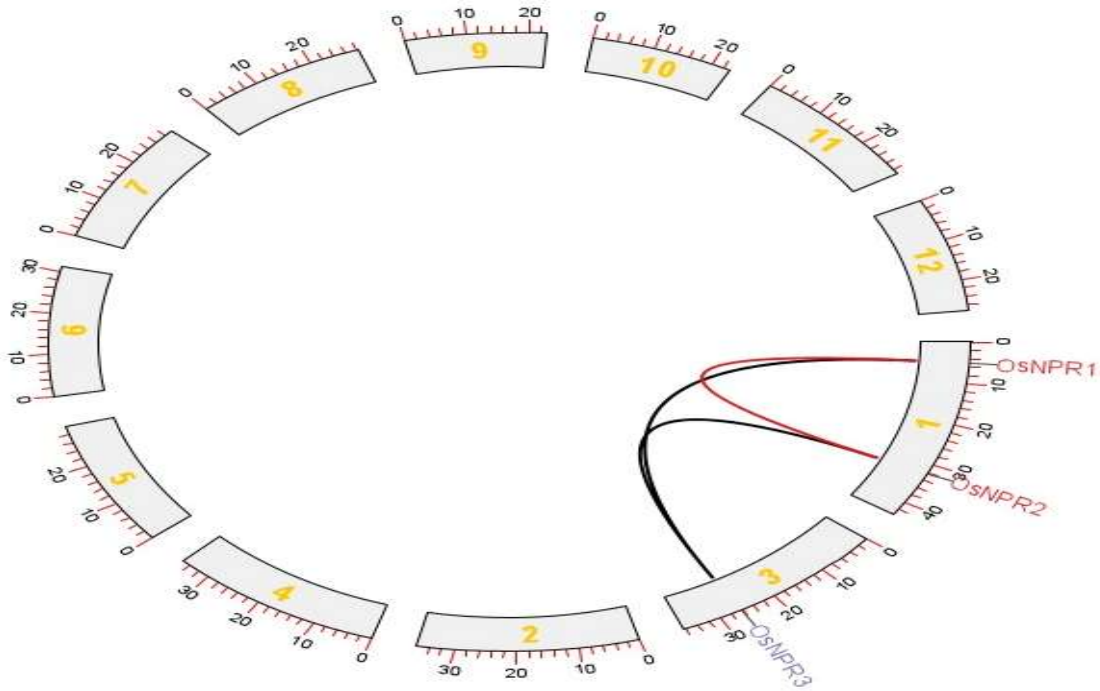

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Figure 5 Synteny analysis of OsNPR1 genes within the rice genome in dicating segmental duplication events

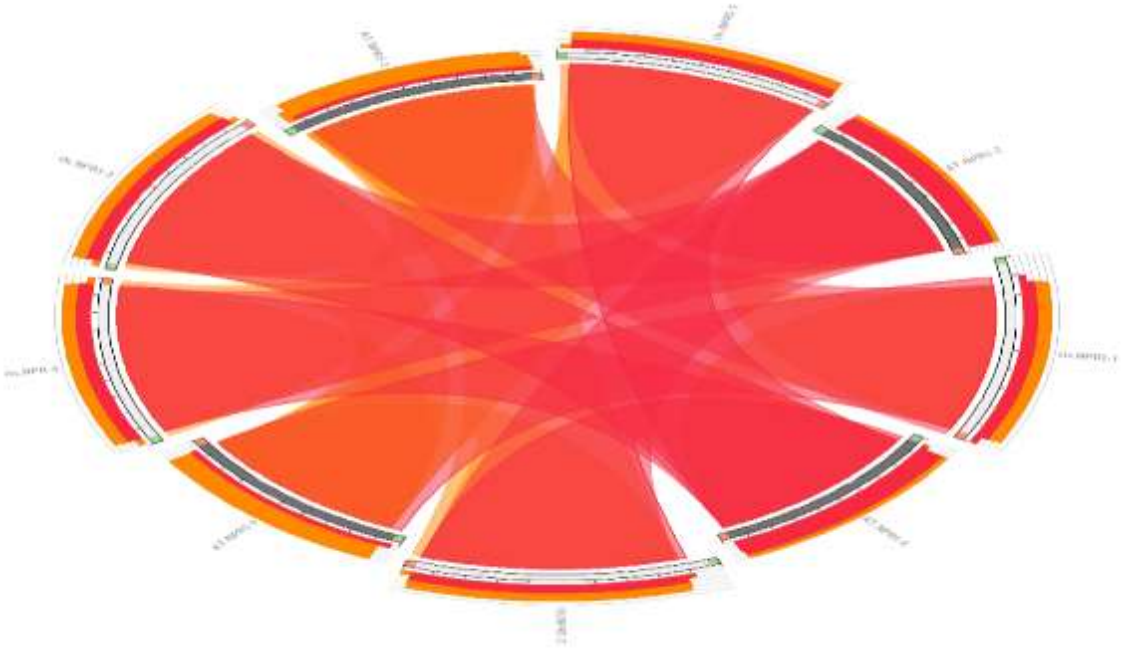

Figure 6 Comparative synteny analysis between Oryza sativa and Arabidopsis thaliana highlighting orthologous gene relationships

The calculation of the synonymous (Ks) and nonsynonymous (Ka) substitution rates is an important metric in molecular analysis. The rates measure the number of synonymous and nonsynonymous substitutions per site per year, providing useful insights into molecular evolutionary dynamics. If the ratios of Ka/Ks=1, Ka/Ks>1, and Ka/Ks<1 indicate neutral, positive, or purifying selection, respectively. The Ka/Ks ratio and Time of Divergence (T) were estimated for NPR1 gene members in *Oryza sativa* to
better understand their genetic diversity among them. The results depicted that all Ka/Ks ratios were smaller than one, ranging from 0.20 to 0.45, indicating purifying selection (Fig 7, Table S4). The duplication period for NPR1 gene pairs in *Oryza sativa* ranged from 88.60 to 112.48 MYA (Table S4).

