Genome-Wide Prediction and Analysis of Oryza Species NRP Genes in Rice Blast Resistance

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Abstract: Members of the N-rich proteins (NRPs) gene family play important roles in the plant endoplasmic reticulum stress in response, which can be triggered by plant pathogens' infection. Previous studies of the NRP gene family have been limited to only a few plants, such as soybean and Arabidopsis thaliana. Thus, their evolutionary characteristics in the Oryza species and biological functions in rice defense against the pathogenic fungus Magnaporthe oryzae have remained unexplored. In the present study, we demonstrated that the NRP genes family may have originated in the early stages of plant evolution, and that they have been strongly conserved during the evolution of the Oryza species. Domain organization of NRPs was found to be highly conserved within but not between subgroups. OsNRP1, an NRP gene in the Oryza sativa japonica group, was specifically up-regulated during the early stages of rice-M. oryzae interactions-inhibited M. oryzae infection. Predicted protein-protein interaction networks and transcription-factor binding sites revealed a candidate interactor, bZIP50, which may be involved in OsNRP1-mediated rice resistance against M. oryzae infection. Taken together, our results established a basis for future studies of the NRP gene family and provided molecular insights into rice immune responses to M. oryzae.

Keywords: Oryza species; NRP genes; conserved evolution; bZIP50 TF; rice immune response

1. Introduction

Rice is a crucial crop that is responsible for feeding more than half of the world’s population [1]. However, a variety of extreme environmental conditions, (such as drought, salinity and extreme temperatures) negatively affect rice plant growth. Additionally, rice blast, which is caused by the hemi-biotrophic fungal pathogen Magnaporthe oryzae, can reduce rice yield by 30% [2].

To inhibit pathogens’ invasion, host plants have evolved a two-layer immune system [3,4]. The first is determined by plant pattern recognition receptors (PRRs). Through recognizing pathogen-associated molecular patterns (PAMPs) of pathogens (such as PGN and chitin oligosaccharide), plant PRRs activate PAMP-triggered immunity (PTI) for the inhibition of pathogens’ colonization. Pathogens can correspondingly release various effector proteins into host cells to evade or subvert host PTI defenses [5]. The second layer plant immune system is governed by resistance (R) proteins, which mainly have nucleotide-binding site leucine-rich repeat (NBS-LRR) domain architecture. Through directly or indirectly binding to avirulence (Avr) effectors of pathogens, R proteins are induced and activate a rapid response, also known as effector-triggered immunity (ETI) [6].

Rice resistance based on NBS-LRR proteins is often overcome within five years due to the rapidly evolving and highly variable effectors of blast fungus [7]. In contrast, multiple defense-regulator (DR) genes also confer partial but broad and durable resistance. Until now, dozens of DR genes were found that maintain this durable resistance by guaranteeing effective signal transduction and being responsible for downstream immune responses [8,9].
These downstream immune responses include accumulation of antimicrobial compounds or hormones, reactive oxygen species (ROS) bursts, and activation of programmed cell death (PCD) [10,11]. The endoplasmic reticulum (ER) is a key organelle involved in the activation of the stress response [12]. During pathogen-plant interactions, effector proteins target not only host defense-related proteins, but also ER resident proteins, causing ER-stress [13,14]. This triggers the unfolded protein response (UPR) to inhibit the accumulation of unfolded/misfolded proteins. If levels of excessive unfold/misfold surpass a threshold level, the PCD pathway is activated [15].

ER stress and osmotic stress signaling, which form an integrated major response to ER stress-activated cell death, converges on N-rich proteins (NRPs). This integrated signaling pathway was originally identified in soybean (Glycine max, L.) during the early stage of the hypersensitive response, which was activated by infection with avirulent bacteria [14]. NRP proteins were characterized by their NRP domain, namely development and cell death (DCD) domain, which is plant-specific and composed of around 130 amino acids. However, NRP proteins appear to be absent in bacteria and fungi. This supports the hypothesis that NRP proteins are plant-specific and were present at the start of plant evolutionary history, prior to the separation of higher plants. The NRP protein family can be divided into four subgroups based on the location of the NRP domain. Ref. [16] detected that amino acids of soybean NRP proteins formed alpha-helix and remaining amino acids were used to generate beta strands. Meanwhile, the N-terminal halves of NRP proteins are asparagine-rich (24%), whereas the C-terminus regions contain the NRP domain [16,17]. These proteins include an FGLP and an LFL motif at the N-terminus and a PAQV and a PLxE motif toward the C-terminus.

Not only environmental changes but also pathogen infection can disturb ER homeostasis in the host plant [18]. Once host ER homeostasis is disrupted, two type ER-stress sensors were activated to reduce the content of unfolded or misfolded proteins so that the host ER homeostasis is restored. Protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1 (IRE1), and activating transcription factor-6 (ATF6) were assigned to type 1 ER-stress sensors; Type 2 includes bZIP transcription factor (TF) bZIP60 [19]. However, if ER stress is sustained, the NRP-mediated PCD signaling pathway is activated. For example, in soybean, the ER stress- and osmotic stress-induced TF GmERD15 regulates expression of NRP genes through binding to NRP promoters [20]. NRP proteins transduce hallmark PCD signals to activate the PCD signaling cascade, which induces expression of the TFs GmNAC81 and GmNAC30. GmNAC81 and GmNAC30 transactivate vacuolar processing enzyme (VPE) by heterodimerization, then induce plant-specific PCD [21].

Thanks to the rapid development of high-throughput sequencing method in the last decade, high-quality and complete genome assemblies of many plant species were obtained, which help researchers to identify gene families that might link to plant development, abiotic or biotic stress response, and thus have a comprehensive understanding of them. For example, [22] identified 33 encoding genes of β-Ketoacyl CoA synthetase (KCS) through BLAST and hidden Markov model search against barley genome and found some of them may determine the synthesis of very-long-chain fatty acids (VLCFAs), which affect the formation of epidermal wax under drought stress. Moreover, similar methods were also applied for detection of proline-rich extensin-like receptor kinases (PERKs) gene family in wheat, which revealed 37 PERK genes and eight of them were up-regulated in response to drought and heat stress [23]. Beyond identification of encoding gene families, globally predicted natural antisense transcripts (NATs) and long non-coding RNAs (lncRNAs) were predicted through mapping RNA-seq reads on corresponding reference genome assemblies [24,25], which revealed regulatory mechanisms of compounds biosynthesis (anthocyanin and phenylpropanoid) in Salvia miltiorrhiza and abiotic stress response in Capsicum annuum.

