Plant Genetic Resources: Characterization and Utilization

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Polymorphism and resistance spectrum to *Magnaporthe oryzae* analysis of *Pi-d2* haplotypes in rice (*Oryza sativa* L.) resource from Yunnan province of China

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Research Article

Key message: Multiple *Pi-d2* haplotypes were found in rice (*O. sativa*) resources of Yunnan. B-lectin domain and PAN domain of PI-D2 may be co-involved in the identification of effector of *M. oryzae.*

Cite this article: Yang Y, Li J, Ma J, Tao J, Li C, Sun Y, Xu M (2024). Polymorphism and resistance spectrum to *Magnaporthe oryzae* analysis of *Pi-d2* haplotypes in rice (*Oryza sativa* L.) resource from Yunnan province of China. *Plant Genetic Resources: Characterization and Utilization* 1–9. https://doi.org/10.1017/S1479262124000248

Received: 20 August 2023
Revised: 24 April 2024
Accepted: 24 April 2024

Keywords:
blast disease; broad-spectrum resistance; *Pi-d2*

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Abstract

Pi-d2, which encodes a potential serine-threonine receptor-like kinase (RLK) membrane-spanning protein consisting of 825 amino acids, confers resistance to *Magnaporthe oryzae* strain *ZB15* via an unidentified recognition mechanism. In this study, the *Pid2* alleles of 303 rice (*O. sativa*) varieties from China's Yunnan region were amplified and sequenced in order to produce 24 haplotypes and 16 translation variants. Six of twenty-four alleles possessing the resistant site at the 441st amino acid were chosen for evaluating blast resistance by transforming into the blast-vulnerable rice variety Nipponbare. After being infected with 11 strains of *M. oryzae*, all transgenic lines exhibited resistance to *ZB-15*, whereas resistance to other strains varied. Notably, *Pi-d2_H23* and *Pi-d2_H24* exhibited resistance to all *M. oryzae* strains tested, indicating that these two alleles may have a broader resistance spectrum to *M. oryzae*. Alignment of these alleles' amino acid sequences revealed that the differences in blast resistance spectra were primarily related to the amino acids present in the PAN domain at position 363 (valine/alanine). These findings suggested that the two extracellular signal recognition domains of *PI-D2*, B-lectin and PAN, may play a role in the identification of *M. oryzae* effectors. The present results provide insight into the mechanism of interaction between RLKs and *M. oryzae*.

Rice blast, which is caused by the filamentous ascomycetous (*Magnaporthe oryzae, M. oryzae*), is one of the most destructive diseases of rice on a global scale (Dean *et al.*, 2012). Rice plants utilise a wide variety of disease resistance genes (*R* genes) to detect the presence of pathogens and initiate subsequent defence responses. Resistance mediated by the major *R* gene is effective at identifying fungal strains that carry the matching avirulence (AVR) gene (Woolhouse *et al.*, 2002). With the exception of chromosome 3, at least 84 significant blast resistance genes (*R* gene) have been found and genetically mapped on 11 rice chromosomes. At least 26 of the identified blast R genes have been cloned and functionally investigated: *Pb1*, *Pia*, *Pib*, *Pi-d2*, *Pid3*, *Pik*, *Pikh/Pi54*, *Pikm*, *Pikp*, *Pish*, *Pit*, *Pita*, *Pizt*, *Pi1*, *Pi2*, *Pi21*, *Pi25*, *Pi36*, *Pi37*, *Pi56*, *Pi63*, *PiCO39* (http://www.ricedata.cn), *Pi64* (Ma *et al.*, 2015) and *Pigm* (Deng *et al.*, 2006). The majority of cloned *R* genes are members of the nucleotide-binding site leucine-rich repeat (NBS-LRR) family, with the exception of *Pi-d2* and *pi21*, which encode a receptor-like kinase and a proline-rich protein, respectively.