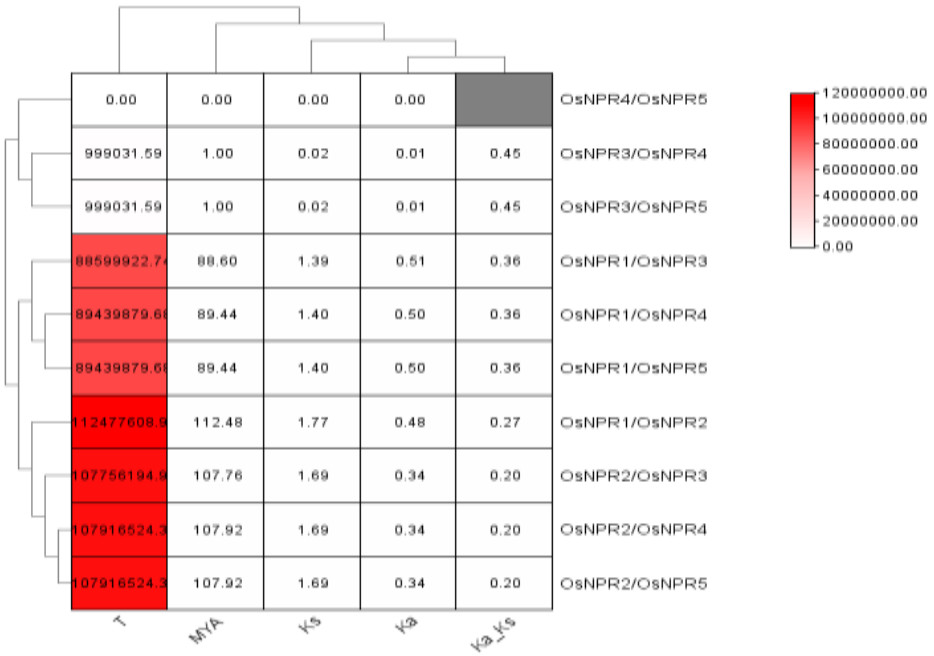

Figure 7 Ka/Ks ratio analysis of duplicated OsNPR1 gene pairs indicating purifying selection and evolutionary conservation

Analysis of Cis-regulatory elements

Cis-regulatory elements on promoter sites regulate gene transcription. In-silico analysis is used to investigate the diverse functions of genes by discovering cis-regulatory regions. *Oryza sativa* exhibits elements-related critical processes such as light response, stress, hormone-specific, seed-specific, embryogenic signals, drought, cell-specific, and meristem-specific (Figure 8, Table S5).

The A-box, AC-II, AE-box, Box 4, GATA-motif, G-box, GT1-motif, and TCT-motif are light-sensitive motifs that control gene expression in response to light signals. Motifs such as ABRE, ABRE3a, and ABRE4 are fundamental elements of the abscisic acid (ABA) signaling pathway and play vital roles in responses to drought and salinity stress. Likewise, the DRE core plays a crucial role in dehydration and cold stress signaling, frequently partnering with ABREs. ARE motif is involved in the antioxidant response, whereas STRE serves as a general stress response element. Hormonal regulation involves various motifs, such as the CGTCA-motif and TGACG-motif, which react to methyl jasmonate (MeJA), the TCA-element and CARE for salicylic acid (SA) pathways, the P-box and GARE-motif for gibberellin (GA) interactions, and the TGA-element for auxin signaling. The MYB, MBS, Myb-binding site, and MYC motifs have been identified to interact with MYB and bHLH transcription factors, regulating genes that respond to drought, ABA, and jasmonate stimuli. Moreover, the W box and WRE3 serve as crucial binding sites for WRKY transcription factors, which are mainly engaged in defense against biotic and abiotic stresses, as shown in Table S5.

Core elements of promoters, including the TATA-box, CAAT-box, and AT~TATA-box, are essential for starting transcription, offering binding sites for transcription machinery such as TFIID and RNA polymerase II. The Sp1 and GC-motif operate comparably by promoting transcription initiation via GC-rich binding areas. Responses to metal and nutrient stress are regulated by motifs such as MRE (metal-responsive element) and O2-site, the latter playing a role in the regulation of storage protein genes in seeds. Elements like the ERE react to ethylene, whereas the LTR motif is responsive to cold. The WUN-motif is linked to gene expression triggered by wounds. Motifs that are rare or less understood, including the AACA_motif, AAGAA-motif, CCGTCC motif, and CCGTCC-box, could play regulatory roles in development or under stress, but their precise functions are still under investigation. Finally, the as-1 element serves as a pathogen-responsive motif that interacts with bZIP transcription factors such as TGA, significantly contributing to the activation of defense genes. Different numbers of motifs present in the given heatmap by using TBtool, Figure 8.