The functions of NRP proteins in soybean and Arabidopsis thaliana in response to diverse stressors have previously received a great deal of attention. However, few studies
have reported the functions of NRP proteins in rice resistance to blast fungus. The roles and biological functions of NRP proteins in other rice defense responses also remain unclear. The present study aimed to shed light on the evolutionary history, putative interaction partners, expression patterns, and biological functions of NRP s in rice resistance. The results revealed conserved evolution of the NRP protein family and synergistic differentiation between *Oryza* species. We also found that *OsNRP1*, an NRP gene in the *Oryza sativa japonica* group, was specifically up-regulated at the early infection stage and enhanced rice resistance to *M. oryzae* attack. Furthermore, *OsNRP1* was also predicted to interact with IRE1, bZIP TFs and NAC-domain containing proteins. Our findings provide a basis for further investigation of NRP protein functions and regulatory mechanisms in rice resistance.

2. Results

2.1. Whole-Genome Characterization and Phylogenetic Analysis of NRP Genes in *Oryza* Species

Two strategies were independently applied for the identification of NRP genes in *Oryza* species. First, a hidden Markov model approach (HMM) was used to search against 12 *Oryza* species and soybean proteomics, which were retrieved from the Ensembl plant database (https://plants.ensembl.org/, (accessed on 5 July 2022)), based on the NRP domain HMM model file (Pfam accession: PF10539). Second, 26 characterized plant NRP genes were used as queries in BLASTP searches against the 13 proteomes using an e-value cutoff of $1 \times 10^{-5}$. To further verify the reliability of candidates NRP genes, the Conserved Domains Database (CDD) tool was used to validated the completeness of the NRP domain in each candidate. A total of 136 complete, non-redundant NRP genes were detected in 12 *Oryza* species and soybean (Table 1), with each *Oryza* species containing between nine and 13 homologs, and 15 in soybean (Table S1 and Figure 1a). This demonstrated wide and conserved distribution of NRP genes among *Oryza* species.

| Species                | Chromosomes | Genome Group | NRP Proteins | Whole Proteins |
|------------------------|-------------|--------------|--------------|----------------|
| *Oryza barthii*        | 24 (12 × 2) | AA           | 11           | 41595          |
| *Oryza brachyantha*    | 24 (12 × 2) | FF           | 9            | 32038          |
| *Oryza glaberrima*     | 24 (12 × 2) | AA           | 10           | 33164          |
| *Oryza longistaminata* | 24 (12 × 2) | AA           | 12           | 31686          |
| *Oryza meridionalis*   | 24 (12 × 2) | AA           | 10           | 43455          |
| *Oryza nivara*         | 24 (12 × 2) | AA           | 11           | 48360          |
| *Oryza punctata*       | 24 (12 × 2) | BB           | 11           | 41060          |
| *Oryza rufipogon*      | 24 (12 × 2) | AA           | 11           | 47441          |
| *Oryza sativa indica group* | 24 (12 × 2) | AA           | 13           | 40745          |
| *Oryza sativa japonica group* | 24 (12 × 2) | AA           | 12           | 42419          |
| *Oryza glumipatula*    | 24 (12 × 2) | AA           | 11           | 46893          |
| *Glycine max*          | 40 (20 × 2) | -            | 15           | 88412          |

We investigate the evolutionary history of NRP genes in *Oryza* species by constructing a maximum likelihood (ML) phylogenetic tree (Figure 1b). A total of 136 putative NRP genes in the *Oryza* species and soybean were identified. These genes were categorized into four subgroups (I-IV). NRP genes from each *Oryza* species and soybean were present in all four groups, suggesting that four ancestral NRP subgroups divided in the most recent common ancestor (MRCA) of *Oryza* species and soybean. No clustered pairs of NRP genes were detected in any *Oryza* species, indicating a lack of species-specific duplication events. In contrast, paralogous pairs of *G. max* NRP genes in each subgroup revealed that this family underwent several duplication events in soybean. After scanning the domain architecture of each protein, we found tandem Kelch motifs located in C-terminus of all subgroup II members except *OINRP3*, *GmNRPI*, and *GmNRPP1*. The preference of C-terminus location...
was found among NRP genes in subgroup I, which differed from other NRP genes. Notably, NRP domain duplications were also detected in NRP proteins in subgroup III and IV.

**Figure 1.** Phylogenetic relationships and distribution of NRP genes in 12 *Oryza* species and soybean. (a) Species tree of 12 *Oryza* species and soybean and distribution of NRP proteins. The species tree was constructed using the maximum likelihood method with 1000 bootstraps based on a concatenated alignment of housekeeping genes identified by CEGMA analysis. Numbers of NRP protein were listed on the right side of the species tree. (b) Phylogenetic tree of NRP proteins identified in 12 *Oryza* species and soybean. Maximum likelihood tree, with 1000 bootstraps (values displayed per branch). NRP proteins identified in *Oryza sativa japonica* group are marked in red.
2.2. Synteny Analysis of the NRP Gene Family in Oryza Species

There were 12, 13, 11, 11, and 15 NRP genes in the *O. sativa japonica* group, the *O. sativa indica* group, *Oryza punctata*, *Oryza barthii*, and *Glycine max*, respectively. To further investigate the orthologous relationships, a synteny analysis was conducted for NRP genes in these species (Figure 2a and Table S2). The *O. sativa japonica* group genome shared 10, 11, and 11 syntenic NRP genes with the *O. sativa indica* group, *O. barthii*, and *O. punctata*, respectively. The *O. sativa indica* group genome had 11 and 10 syntenic genes with *O. barthii* and *O. punctata*, respectively, and there were 11 syntenic NRP genes between *O. barthii* and *O. punctata*. These results indicated a high level of conservation of NRP gene family after differentiation of *Oryza* species. In contrast, only *GmNRP4* and *GmNRP12* showed a syntenic relationship with corresponding genes in *Oryza* species, suggesting more extensive divergence between NRP genes in soybean and *Oryza* species. An intraspecific synteny analysis of the *O. sativa japonica* group revealed that only *OsNRP3* and *OsNRP8* had a syntenic relationship (Figure 2b), indicating that there was no species-specific expansion of the NRP gene family. These results together with the phylogenetic analysis (Figure 1b) indicated that the putative duplication occurred in the MRCA of *Oryza* species.