Long regarded as the most efficient and successful means of disease control, a deeper comprehension of the mechanisms behind the interaction between pathogens and host plants and the selection of stable, disease-resistant novel types are becoming increasingly important (Dodds and Rathjen, 2010; Peng *et al.*, 2018). However, *AVR* genes in *M. oryzae* are known to be highly variable and diverse (Selisana *et al.*, 2017), which frequently threatens the efficacy of resistant cultivars with a single *R* gene a few years after their release. It is widely documented that mutations in the *AVR* gene *AVR-Pita1* in historical and contemporary field isolates overcome a key *R* gene-mediated resistance (Dai *et al.*, 2010). Pyramiding more blast resistance genes in a rice cultivar (Arunakumari *et al.*, 2016; Jiang *et al.*, 2019; Wu *et al.*, 2019) or mixture planting different varieties in a region (Zhu *et al.*, 2000) may control rice blast effectively, but both methods have production limitations because pyramiding is a time-consuming and difficult task, and mixture planting is difficult to plant and harvest.

Some *R* genes with a relatively broad spectrum of blast resistance, such as *Pi1* (Hua *et al*., 2012), *Pi37, Pit, Pikm, Pi5,* and *Pb1*, have been extensively utilised in rice disease resistance breeding. Some blast *R* genes have a relatively narrow spectrum of resistance; however, their alleles and orthologs from various rice resources may exhibit varying blast resistance spectra. This is due to the coevolution of rice and the blast pathogen in various rice-growing locations, which has resulted in an abundance of allelic variety in rice resources. For example, *Pi9/Pi2/Piz-t* were cloned from the same chromosome location in different varieties and showed different resistance spectra (Zhou *et al*., 2006). The same condition was also observed in the *Pik* locus, which contains five rice blast *R* genes (*Pik, Pik-m, Pik-p, Pi1k-h* and *Pik-s*) (Wang *et al*., 2009). Therefore, studying natural variation in rice resistance genes from various rice resources can not only predict the stability of resistance to rice blast fungus, but can also be a crucial technique for rice blast resistance breeding. Four types of diversification of *R* genes can be defined on the basis of the polymorphism level (pi value) : conserved (type I; $pi < 0.5\%$), intermediate-diversified (type II; $0.5\% < pi < 5\%$), highly diversified (type III; $pi > 5\%$), and present/absent genes (type IV; P/A).

Receptor-like kinases (RLK) are members of a vast gene family and participate in numerous cell-cell communication activities (Barre *et al*., 2002; Bellande *et al*., 2017). In the preceding decades, a number of studies suggested a variety of possible functions for these genes. First, RLKs participate in plant growth, such as the CLV1 receptor in meristem signalling (Clark *et al*., 1997) and the SRK receptor in pollen development signalling (Clark *et al*., 1997). (Nasrallah, 2000). In addition, RLKs participate in biotic and abiotic stress signal transduction, such as XA21 (Song *et al*., 1995) in rice blight resistance. The TM domains of RLKs play a significant role in the correct signal transduction from ligand reception by the extracellular region to the production of responses by the intracellular region. One amino acid variation in the TM region of *Pi-d2* determines sensitivity or resistance to the AvrPi-containing rice blast fungus (Chen *et al*., 2006).

Compared to Arabidopsis, RLKs exhibits expansionary patterns in rice, which are believed to be associated with recent lineage-specific expansions of resistance/defence-related genes (Shiu *et al*., 2004). The majority of plant RLKs involved in disease resistance, including XA21 (Song *et al*., 1995), XA26 (Sun *et al*., 2004), and FSL2, belong to this subclass (Gomez-Gomez *et al*., 2001). *Pi-d2*, a single copy gene identified in the rice genome encoding a potential serine-threonine RLKs membrane-spanning protein with 825 amino acids, gives resistance to *M. oryzae* strain ZB15 in the indica cultivar Digu (Chen *et al*., 2004, 2010). At position 441 of the vulnerable *pi-d2* allele, the amino acid methionine (M) was identified, while the resistant allele included isoleucine (I) (Chen *et al*., 2006). *Pi-d2* represents a new class of blast resistance genes since its structure differs from that of existing *R* genes, indicating that it may employ a novel pathogen recognition mechanism. The intracellular serine/threonine kinase domain of *Pi-d2* has been discovered to interact directly with the E3 ligase OsPUB15 to regulate plant cell death and blast disease resistance (Wang *et al*., 2015). Although some allelic variations have been discovered at the *Pi-d2* locus in diverse rice varieties (Chen *et al*., 2006; Li *et al*., 2015). However, the corresponding evolutionary analysis could not be performed due to the small number of the found *Pi-d2* haplotypes. Moreover, the disease resistance spectra among the different haplotypes were not determined yet.