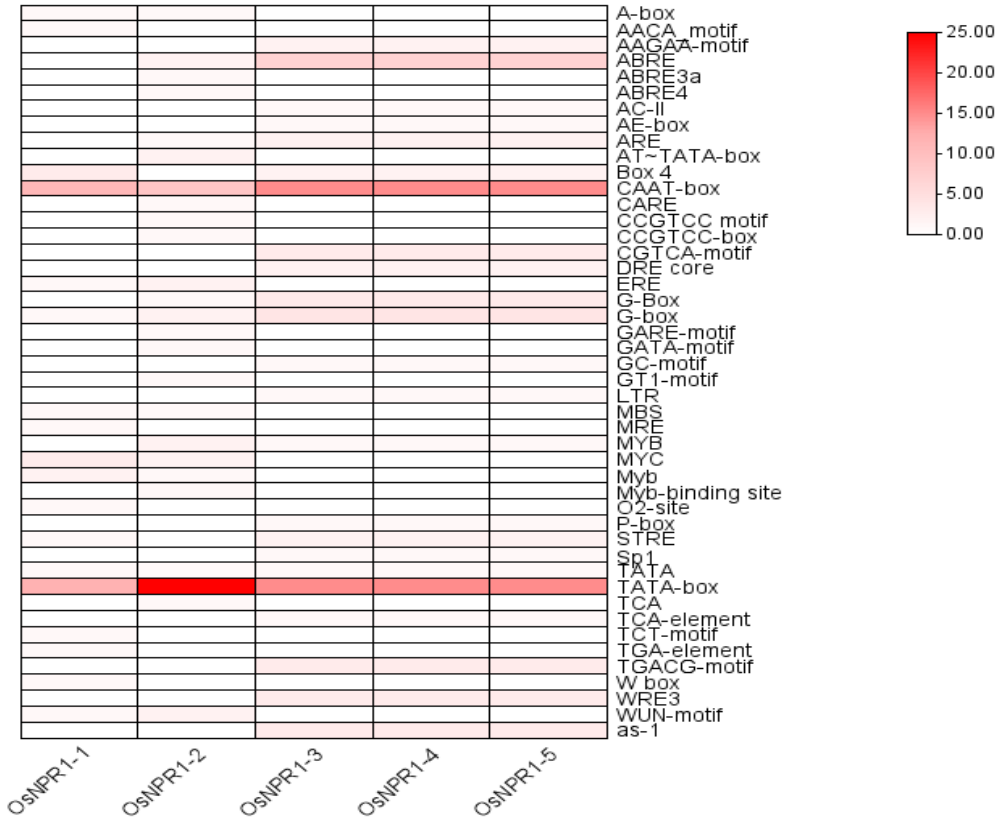

Figure 8 Cis-regulatory element analysis of promoter regions of OsNPR1 genes showing distribution of light-, stress-, and hormone-responsive elements

Analysis of Sub-cellular localization

Online web tool WOLF PSORT was used to identify the Subcellular localization of *NPR1* genes (https://wolfpsort.hgc.jp). The prediction of protein localization within the cellular environment is based on the analysis of sorting signals and the composition of amino acids. The results suggested most of the *OsNPR1* genes were predicted in nucleus, and few genes were present in chloroplast and plasmid. While the remaining *OsNPR1* were present in mitochondria, golgi bodies, extracellular membrane, and cytoplasm, as shown in (Figure 9).

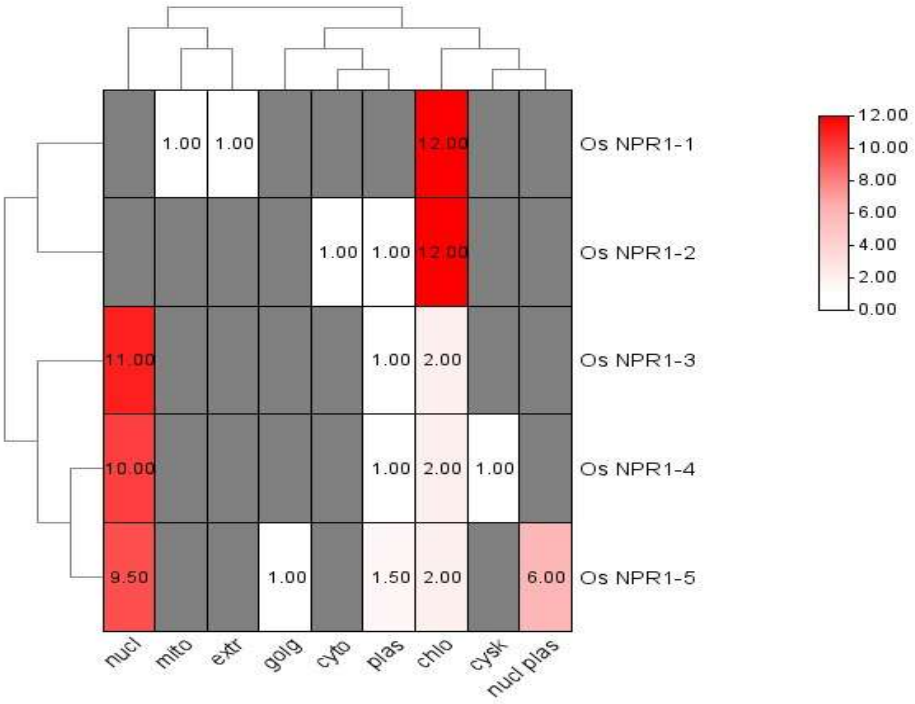

Figure 9 Predicted subcellular localization of OsNPR1 proteins showing distribution across nucleus, cytoplasm, chloroplast, and other cellular compartments.