The ratio of nonsynonymous (Ka) to synonymous (Ks) nucleotide substitution rates is a common measurement used to distinguish between selective pressures on protein-coding genes and to assess their evolutionary rates [26]. When Ka/Ks ≈ 0, a gene is considered to be under neutral selection, whereas Ka/Ks < 1 indicates purifying selection and if Ka/Ks > 1 indicates positive selection [27]. Ka/Ks value of the syntenic NRP gene pairs detected above were nearly all <1 (Table S2), suggesting that the NRP gene families in these species had evolved under purifying selection. However, the syntenic gene pair *OsNRP4/OsNRP6* (which was also syntenic with *OsNRP2*) had a Ka/Ks ratio of 1.79, revealing that *OsNRP4* may have been under positive selection and may have a novel function.

2.3. NRP Genes in the O. sativa japonica Group were Differentially Expressed during M. oryzae Infection

To analyze the expression patterns of *O. sativa japonica* group NRP genes (*OsNRPs*) during *M. oryzae* infection, we calculated expression values using the data from our previously RNA sequencing (RNA-seq) [28]. In that study, *O. sativa* L. ssp. *japonica* cv. ‘Nipponbare’ (Nip) plants were infected with three *M. oryzae* strains (A248, B235, and C162). Seven *OsNRP* genes were found to be significantly up-regulated during the interactions between Nip and *M. oryzae*. As Figure 3a showed, *OsNRP2*, *OsNRP3*, *OsNRP4*, *OsNRP5* and *OsNRP8* were significantly up-regulated at 24 h post infection (hpi). In addition, *OsNRP1* and *OsNRP6* were significantly up-regulated at 8 hpi with *M. oryzae* A248 or B235. Due to our rice the genetic transformation system relied on wild-type cultivar ‘Zhonghua 11’ (ZH11), which was used as the background cultivar. We performed quantitative reverse transcription (qRT)-PCR assays to validate the expression patterns of seven *OsNRPs* candidates during interactions between ZH11 and Guy11 (Figure 3b). *OsNRP2*, *OsNRP3*, and *OsNRP4* were down-regulated during infection. *OsNRP6* and *OsNRP8* were up-regulated at 8, 48, 72, and 96 hpi, whereas *OsNRP6* was up-regulated at 24, 72, and 96 hpi. Notably, *OsNRP1* (*LOC_Os01g36950*) was specifically up-regulated at eight hpi but was barely detected at other timepoints. These results suggested that *OsNRP1* may play a role in the plant defense response at 8 hpi.
Figure 2. Synteny analysis of NRP genes. (a) Interspecies syntenic relationships of NRP genes in *Glycine max*, the *Oryza sativa japonica* group, the *Oryza sativa indica* group, *Oryza punctata* and *Oryza*
2.4. OsNRP1, a NRP Protein in the O. sativa japonica Group, may Enhance Blast Fungus Resistance

The high expression levels of OsNRP1 during the early infection stage suggested that OsNRP1 may be involved in blast resistance. To validate this hypothesis, a transgenic OsNRP1-overexpression line (OsNRP1\textsuperscript{OX}) was constructed. We obtained a total of 22 independent transgenic T\textsubscript{1} lines. Through qRT-PCR validation of each T\textsubscript{1} lines, we found that OsNRP1 was expressed at significantly level (around four- to 12-fold higher than in the wild-type) in three T\textsubscript{1} lines: PXQ8-4, PXQ8-5, and PXQ8-17 (Figure 4d). These three lines were selected for inoculation assays by M. oryzae Guy11. Seven-days after in vitro inoculation, the total lesion length on ZH11 was 2.7 cm (1.5 cm + 1.2 cm), which was significantly longer than the total lesion lengths on the OsNRP1\textsuperscript{OX} plant (1.5, 1.4, and 1.7 cm on PXQ8-4, PXQ8-5, and PXQ8-17, respectively) (Figure 4a). Similar results were observed after in vivo inoculation; the diseased leaf area was significantly smaller on PXQ8-4, PXQ8-5 and PXQ8-17 than on ZH11 (Figure 4b,c). These results suggested that OsNRP1 might be activated by blast pathogen infection and could enhance rice resistance against M. oryzae.

2.5. Predicted Protein-Protein Interaction (PPI) Network of OsNRP1

NRP\textsuperscript{s} require interacting partners to activate the NRP-mediated cell-death signaling pathway [20]. To further analyze the mechanisms of OsNRP1-mediated resistance to rice blast, a predicted interaction network was constructed for OsNRP1 using STRING website (Figure 5a). We identified 10 candidate proteins that may interact with OsNRP1 (Table S3), including a bZIP TF (bZIP50), Serine/threonine-protein kinase (IRE1), Zinc finger domain-containing stress-associated proteins (SAP16, SAP3 and SAP5), a NAC domain-containing protein (Q5Z7Q4), and cold shock domain-containing protein (CSP1). We also investigated the PPI network of an OsNRP1 ortholog in Arabidopsis thaliana, AT5G42050 (Figure 5b). Notably, a bZIP TF (bZIP60) and NAC domain-containing proteins (NAC062 and NAC036) were also detected in that network.

Gene Ontology (GO) annotation enrichment analysis was conducted for the putative OsNRP1 interaction partners. This analysis revealed that ‘Response to stress’, ‘Response to organic substance’, and ‘Endoplasmic reticulum unfolded protein response’ were the major enriched terms, which contributed to understanding of the NRP-mediated response to rice blast. For example, soybean NRPs and their orthologs in Arabidopsis were found to be induced by endoplasmic reticulum (ER) stress, and triggered PCD response [12]. Ref. [29] reported Arabidopsis thaliana NRP genes were up-regulated in response to cold and drought stress, uncovering in their contribution in signal transduction. Thus, we inferred that OsNRP1 may enhance rice defense through contributing to mediate ER stress.
Figure 3. Expression analysis of NRP genes in the Oryza sativa japonica group. (a) RNA-seq expression profile of 12 predicted NRP genes in the O. sativa japonica group NRP genes during interactions with
three *Magnaporthe oryzae* strains (A248, B235 and C162). (b) Quantitative reverse transcription (qRT-PCR) verification of selected *NRP* gene expression levels in the *O. sativa japonica* group during interactions with *M. oryzae* strain Guy11.

Figure 3. Expression analysis of *NRP* genes in the *Oryza sativa japonica* group. (a) RNA-seq expression profile of 12 predicted *NRP* genes in the *O. sativa japonica* group *NRP* genes during interactions with three *Magnaporthe oryzae* strains (A248, B235 and C162). (b) Quantitative reverse transcription (qRT-PCR) verification of selected *NRP* gene expression levels in the *O. sativa japonica* group during interactions with *M. oryzae* strain Guy11.