Gene Ontology (GO) annotation of *NPR1* genes

Protein-protein interaction study revealed substantial links between *OsNPR1-1*, *OsNPR1-2*, and *OsNPR1-5*, indicating a highly integrated functional network. Multiple interaction data suggests that these *NPR1* homologs may cooperate to modulate salicylic acid-mediated defense signaling in *Oryza sativa* (Fig 10).

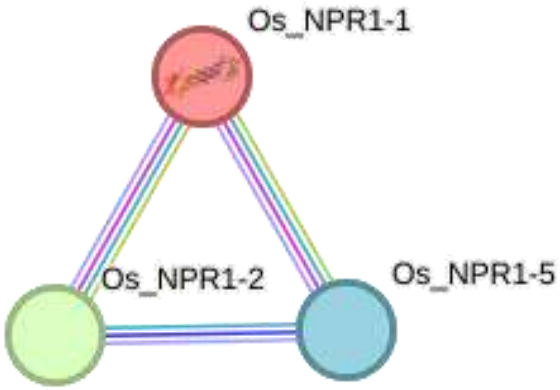

Figure 10 Protein–protein interaction network of OsNPR1 proteins illustrating functional associations and regulatory interactions.

Using GO categories, we identified the activities of all *NPR1* proteins, including biological processes, molecular functions, and cellular components. Within the biological process category, the vast majority of proteins were implicated in salicylic acid signaling pathway with the GO terms (GO:0009862) & (GO:2000031) and the jasmonic acid signaling pathway (GO:2000022) (Fig 11). Molecular process category revealed the two proteins to be associated with monocarboxylic acid binding (GO:0033293). The findings showed some proteins to be related to monocarboxylic acid binding, whereas others were associated with organic acid binding (GO:0043177). As far as cellular components are concerned, significant enrichment of the INA Complex (GOCC:1990524) was observed. In this way, the results have pinpointed a few diverse roles of these NPR1 proteins in cellular metabolism.

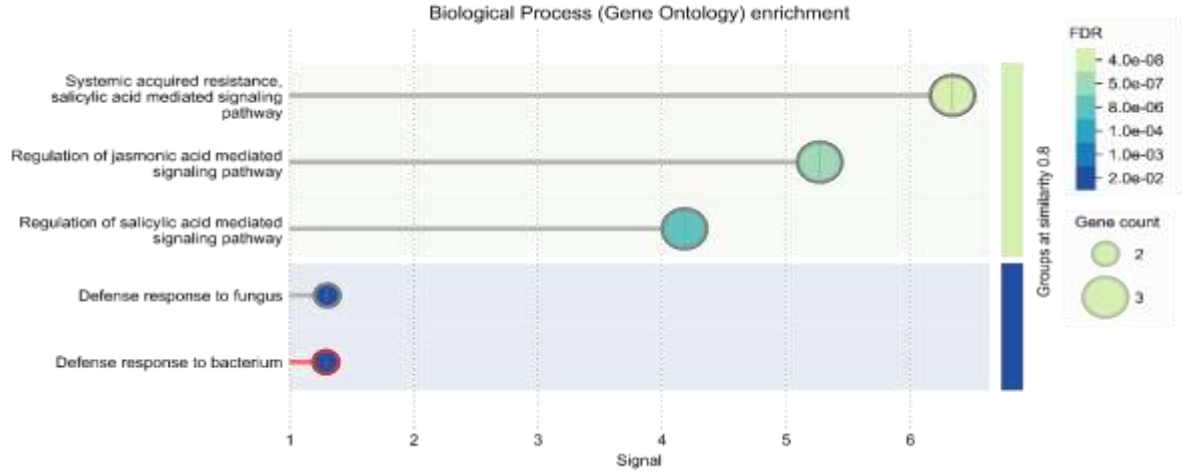

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Figure 11 Gene Ontology (GO) classification of OsNPR1 proteins categorized into biological process, molecular function, and cellular components.

Functional genomics analysis of the *NPR1* gene in rice indicate its involvement in defense signaling during fungus infection through dual RNA-seq

By analyzing transcriptome datasets of the rice cultivar Kasalath, acquired from a public repository, this research aimed to study the expression profiles of OsNPR1 genes in response to nitrogen fertilization and *Pyricularia oryzae* infection. Seedling shoots of 3-week-old exposed to different treatments (nitrogen fertilization, *P. oryzae* inoculation, or the combination of both) were harvested and analyzed at 0 and 2 days after treatments, while the distilled water treated samples served as controls. In order to understand the crosstalk of nitrogen and pathogen stress in the regulation of these genes, the study measured the transcriptional response of *OsNPR1* genes (fold change and p-value) across five treatment comparisons (Table S7).