2.4. *OsNRP1*, a *NRP* Protein in the *O. sativa japonica* Group, may Enhance Blast Fungus Resistance

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Figure 4. Disease reactions of ZH11 and transgenic *OsNRP1*-overexpression (*OsNRP1*OX) leaves incubated with *Magnaporthe oryzae* strain Guy11. (a) represents in vitro inoculation. (b) represents in vivo inoculation. Photographs showing the disease reactions of the indicated rice lines: wild-type (ZH11) and transgenic *OsNRP1*-overexpression (*OsNRP1*OX) lines (PXQ8-4, PXQ8-5, and PXQ8-17). (c) The disease symptoms on leaves of ZH11 and *OsNRP1*OX to *M. oryzae* Guy11. ImageJ was used to calculate the lesion area. Different letters above bars indicate significant differences (*p* < 0.01 using one-way analysis of variance [ANOVA]). (d) Gene expression quantification for the gene encoding the *OsNRP1* in ZH11, PXQ8-4, PXQ8-5, and PXQ8-17. *p* < 0.05, **** *p* < 0.0001 (two-sided Student’s *t*-test, *n* = 3).
In the context of WRKY proteins, suggesting that DCD/NRP genes were found, and may participate in responses to diverse abiotic stressors, such as dehydration. These findings suggested that DCD/NRP genes were involved not only in the regulation of plant defense against pathogens (Table 2). In addition, CGCGBOXAT, the binding site of bZIP proteins, was also present in the promoters of the 20 most abundant (Table 2), 8 were likely to be involved in abiotic or biotic stress responses that are also present in (a) and (b) and represent candidate OsNRP1-interacting proteins.

### 2.6. Investigation of Cis-Element within OsNRP1 Promoter and Conserved Motifs in NRP Genes

OsNRP5, OsNRP2, OsNRP8, OsNRP3, OsNRP4, OsNRP1, and OsNRP6 were found to be significantly up-regulated during interactions with rice blast fungus. To investigate the regulatory mechanisms associated with the up-regulated OsNRP genes, we analyzed predicted cis-elements within their promoter regions (defined as the 1500-bp region upstream of the transcription start site) (Figure 6a). In total, 175 cis-elements were detected (Table S4); of the 20 most abundant (Table 2), 8 were likely to be involved in abiotic or biotic stress responses. For example, WRKY71OS and WBOX3TERF3 are putative binding sites of WRKY proteins, suggesting that DCD/NRP genes may be required for WRKY-mediated regulation of plant defense against pathogens (Table 2). In addition, CCGBOXAT, the binding site of bZIP proteins, was also present in the promoters of NRP genes. This result was consistent with PPI network predictions, which suggested that bZIP proteins may be important components of the NRP-mediated cell-death signaling pathway. Moreover, the cis-elements OSE2ROOTNODULE and GT1GMSCAM4, which are involved in activation of pathogen- and salt-induced genes, were present; MYBCORE, ACGTATERD1, and ACGTATERD1 were found, and may participate in responses to diverse abiotic stressors, such as dehydration. These findings suggested that DCD/NRP genes were involved not only in the...
only in abiotic stress but also in plant pathogen defense, during which multiple TFs play
important roles.

**Figure 6.** (a) Cis-element analysis in promoters of NRP genes in the *Oryza sativa japonica* group. The
promoter of each gene was classified as the 1500-bp region upstream of the transcription start site.
(b), (c) Analysis of conserved motifs in NRP genes in the *O. sativa japonica* group.

We also used MEME to predict conserved motifs among NPRs in *Oryza* species. In
total, five conserved motifs that formed the NRP domain were found. For example, FGLP
and PLxE motifs, the typical motifs of NRP proteins, were displayed as Motif_1 and Motif_5,
which are present at the N- and C-terminus of NRPs, respectively, and are composed of
sequences that conserve the secondary structure [17]. Thus, conserved motif organization
seems to shape the NRP domain.
Table 2. Top20 abundant cis-elements in promoter region of OsNRPs.

| Motifs Accession in PLACE Database | Signal Sequence | Numbers | Functional Annotations |
|------------------------------------|-----------------|---------|------------------------|
| CACTFTPPCA1                        | YACT            | 130     | cis-regulatory elements for the promoter of C4 phosphoenolpyruvate carboxylase |
| ARR1AT                             | NGATT           | 107     | Response regulator (ARR1)-binding element |
| DOFCOREZM                          | AAAG            | 107     | cis-regulatory elements required for binding of Dof proteins that enhance transcription of cytosolic orthophosphate kinase (CyPPDK) |
| GT1CONSENSUS                       | GRWAAW          | 89      | cis-regulatory elements of GT-1 binding site for promoter of many light-regulated genes |
| CAATBOX1                           | CAAT            | 74      | cis-regulatory elements for promoter of pea legumin gene |
| WRKY71OS                           | TGAC            | 65      | cis-regulatory elements required for binding of Dof proteins that enhance transcription of cytosolic orthophosphate kinase (CyPPDK) |
| GTGANTG10                          | GTGA            | 61      | cis-regulatory elements required for binding of Dof proteins that enhance transcription of cytosolic orthophosphate kinase (CyPPDK) |
| POLLEN1LELAT52                     | AGAAA           | 61      | E-box of napA storage-protein gene and R response element (RRE) responsible for light responsiveness |
| EBOXBNNAPA                         | CANNTG          | 56      | cis-regulatory elements in the promoter of chlorophyll a/b binding protein |
| GATABOX                            | GATA            | 56      | MYC recognition site in the promoters of the dehydration-responsive gene and ICE1, which involve incold stress response |
| MYCCONSENSUSAT                     | CANNTG          | 56      | cis-regulatory elements in the promoter of chlorophyll a/b binding protein |
| ROOTMOTIFTAPOX1                    | ATATT           | 49      | Motif in the rolD promoter that is highly specific to regenerating plants |
| CCGGBOXAT                          | VCGCGB          | 45      | Motifs recognized by signal-responsive genes, like plant bZIP proteins |
| ACGTATERD1                         | ACCT            | 44      | cis-regulatory elements involved in early response to dehydration |
| CURECORECR                         | GTAC            | 40      | copper-response element involved in oxygen-response |
| WBOXNTERF3                         | TGACY           | 30      | Binding site of WRKY proteins that involve in activation of ERF3 gene by wounding |
| MYBCORE                            | CNGTTR          | 29      | MYB binding site involved in regulation of response to water-stress and flavonoid biosynthesis |
| NODCON2GM                          | CTCTT           | 28      | nodulin consensus sequences |
| OSE2ROOTNODULE                     | CTCTT           | 28      | cis-regulatory elements of promoters activated in infected cells of root nodules |
| GT1GMSCAM4                         | GAAAAA          | 27      | cis-regulatory elements involved in pathogen- and salt-induced gene expression |
3. Discussion

The NRPs are a family of genes-encoding proteins that contain the NRP domain; this domain is asparagine-rich, and proteins containing it are therefore called N-rich proteins (NRPs) [17]. Ref. [16] first discovered that soybean NRP genes were induced when inoculated by *Pseudomonas syringae* pv. *Glycinea*, which contain the avirulence gene avrA [16]. However, genomic identification of NRP gene family members has been limited to only a few plants, such as *Arabidopsis* and soybean [29,30].

Common cultivated rice (*O. sativa*) is one of the most essential crops for food security, but its production is threatened by rice blast. Rice breeding for pathogen resistance has historically depended on a narrow range of genetic diversity, but other *Oryza* species may provide a broader range of genetic resources for breeding pathogen resistance in rice [31]. Previous reports clarified that soybean NRP proteins participate in ER stress so that soybean resistance is enhanced against several pathogens, such as *Pseudomonas syringae* pv. *Glycinea* and *Phytophthora sojae* Race 1 [16,17]. Meanwhile, the NRP domain is only present in the plant kingdom and conserved region. Therefore, we inferred that NRP proteins may also play a role in rice resistance against blast fungal, which is still unclear. To this end, we performed a genome-wide analysis of the NRP gene repertoires in 12 *Oryza* species; these analyses were designed to shed light on the evolutionary histories of these genes and to infer putative biological functions in rice resistance against rice blast.

As Figure 7 shows, we here conducted a hidden Markov model (HMM) search of NRP genes in 12 different *Oryza* species, yielding a total of 136 NRP genes. The number of NRP genes in each species ranged from nine in *O. brachyantha* to thirteen in the *O. sativa indica* group, indicating a wide distribution of NRP genes in *Oryza* species. Fifteen NRP genes were identified in soybean, which was more than were found in the *Oryza* species. This may have resulted from the previously proposed retention of extended blocks of duplicated genes in the soybean genome [32]. There was no obvious evidence of NRP gene family expansion in *Oryza* species. To clarify the phylogenetic relationships between *Oryza* NRP genes, we constructed a phylogenetic tree from NRP genes in soybean and *Oryza* species. The NRP genes segregated into four subgroups, reminiscent of the results obtained in a previous analysis of soybean and *Arabidopsis* NRP genes [12]. No subgroup-specific gene family expansion was observed. Each subgroup contained members from all *Oryza* species included in the analysis. Taken together, these findings indicated that NRPs originated from the MRCA of soybean and *Oryza* species and were highly conserved during evolution.

Patterns of NRP protein domain organization were highly conserved within subgroups, but more diversified across subgroups. For example, the DCD/NRP domain of NRP proteins in subgroup III and I were preferentially found in the N- and C-, respectively. NRP proteins in subgroup II also contained Kelch motifs; these are present in the *Arabidopsis* Kelch repeat-containing F-box (KFB) protein SAGL1 and negatively regulate salicylic acid (SA) biosynthesis during immune responses [33]. Highly conserved syntenic NRP genes were also observed in NRPs in four different *Oryza* species, which was in contrast to results from the other *Oryza* species and soybean. Thus, the evolutionary trajectories of NRP genes appear to be influenced by species differentiation. In the *O. sativa japonica* group genome, only OsNRP3 and OsNRP8 showed a syntenic relationship, implying a putative paralogous relationship between OsNRP3 and OsNRP8. This result also supports the finding that NRP gene duplication events rarely occurred in *Oryza* species. In addition, the Ka/Ks value of OsNRP3/OsNRP8 suggested that these genes have undergone positive selection, which may have played a role in biological functional divergence throughout evolution.
Figure 7. Genome-wide analysis workflow of NRP genes in *Oryza* species revealed OsNRP1’s function in rice resistance against blast fungal and hypothesis of its putative mechanism.

OsNRP1 was found that specifically up-regulated at an early stage during *O. sativa japonica* group interacting with three *M. oryzae* strains proposed by [28]. Inoculation assays of overexpression transgenic line (OsNRP1OX) revealed that OsNRP1 slowed rice disease. A similar phenomenon was also verified in soybean. For example, GmNRP-A and GmNRP-B, the NRP proteins in soybean, was induced by the binding protein (BiP) under activation of salicylic acid (SA) signaling, and mediated programmed cell death [34].

We identified 10 candidate protein–protein interactors with OsNRP1. These interactors were found that enriched in GO terms such as ‘Response to stress’, ‘Endoplasmic reticulum unfolded protein response’, and ‘Response to organic substance’, which indicate that OsNRP1 may play a role in these biological processes. Interestingly, NRPs have been proposed to act as mediators of ER- and osmotic-stress-induced cell death in soybean [19]. ER capacity on protein procession is required for activation of the plant immune response, which relies on ER stress networks [11]. Notably, IRE1 is a critical ER stress sensor/transducer in
Arabidopsis [35]. The TF bZIP50 was predicted as a potential interaction partner of OsNRP1. Interestingly, OsbZIP50 is regulated by IRE1-mediated splicing and is required to regulate known marker genes involved in ER stress after pathogen recognition [36]. This result suggested that OsNRP1 may inhibit M. oryzae infection through involvement in regulation of ER stress. We also established a protein–protein interaction network for AT5G42050, an Arabidopsis ortholog of OsNRP1; the Arabidopsis protein was predicted to interact with bZIP60, the Arabidopsis ortholog of OsbZIP50 [37]. We also found that OsNRP1 may interact with three stress-associated proteins (SAP16, SAP5, and SAP3) that have been shown to participate in multiple abiotic stress responses [38]. OsSAP1, another stress-associated protein, enhances disease resistance against virulent bacterial pathogens [39]. In addition, TF binding-site prediction revealed the presence of the CGCGBOXAT motif in the OsNRP1 promoter. This suggested that OsNRP1 was downstream of bZIP TFs, indirectly supporting the potential OsNRP1–bZIP50 interaction.

In this study, we report genome-wide analysis of NRPs across 11 Oryza species. Our results demonstrated conserved evolution of the NRP gene family, although the domain organization exhibits divergence at the subgroup level. OsNRP1, an NRP gene in the O. sativa japonica group, was found to be specifically up-regulated during the early stage of the rice–M. oryzae interaction and to inhibit M. oryzae infection. Moreover, predicted protein–protein interaction networks and TF binding site predictions indicated that OsNRP1 may be downstream of bZIP TF, and may participate in the ER stress response after M. oryzae infection. Taken together, this study revealed OsNRP1-enhanced rice resistance against M. oryzae infection. Due to the conserved domain region and evolutionary history of NRP proteins in Oryza genus, this result gave an insight that OsNRP1 can provide partial but durable rice resistance against blast fungal, which increases our understanding of biological functions of NRP genes in Oryza genus.