Among all the genes, *OsNPR1-1* showed the greatest upregulation with the highest fold change value of 1.80 in T2 and other values are T1 (1.50), T5 (1.10), and T3 (0.90), indicating a strong induction in both conditions of nitrogen availability and *Pyricularia oryzae* infection. *OsNPR1-3* demonstrated moderate upregulation as well, especially at T1 (0.90), indicating its potential role in initial nitrogen response. In contrast, *OsNPR1-2* and *OsNPR1-5* showed modest or inconsistent expression alterations, with fold changes ranging from -0.30 to 0.60, and none of them exceeded a significant regulatory threshold. *OsNPR1-4* expression remained largely steady throughout all treatments, with just a modest increase found, peaking at 0.50 under T1 (Table S7).

Table 2 Expression analysis of OsNPR1 genes under different treatments showing fold change values and corresponding p-values.

Gene ID	Gene Name	T1.fC	T1, pValue	T2, fC	T2, pValue	T3, .fC	T43, .pValue	T4' .fC	T4, pValue	T5, .fC	T5, pValue
Os01g0194300	*OsNPR1*	1.5	4.50E-04	1.8	1.07E-26	0.9	1.82E-04			1.1	0.020303157
Os03g0767900	*OsNPR2*	0.6	0.222918087			-0.3	0.3460047				
Os02g0667100	*OsNPR3*	0.9	0.298052301	0.3	0.565111514	0.2	0.801512672				
Os03g0667100	*OsNPR4*	0.5	0.358163358	0.2	0.336116266	0.4	0.119401515	-0.2	0.999991062	0.1	0.969982164
Os04g0667100	*OsNPR5*	-0.3	0.470013784	0.2	0.597654687	-0.1	0.741076392	0.2	0.999991062	0.4	0.602153745

A p-value heatmap analysis was performed to evaluate the significance of differential expression across members of the *OsNPR1* gene family under different treatments. The findings demonstrated a substantial transcriptional response, with *OsNPR1-1* displaying statistically significant expression changes under four treatment conditions: T2 (p = 0.00), T1 (p = 0.00), T3 (p = 0.00), and T5 (p = 0.02). Under T3, *OsNPR1-3* also demonstrated a substantial change (p = 0.03). However, as all related p-values were higher than 0.05, the other genes *OsNPR1-2*, *OsNPR1-4*, and *OsNPR1-5* did not exhibit statistically significant expression across any treatments (Table S7). Thus, *OsNPR1-1* exhibits significant overexpression in response to T1, T2, T3, and T5, suggesting a crucial role in defense mechanisms. Its heightened sensitivity to biotic and abiotic influences positions it as a promising subject for future functional research,

distinguishing it from other family members with less pronounced expression changes.

Putative miRNA Targets in *Oryza sativa*

A genome-wide analysis of microRNAs found 59 distinct miRNAs targeting *OsNPR1-1*, *OsNPR1-2*, and *OsNPR1-3* genes in rice Table S8. *OsNPR1-1* was the most commonly targeted gene, with 41 unique mature miRNAs targeting different areas of its transcript. *OsNPR1-2* was regulated by 14 mature miRNAs, suggesting moderate post-transcriptional regulation. The study also found a strong level of sequence conservation and functional redundancy within miRNA families, suggesting a coordinated regulatory process among family members. Since it is heavily involved in signaling salicylic acid and activating plant defenses, the most influenced gene *OsNPR1-1* may be under very tight post-transcriptional regulation.

Discussion

The study of the pathways through which *NPR1* homologs operate in *Oryza sativa* was initiated, putting a spotlight on the nitrogen response and interaction with biotic factors. The *Arabidopsis thaliana NPR1* sequence, known for its role in salicylic acid induced defense, served as the reference for blasting homologous sequences in the rice genome (Tang et al., 2025). This was done by identifying conserved domains, i. e. molecular signatures that are essential for protein interactions and co-activation, which quite literally came handy from the use of the resources such as Phytozome v13 and MEME Suite. A BLASTP inquiry of rice proteome in the Phytozome and NCBI databases showed the potential rice homologs by way of sequence similarity, domain conservation, and the functional annotation criteria (Shrestha and Huang, 2022).

A total of five *OsNPR1* genes (*OsNPR1-1 to OsNPR1-5*) were found, with two later suspected to be duplicates because of close domain structures and coding sequences. Nevertheless, chromosome mapping and analysis of the flanking regions revealed that these are separate genomic loci and gene names were standardized according to the chromosomal positions as per the genome-wide conventions. This was the basis for structural, evolutionary, and functional aspects to be, among others, studied in connection with responses to the nitrogen treatment (Saukkonen and Hemminki, 2004). The present study was focused on analyzing gene structures of five *Oryza sativa NPR1* homologs (*OsNPR1-1 to OsNPR1-5*) for unraveling their genomic order and variations. Substantial change in lengths of genes indicated by variation in their nucleotide composition opening a window for differential gene expression, regulation, and functions in these genes. Initially, the gene lengths were determined by using annotate features available in the Phytozome database and the results were double checked by analyzing exon-intron structures using the Gene Structure Display Server (GSDS) (Roy, 2016). Physiochemical traits such as amino acid length, molecular weight (MW), and isoelectric point (pI) of the 5 *OsNPR1* protein isoforms discovered in *Oryza sativa*, were studied with the result that the amino acid sequences were in the range of 582 and 635 residues. A large size like this is totally in line with the type of the *NPR1* family proteins, which are known to have BTB/POZ and ankyrin repeats type of domains that are indispensable for protein-protein interactions during salicylic acid defense signaling. The *OsNPR1* proteins identified here have a molecular weight range of 63.76 kDa to 68. 49 kDa and their pI (isoelectric strength) values lie between 5.41 and 6. 04. Such molecular masses align with their function as transcriptional scaffold proteins, aiding the assembly of TGA transcription factors and cofactors for systemic acquired resistance (SAR). These protein sizes also infer a capacity for nuclear entry (Papadakis et al., 2004; Roy, 2016).

The analysis of *OsNPR1* gene family by exon - intron organization in rice shows that the number of exons and their positions are pretty much conserved among all members. Similar exon numbers and sequences are found in *OsNPR1-1* and *OsNPR1-3* (Figure 1, Table S1), so one can speculate gene duplication in the biological past of at least one the pair members. Remarkably, *OsNPR1-5* is an intronless gene which is a classical feature of genes that are involved in fast responses to environmental signals. Gene structures largely match phylogeny which means that function and co-evolution are linked and identical exon patterns help point to evolutionary lineages that have been maintained, especially in relation to genes involved in defense. The OsNPR1 gene family (*OsNPR1-1 to OsNPR1-5)* has conserved compositions and mostly comprise exon-only or intronless genes. This intron-free feature of the gene not only facilitates a quicker and more efficient gene expression, which is a characteristic very important for survival and defense of plants, particularly in the case of salicylic acid signaling - other hand, structural similarities are barely enough to say that they have a common origin and function; changes in expression patterns are the proof that these genes are regulated differently at transcription and post-transcription level. Intronless genes are well-known for their quick and reliable stress-induced expression, which is exactly what *NPR1* is, as a stressed-induced gene, to biotic and abiotic stimuli scenarios (Lowry and Zitomer, 1984; Nettelbeck et al., 2002).

In order to assess the evolutionary relationship and functional conservation of *OsNPR1* genes, a maximum likelihood phylogenetic tree was built with MEGA X, seeded with 1000 bootstrap replicates, by protein sequence comparison of rice Arabidopsis *Daucus carota*, and two other species. The *25 NPR1* proteins were divided into five

classes, namely Group-1 to Group-5 (Fig. 3, Table S3). *OsNPR1* genes appeared in all groups with four rice genes in Group-2, and *OsNPR1-1* in Group-5. Group-3 had the highest number of genes (9) which might be considered as evidence of lineage-specific duplications. According to this analysis, *OsNPR1* proteins(cpatable with rice) share a common ancestor with related *NPR1* proteins in dicots and monocots which is also true for proteins that have been conserved over time and for the ones which are unique to rice (Lee and Seo, 2022; Lenhard et al., 2012).

The MEME suite identified 20 conserved motifs in the *OsNPR1* proteins. Genes that are closely related in the evolutionary tree have, by and large, the same arrangement and frequency of motifs, especially within the same group. All the *OsNPR1* proteins had motifs linked with the *NPR1-like*_C_superfamily domain (Fig 2), which plays a key role in interaction between proteins and regulation of transcription during the SA-mediated defense. Functional similarity and overlapping regulatory functions are inferred from motif redundancy, while unique motifs, for instance motif 7 in *OsNPR1-1*, are considered as a manifestation of sub functionalization for a specialized signaling (Fig 2). This is based on the observation that the OsNPR1 gene family members have not only been under purifying selection but also have diverged, thus leading to the complexity of plant immune responses. The five OsNPR1 proteins are very similar in their sequences and feature the same domains, suggesting that they carry out essentially the same function in different conditions and tissues. Phylogenetically they cluster together and share the conserved motifs. More specific characteristics are given in Table S2(Kawakami et al., 2024; Lee and Kaplan, 1992).

The results of *OsNPR1* protein subcellular localization prediction by WOLF PSORT showed that majority of these proteins are located in the nucleus (Figure 9). This corroborates that they act as transcription co-activators in the salicylic acid (SA) defense pathway. The other possible localizations predicted to be chloroplast cytoplasm mitochondria, Golgi apparatus, extracellular space, and plasma membrane also imply functional diversity and involvement in inter-organellar communication or stress response. These data match those of Arabidopsis, where *NPR1* proteins are known to undergo redox-driven conformational status changes for nuclear translocation during the signaling process (Guillermo et al., 2017; Harshith et al., 2024).

Rice analysis revealed ten OsNPR1 gene pairs as segmentally duplicated, while none was identified as tandem duplicated, thus the whole genome duplications (WGD) likely played a major role in the expansion of the NPR1 gene family. These segmental duplications are evolutionarily very old with the divergence time ranging from 88. 60 to 112. 48 million years ago. Ka/Ks ratios for all pairs were less than 1 and ranged between 0. 20-0. 45 (Table S4, Figure 7), which is indicative of strong purifying selection. Such a purifying selection means that these duplicated OsNPR1 genes have preserved their essential functions, maybe by undergoing sub-functionalization or functional redundancy to ensure robustness in plant immune responses and defense signaling pathways (Farrera-Sal et al., 2020; Geffers et al., 2000).