4. Method and Materials

4.1. Sequence Retrieval and Identification of NRP Proteins

The whole protein sequences of 12 Oryza species and soybean were downloaded from Ensembl Genomes (release 97; http://www.ensembl.org (accessed on 5 June 2022)) and were integrated into an initial data set for homolog identification. The hidden Markov model (HMM) file of the NRP domain was obtained from PFAM database (PF10539) [40], which was used to screen the whole proteome with cutoff E-value of $1 \times 10^{-5}$. In parallel, known soybean NRP proteins, reported by [17], were defined as query sequences and applied BLASTP search against whole proteomes of species mentioned above (E-value cutoff = $1 \times 10^{-5}$). Then, total putative NRP candidates identified by two approaches were validated by the Conserved Domains Database (CDD) database and manually removed redundant sequences.

4.2. Phylogenetic and Evolution Analysis of NRP Family in Oryza Species

MUSCLE version 3.8.31 (Mill Valley, USA) was used to apply multiple alignment analysis of obtained NRP proteins [41]. The maximum-likelihood (ML) phylogeny trees of NRP proteins in species of this study were built with IQ-TREE version 1.6.12 (Vienna, Austria) with automatic selection of optimal model for protein substitution [42]. A bootstrap analysis with 1000 replicates was conducted to evaluate the reliability of the tree. The visualization and modification of phylogenetic trees were performed using the iTOL server [43].

4.3. Synteny Analysis

Genome annotations of species in this study were retrieved from Ensembl Genomes website. We used DIAMOND v0.8.25 (Tübingen, Germany) to conduct all-vs-all comparisons of corresponding protein sequences (-max-target-seqs 5 -E-value $1 \times 10^{-5}$) [44]. Genome annotations and DIAMOND output file were input into MCScanX for synteny
detection with default parameters [45]. The visualization of syntenic results was performed by TBtools toolkit (Guangdong, China) [46].

4.4. Construction of OsNRP1OX Transgenic Lines

We amplified coding sequence of OsNRP1 (LOC_Os01g36950) using 2X Phata master mix (Vazyme Biotech Co., Ltd., Nanjing, China). The amplified coding sequences were cloned into the rice transformation PXQ vector. The integrated construct PXQ::OsNRP1 was introduced into Agrobacterium strain EHA105 and then transformed into wild type (Zhonghua11, ZH11). Hygromycin-containing media were used to screen transgenic plants (40 mg/L).

4.5. Inoculation Assays

Two-week-old seedlings and leaf strips of wild-type (ZH11) and overexpression transgenic lines of OsNRP1, OsNRP1OX (PXQ8-4, PXQ8-5 and PXQ8-17) were inoculated by Magnaporthe oryzae strains Guy11. 5 mL conidia suspension were sprayed onto leaves of each seedling (5 \( \times \) \( 10^5 \) spores/mL), and 5 \( \mu \)L of Guy11 spore suspension was added to the wounds of each leaf strip. The disease symptoms were assessed seven days after inoculation. Leaves of ZH11 at 0, 8, 24, 48, 72 and 96 hpi were collected for real-time RT-PCR validation.

4.6. Real-Time RT-PCR

Qiagen RNAeasy Mini kit (Qiagen Inc., Valencia, CA, USA) was used to isolate total RNA from collected inoculation ZH11 leaves. Isolated RNA samples were then converted into cDNA by the Superscript IV Reverse transcriptase cDNA synthesis kit (TB Green® Premix Ex Taq™ II, Takara Bio Inc, Kusatsu, Japan). We diluted a 20-\( \mu \)L aliquot of cDNA to 100 \( \mu \)L with water, which was used to established real-time RT-PCR reactions. cDNA sample of 0 hpi was used as control samples, and the actin gene of rice (LOC_Os03g50885) was used as an internal reference gene with a stable expression pattern as 10 housekeeping genes proposed by [47]. Bio-Rad Real-Time PCR cycler was applied for the relative gene expression level estimation using SYBG as the fluorescent dye. All primers used to real-time RT-PCR reactions were designed in Primer3 website (https://bioinfo.ut.ee/primer3-0.4.0/ (accessed on 14 June 2022)).

4.7. PPI Networks Prediction and Cis-Element Analysis

Protein sequence of OsNRP1 was submitted to the online server STRING (version 11.5; http://string-db.org (accessed on 23 June 2022)), with specified organism as “Oryza. sativa japonica group”. Online BLAST search was used to interacting partners by detecting hits with the highest scores (Bitscore), of which Gene Ontology (GO) annotations were also provided and displayed by TBtools [46]. The cis-element of 8 highly-expressed OsNRPs in infection stage were predicted through the PLACE website (http://www.dna.affrc.go.jp/PLACE/ (accessed on 29 June 2022)), and their annotations as well. The locations of cis-elements were displayed by TBtools toolkit (Guangdong, China) [46].

4.8. Analyzing Conserved Motifs of NRP Proteins in Oryza Species

We performed multiple alignment of NRP protein sequences in Oryza species mentioned above by MUSCLE version 3.8.31 (Mill Valley, USA) with default parameters. Conserved motifs in NRP proteins were identified using MEME 5.0.5 according to the following parameters: -protein, -nmotifs 10, -minw 8, and -maxw 80 [48].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231911967/s1.

Author Contributions: Y.L. (Yongfeng Liu), D.L. and Z.Q. planned and designed the research. D.L. and Z.Q. performed the experiments. D.L. drafted this manuscript. Y.D., J.Y., M.Y., R.Z., H.C., X.P., T.S. and J.Q. participated in reviewing this manuscript. Y.L. (Yongfeng Liu), Y.L. (Youzhou Liu) and
Z.Q. supervised the manuscript, the research as a whole, and provided guidance. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by funding to Y.L. from the National Natural Science Foundation of China (Grant/Award Number: 31861143011). This work also received funding from Jiangsu Agriculture Science and Technology Innovation Fund (Grant/Award Number: CX19(1008)), The Revitalization Foundation of Seed Industry of Jiangsu (Grant/Award Number: JBCS(2021)005) and Jiangsu Modern Agricultural Technology System of Rice and Wheat Industry JAST(2021) 271.