Chromosomal localization analysis shows that 5 *OsNPR1* genes in rice are unevenly distributed on chromosomes 1 and 3, with *OsNPR1-1* & *OsNPR1-2* on chromosome 1, and *OsNPR1-3*, *OsNPR1-4*, and *OsNPR1-5* on chromosome 3. The rest 2 4 5, 6 7 8, 9 10 11, and 12 chromosomes do not have OsNPR1 genes (Figure 4, Table S5). This localized distribution implies gene aggregation, the possible presence of common regulatory systems, and segmental duplication events, which are all indicators of the limited evolutionary diversification of the *NPR1* gene family. Physical map data could help us reveal the structural organization of the *NPR1* gene family and at the same time could be used for synteny and co-expression studies which may associate these genes with stress resistance QTLs for rice breeding (Bowen and Hassan, 1993; Chen et al., 2025).

To look at the evolutionary history and conservation of *OsNPR1* genes in rice and other species, mainly Arabidopsis thaliana, synteny analysis was performed with TBtools and Circoletto. The study was based on both intra-genomic and inter-genomic synteny. Within the rice genome (Figure 5), it is shown that the primary mode of expansion of the *OsNPR1* gene family has been through segmental duplication as the evidence to be the linkage of homologous loci in synteny analysis. This is unlike tandem duplication which is usually the way gene families expand in plants. Segmental duplication has also been the major mode of duplication in rice which fits with the fact that it is an ancient polyploidy (Buttar et al., 2020).

The result of a dual synteny comparison between *Oryza sativa* (rice) and Arabidopsis thaliana (Figure 6) showed that a part of gene pairs were conserved while a part was divergent. OsNPR1-4 was strongly conserved in syntenic regions with *AtNPR1-3* and *AtNPR1-6*, which implies that they have kept the same ancestral role and structural integrity that is probably associated with immune signaling and stress responses. However, *OsNPR1-1* and *OsNPR1-3* have shown a fragmented syntenic relationships which may indicate functional divergence and species-specific adaptations. This research highlights the evolutionary conservation of the main NPR1 genes but at the same time reveals the existence of unique functions that specific rice NPR1 members have probably gained through evolution. Thus, it supports the idea of a conserved NPR1 module and at the same time, it exemplifies the complexity of the evolution of plant defense regulation (Eshed et al., 2004).

The analysis of the cis-regulatory elements of *OsNPR1* gene promoters exhibited various regulatory elements that affect gene expression (Figure 8). All the 5 *OsNPR1* genes were equipped with light-responsive elements such as G-box, GT1-motif, Box 4, AE-box, and TCT-motif, suggesting their role in light-regulated functions (Jenkins, 2009). Besides that, the presence of stress-responsive elements *ABRE DRE* core, *ARE*, and *STRE*, points to the possibility that OsNPR1 genes might be regulated at the transcriptional level by abiotic stresses like drought and cold. Hormonal control was evident through the appearance of MeJA-associated motifs (CGTCA-motif, TGACG-motif), salicylic acid-related motifs (TCA-element, CARE), and gibberellin (GARE-motif, P-box), thus indicating that these OsNPR1 genes play roles in the integration of hormone signaling pathways, in aspects of plant defense and development. Along with these, numerous transcription factor binding sites such as *MYB MYC* W box, and *WRE3*, binding sites were identified, linking *OsNPR1* to major transcriptional regulators including WRKY and bHLH families which are essential for stress and defense responses. This detailed analysis clearly shows that *OsNPR1* genes are tightly regulated by multiple factors and therefore function as major players in the gene regulatory networks of rice (Horton et al., 2006).

A genome-wide study of potential miRNA binding sites revealed that 59 distinct miRNAs target the *OsNPR1* gene family *(OsNPR1-1, OsNPR1-2, OsNPR1-3*) (Table S8). OsNPR1-1 was targeted most, with 41 miRNAs, among which was the miR395 family, implying regulation by stress-responsive pathways. *OsNPR1-2* was targeted by 14 miRNAs. miR5075 notably targeted both *OsNPR1-1* and OsNPR1-2, pointing to shared regulatory mechanisms. *OsNPR1-3* was targeted by the least number of miRNAs 8 which might mean that it has more specialized regulatory functions. The results point to the fact that the heavy miRNA targeting of *OsNPR1-1* highlights its major involvement in salicylic acid signaling and pathogen defense, the elaborate regulation ensuring accurate responses to biotic and abiotic stresses (Adak et al., 2023; Akbudak et al., 2020).

The protein-protein interaction network of *Oryza sativa NPR*1 homologs (*OsNPR1-1, OsNPR1-2, and OsNPR1-5*) (Figure 10 & 11) reflects a very close functional relationship that hints at a conserved regulatory mechanism of rice defense signaling. The solid interactions among these proteins indicate that they likely collaborate to affect the salicylic acid (SA)-dependent pathways and systemic acquired resistance (SAR), just like the NPR1-mediated responses in Arabidopsis thaliana. Among these, *OsNPR1-1* was seen as a central node, which may indicate its major role as a regulator of downstream defense gene expression. This kind of clustering also implies that there could be both redundancy and specialization within the *NPR1* family members who individually might be fine-tuning defensive responses to different stress situations. In conclusion, our data highlight the functional importance of *NPR1* homologs in rice immunity and provide a basis for further experimental verification of their interactions and roles in biology (Sherazi et al., 2026).