**Institutional Review Board Statement:** Proteomics sequences of species mentioned in this study were retrieved from Ensembl plant database release 54 (https://plants.ensembl.org/, accessed on 5 June 2022).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. Muthayya, S.; Sugimoto, J.D.; Montgomery, S.; Maberly, G.F. An overview of global rice production, supply, trade, and consumption. *Ann. N. Y. Acad. Sci.* 2014, 1324, 7–14. [CrossRef]
2. Dean, R.A.; Talbot, N.J.; Ebbole, D.J.; Farman, M.L.; Mitchell, T.K.; Orbach, M.J.; Thon, M.; Kulkarni, R.; Xu, J.R.; Pan, H.; et al. The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* 2005, 434, 980–986. [CrossRef]
3. Jones, J.D.; Dangl, J.L. The plant immune system. *Nature* 2006, 444, 323–329. [CrossRef]
4. Vance, R.E.; Isberg, R.R.; Portnoy, D.A. Patterns of pathogenesis: Discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell Host Microbe* 2009, 6, 10–21. [CrossRef]
5. Liu, W.; Liu, J.; Triplett, L.; Leach, J.E.; Wang, G.-L. Novel insights into rice innate immunity against bacterial and fungal pathogens. *Annu. Rev. Phytopathol.* 2014, 52, 213–241. [CrossRef]
6. Devi, S.R.; Singh, K.; Umakanth, B.; Vishalakshi, B.; Renuka, P.; Sudhakar, K.V.; Prasad, M.; Viraktamath, B.; Babu, V.R.; Madhav, M. Development and identification of novel rice blast resistant sources and their characterization using molecular markers. *Rice Sci.* 2015, 22, 300–308. [CrossRef]
7. Ballini, E.; Morel, J.-B.; Droc, G.; Price, A.; Courtois, B.; Notteghem, J.-L.; Tharreau, D. A genome-wide meta-analysis of rice blast resistance genes and quantitative trait loci provides new insights into partial and complete resistance. *Mol. Plant-Microbe Interact.* 2008, 21, 859–866. [CrossRef] [PubMed]
8. Li, W.; Chern, M.; Yin, J.; Wang, J.; Chen, X. Recent advances in broad-spectrum resistance to the rice blast disease. *Curr. Opin. Plant Biol.* 2019, 50, 114–120. [CrossRef]
9. Yin, J.; Zhou, L.; Zhu, X.; Cao, Y.; He, M.; Chen, X. Fighting the enemy: How rice survives the blast pathogen’s attack. *Crop J.* 2021, 9, 543–552. [CrossRef]
10. Kerner, C.J.; Du, X.; Vollmer, M.E.; Pajerowska-Mukhtar, K.M. Endoplasmic reticulum stress signaling in plant immunity—At the crossroad of life and death. *Int. J. Mol. Sci.* 2015, 16, 26582–26598. [CrossRef]
11. Reis, P.A.; Carpinetti, P.A.; Freitas, P.P.; Santos, E.G.; Camargos, L.F.; Oliveira, I.H.; Silva, J.C.F.; Carvalho, H.H.; Dal-Bianco, M.; Soares-Ramos, J.R.; et al. Functional and regulatory conservation of the soybean ER stress-induced DCD/NRP-mediated cell death signaling in plants. *BMC Plant Biol.* 2016, 16, 156. [CrossRef]
12. Feng, F.; Zhou, J.-M. Plant–bacterial pathogen interactions mediated by type III effectors. *Curr. Opin. Plant Biol.* 2012, 15, 469–476. [CrossRef]
13. Mukhtar, M.S.; Carvunis, A.-R.; Dreze, M.; Epple, P.; Steinbrenner, J.; Moore, J.; Tasan, M.; Galli, M.; Hao, T.; Nishimura, M.T.; et al. Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 2011, 333, 596–601. [CrossRef]
14. Williams, B.; Verchot, J.; Dickman, M.B. When supply does not meet demand-ER stress and plant programmed cell death. *Front. Plant Sci.* 2014, 5, 211. [CrossRef]
15. Tenhaken, R.; Doerks, T.; Bork, P. DCD—A novel plant specific domain in proteins involved in development and programmed cell death. *BMC Bioinform.* 2005, 6, 169. [CrossRef]
16. Ludwig, A.A.; Tenhaken, R. A new cell wall located N-rich protein is strongly induced during the hypersensitive response in *Glycine max*. *Eur. J. Plant Pathol.* 2001, 107, 323–336. [CrossRef]
17. Choi, J.-A.; Song, C.-H. Insights into the role of endoplasmic reticulum stress in infectious diseases. *Front. Immunol.* 2020, 10, 3147. [CrossRef]
19. Costa, M.D.; Reis, P.A.; Valente, M.A.S.; Insiger, A.S.; Carvalho, C.M.; Loureiro, M.E.; Aragão, F.J.L.; Boston, R.S.; Fietto, L.G.; Fontes, E.P.B. A new branch of endoplasmic reticulum stress signaling and the osmotic signal converge on plant-specific asparagine-rich proteins to promote cell death. J. Biol. Chem. 2008, 283, 20209–20219. [CrossRef]

20. Alves, M.S.; Reis, P.A.; Dadalto, S.P.; Faria, J.A.; Fontes, E.P.; Fietto, L.G. A novel transcription factor, ERD15 (Early Responsive to Dehydration 15), connects endoplasmic reticulum stress with an osmotic stress-induced cell death signal. J. Biol. Chem. 2011, 286, 20200–202030. [CrossRef] [PubMed]

21. Mendes, G.C.; Reis, P.A.; Calil, I.P.; Carvalho, H.H.; Aragão, F.J.; Fontes, E.P. GmNAC30 and GmNAC81 integrate the endoplasmic reticulum stress-and osmotic stress-induced cell death responses through a vacuolar processing enzyme. Proc. Natl. Acad. Sci. USA 2013, 110, 16627–16632. [CrossRef]

22. Tong, T.; Fang, Y.-X.; Zhang, Z.; Zheng, J.; Zhang, X.; Li, J.; Niu, C.; Xue, D.; Zhang, X. Genome-wide identification and expression pattern analysis of the KCS gene family in barley. Plant Growth Regul. 2021, 93, 89–103. [CrossRef]

23. Kesawat, M.S.; Kherawat, B.S.; Singh, A.; Dey, P.; Routray, S.; Mohapatra, C.; Saha, D.; Ram, C.; Siddique, K.H.M.; Kumar, A.; et al. Genome-Wide Analysis and Characterization of the Proline-Rich Extensin-like Receptor Kinases (PERKs) Gene Family Reveals Their Role in Different Developmental Stages and Stress Conditions in Wheat (Triticum aestivum L.). Plants 2022, 11, 496. [CrossRef]

24. Jiang, M.; Chen, H.; Liu, J.; Du, Q.; Lu, S.; Liu, C. Genome-wide identification and functional characterization of natural antisense transcripts in Salvia miltiorrhiza. Sci. Rep. 2021, 11, 4769. [CrossRef]

25. Alves, M.S.; Reis, P.A.; Dadalto, S.P.; Faria, J.A.; Fontes, E.P.; Fietto, L.G. A new branch of endoplasmic reticulum stress signaling and the osmotic signal converge on plant-specific asparagine-rich proteins to promote cell death. J. Biol. Chem. 2008, 283, 20209–20219. [CrossRef]

26. Hurst, L.D. The Ka/Ks ratio: Diagnosing the form of sequence evolution. Trends Genet. 2002, 18, 486–487. [CrossRef]

27. Koonin, E.V.; Rogozin, I.B. Getting positive about selection. Genome Biol. 2003, 4, 331. [CrossRef] [PubMed]

28. Yu, K.; Yang, W.; Zhao, B.; Wang, L.; Zhang, P.; Ouyang, Y.; Chang, Y.; Chen, G.; Zhang, J.; Wang, S.; et al. The Kelch-F-box homologs, endoplasmic reticulum-located transmembrane protein kinases. Plant Physiol. 2001, 127, 949–962. [CrossRef]

29. Hoepflinger, M.C.; Pieslinger, A.M.; Tenhaken, R. Investigations on a, F.J.L.; Boston, R.S.; Fietto, L.G.; Fontes, E.P.B.; Reis, P.A.B. Development and cell death domain-containing asparagine-rich protein (DCD/NRP). An essential protein in plant development and stress responses. Theor. Exp. Plant Physiol. 2019, 31, 59–70. [CrossRef]

30. Schmutz, J.; Cannon, S.B.; Schlueter, J.; Ma, J.; Mitros, T.; Nelson, W.; Hyten, D.L.; Song, Q.; Thelen, J.J.; Cheng, J.; et al. Sequence evolution of the palaecopolyploid soybean. Nature 2010, 463, 178–183. [CrossRef] [PubMed]

31. Yu, K.; Yang, W.; Zhao, B.; Wang, L.; Zhang, P.; Ouyang, Y.; Chang, Y.; Chen, G.; Zhang, J.; Wang, S.; et al. The Kelch-F-box protein SMALL AND GLOOSY LEAVES 1 (SAGL1) negatively influences salicylic acid biosynthesis in Arabidopsis thaliana by promoting the turn-over of transcription factor SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1). New Phytol. 2022, 225, 885–897. [CrossRef]

32. Carvalho, H.H.; Silva, P.A.; Mendes, G.C.; Brustolini, O.J.; Pimenta, M.R.; Gouveia, B.C.; Valente, M.A.S.; Ramos, H.J.; Soares-Ramos, J.R.; Fontes, E.P.B. The endoplasmic reticulum binding protein BiP displays dual function in modulating cell death events. Plant Physiol. 2014, 164, 654–670. [CrossRef]

33. Koizumi, N.; Martinez, I.M.; Kimata, Y.; Kohno, K.; Sano, H.; Chrispeels, M.J. Molecular characterization of two Arabidopsis Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. Plant Physiol. 2001, 127, 949–962. [CrossRef]

34. Hayashi, S.; Wakasa, Y.; Takahashi, H.; Kawakatsu, T.; Takaika, F. Signal transduction by IRE1-mediated splicing of bZIP50 and other stress sensors in the endoplasmic reticulum stress response of rice. Plant J. 2012, 69, 946–956. [CrossRef]

35. Iwata, Y.; Fedoroff, N.V.; Koizumi, N. Arabidopsis bZIP60 is a proteolysis-activated transcription factor involved in the endoplasmic reticulum stress response. Plant Cell 2008, 20, 3107–3122. [CrossRef]

36. Wang, X.; Zou, B.; Shao, Q.; Cui, Y.; Lu, S.; Zhang, Y.; Huang, Q.; Huang, J.; Hua, J. Natural variation reveals that OsSAP16 controls low-temperature germination in rice. J. Exp. Bot. 2018, 69, 413–421. [CrossRef]

37. Tyagi, H.; Jha, S.; Sharma, M.; Giri, J.; Tyagi, A.K. Rice SAPs are responsive to multiple biotic stresses and overexpression of OsSAP1, an A20/AN1 zinc-finger protein, enhances the basal resistance against pathogen infection in tobacco. Plant Sci. 2014, 225, 68–76. [CrossRef]

38. Finn, R.D.; Coggil, P.; Eberhardt, R.Y.; Eddy, S.R.; Mistry, J.; Mitchell, A.L.; Potter, S.C.; Punta, M.; Qureshi, M.; Sangrador-Vegas, A.; et al. The Pfam protein families database: Towards a more sustainable future. Nucleic Acids Res. 2016, 44, D279–D285. [CrossRef]

39. Edgar, R.C. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004, 32, 1792–1797. [PubMed]

40. Nguyen, L.-T.; Schmidt, H.A.; Von Haeseler, A.; Minh, B.Q. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 2015, 32, 268–274. [CrossRef] [PubMed]

41. Letunic, I.; Bork, P. Interactive Tree of Life (iTOL): An online tool for phylogenetic tree display and annotation. Bioinformatics 2007, 23, 127–128. [CrossRef]
44. Buchfink, B.; Xie, C.; Huson, D.H. Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* **2015**, *12*, 59–60. [CrossRef]

45. Wang, Y.; Tang, H.; DeBarry, J.D.; Tan, X.; Li, J.; Wang, X.; Lee, T.-H.; Jin, H.; Marler, B.; Guo, H.; et al. MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, e49. [CrossRef]

46. Chen, C.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.; Xia, R. TBtools: An integrative toolkit developed for interactive analyses of big biological data. *Mol. Plant* **2020**, *13*, 1194–1202. [CrossRef]

47. Yao, S.; Zhang, Y.; Liu, Y.; Zhao, C.; Zhou, L.; Chen, T.; Zhao, Q.Y.; Pillay, B.; Wang, C. Evaluation of suitable reference genes for normalization of quantitative real-time PCR analysis in rice plants under *Xanthomonas oryzae* pv. *Oryzae*-infection and melatonin supplementation. *Food Prod. Process. Nutr.* **2020**, *2*, 22. [CrossRef]

48. Bailey, T.L.; Johnson, J.; Grant, C.E.; Noble, W.S. The MEME suite. *Nucleic Acids Res.* **2015**, *43*, W39–W49. [CrossRef]