Expression profiling of the *OsNPR1* gene family members in *Oryza sativa* (cultivar: Kasalath) under nitrogen fertilization and *Pyricularia oryzae* inoculation revealed differential responses. *OsNPR1-1* was the most dynamically expressed member. It was significantly up-regulated in both nitrogen and fungal infection indicating its involvement in the merging of nitrogen signaling and immune response. *OsNPR1-3's* up-regulation in relation to nitrogen signaling was moderate and *OsNPR1-4's* expression was quite stable and low level. The changes in *OsNPR1-2* and *OsNPR1-5* fold levels were either low or opposite (Figure 12, Table S7), implying that they might not be responsive to the conditions tested. In addition, their non-responsiveness to the conditions tested could possibly be due to post-transcriptional regulation or that they are involved in different stress situations. These data indicate that the OsNPR1 gene family members have different functions, which is in line with other species studies. The p-value's heatmap validated the fold change results and also brought to light that *OsNPR1-1* is the most significant gene with 0.00 p-values in T1, T2, and T5, and 0.02 in T4 treatments (Figure 13, Table S7). These findings express that the alterations of gene expression changes were statistically significant and they highlight *OsNPR1-1* as the gene that is likely to be involved in the regulation of nitrogen and pathogen-elicited transcription. On the other hand, *OsNPR1-3* had a slight significance (p=0.80 in T5), *OsNPR1-4*, *OsNPR1-2*, and *OsNPR1-5* had p-values of more than 0.20 which suggest their changes were not statistically significant and most probably represent background variation or very low responsiveness to treatments. These findings agree with the earlier studies on *NPR1* orthologs where they show differential expression depending on tissue and treatment (Hayat et al., 2025; Hayyat et al., 2025; TUFAIL et al., 2025).

Rice protein *OsNPR1-1* was found to be an essential protein in the regulation of not only nitrogen but also pathogen response, as its levels change substantially in both nitrogen and pathogen stress conditions. It is also believed that *OsNPR1-1* could be the equivalent of *Arabidopsis thaliana's AtNPR1* in mediating the salicylic acid defense and nutrient response pathways (Yilmaz et al., 2025). Investigating other *OsNPR1* family genes may provide supportive evidence of the stress response function, and *OsNPR1*-1 can be the primary candidate for CRISPR/Cas9 mediated genetic manipulation for functional analysis. Moreover, cis-regulatory motifs, miRNA interactions, and synthetic promoter or

miRNA-resistant constructs are some of the experiment possibilities, while transcriptome and proteome datasets in combination can give a more complete picture of *NPR1*-regulated gene networks.

Conclusion

By referring to the model plant Arabidopsis thaliana, we discovered five rice homologs of *OsNPR1*, named *OsNPR1-1* to *OsNPR1-5*. Gene structure analysis, physicochemical properties, phylogenetic classification, identification of conserved motifs and domains, chromosomal mapping, and subcellular localization prediction studies suggest that rice OsNPR1s are structurally conserved, localize to the nucleus, and carry out the functions of transcriptional regulation. Further evolutionary analysis shows segmental duplication as the major mechanism of gene family expansion along with purifying selection, while synteny analysis demonstrates that OsNPR1-4 is connected to *AtNPR1* orthologues. Promoter studies reveal the presence of cis-elements involved in stress, hormonal, and light response, whereas miRNA targeting analysis shows that OsNPR1-1 is the most regulated at the post-transcriptional level. GO ontology proof that, *OsNPR1-1*, *OsNPR1-2*, and *OsNPR1-5* are functionally interconnected in the salicylic acid-mediated defense response, participate in the immune signaling pathway, localize to the cytoplasmic INA complex, and have organic acid-binding molecular function, all of which contribute to disease resistance in *Oryza sativa*. Expression analysis showed *OsNPR1-1* has the highest transcriptional activity under nitrogen fertilization and *Pyricularia oryzae* infection, suggesting a key role in salicylic acid-mediated defense and nitrogen signaling, with other *OsNPR1* genes having moderate or specific responses. *OsNPR1-1* is a significant candidate for improving rice stress resilience and nutrient utilization, providing tools for developing resilient rice varieties and enhancing understanding of plant immunity and stress responses.

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Statements and Declarations

Authors' Contribution

Tooba Nazish and Muhammad Rizwan Conceptualization, data curation, writing - first draft, formal analysis, investigation, methodology; formal analysis, validation, data curation, software, writing - review and editing. Other authors provide guidance and assistance during the article. All authors made important contributions, reviewed, and approved the published version of the work.

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Conflict of Interest

It is claimed that the authors have no known conflicts of interest for the publication of this work.

Ethical Responsibility

This manuscript is original research, and it is not submitted in whole or in part to another journal for publication.

Data Availability Statement

This study did not generate new datasets. All data used in this investigation were obtained from publically available genetic databases, as mentioned in the report. The Supplementary Data file comprises all data generated or processed during this study, including protein sequences and accession numbers.

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