China's Yunnan province is one of the world's major hotspots of rice genetic variation (Zeng *et al*., 2007). Rice crop diversity has been influenced by the agricultural practises, customs, and traditions of many ethnic groups inhabiting regions of varying altitudes and climatic circumstances. The objective of this study was to investigate the DNA sequence of *Pi-d2* haplotypes from Yunnan landrace varieties and to evaluate their blast resistance via gene transformation and blast inoculation. On the basis of the respective blast resistance spectra of the cloned orthologs, comparative analyses between the amino acid polymorphism sites of the discovered RLK proteins and their respective blast strain-specific resistances were done.

Materials and methods

Plant materials and rice blast strains

The *Pi-d2* coding sequences for Digu (GenBank accession number FJ915121.1) and Nipponbare (NC008399) were retrieved from http://blast.ncbi.nlm.nih.gov. Six (6) wild rice lines' *Pi-d2* haplotypes were retrieved from http://blast.ncbi.nlm.nih.gov. (online Supplementary Table S1), and four (4) wild rice lines' *Pi-d2* haplotypes were amplified from plant seedlings cultivated in the lab's greenhouse. From the main germplasm bank of rice seed resources in Yunnan province, the Institute of Biotechnology and Genetic Resource of Yunnan Academy of Agricultural Sciences chose 299 rice (*O. sativa*) landraces, including 108 indica (*Oryza sativa* L. subsp. *indica* Kato) types and 191 japonica (*Oryza sativa* L. subsp. *japonica* Kato) variants (online Supplementary Table S2). The Agricultural Environment and Resources Research Institute of the Yunnan Academy of Agricultural Sciences supplied the 19 *M. oryzae* strains used in this investigation, which were dominant or high infectivity strains collected from Yunnan province (Table 1). Professor Cailin Lei of the Chinese Academy of Agricultural Sciences supplied ZB15 as the first isolate for the determination of *Pi-d2*.

Gene cloning

Fresh leaves of farmed and wild rice species were used to extract DNA. Forward primer Pid2F: 5′-ATAggatccATGGAAGCTCATG GCAATCG-3′ and reverse primer Pid2R: 5′-ATAcccgggTCATC TGGGACCAGAGAGCC-3′ were constructed and used to amplify the whole coding sequence of *Pi-d2* based on the published data sequence (Li *et al*., 2015). A *BamH*I and a *Sma*I recognition sites with three protective bases (ATA) were added to their respective 5′ ends. Initial DNA denaturation was conducted at 95°C for 5 min, followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 60°C for 40 s, extension at 72°C for 3 min, and a final extension at 72°C for 5 min. The PCR products were respectively purified and sequenced.

Rice transformation

Because the *Pi-d2* lacks an intron, the cloned gene fragments above were inserted into the binary vector pUN1301 via *the BamH*I and *SmalI* cloning sites. The completed construct was injected into *Agrobacterium tumefaciens* EHA105 after sequence verification.

Hiei *et al*. (Hiei *et al*., 1994) performed *Agrobacterium*-mediated transformation on calli obtained from mature embryos of the sensitive rice variety Nipponbare. Positive transgenic plants were identified by amplifying the marker gene hygromycin

https://doi.org/10.1017/S1479262124000248 Published online by Cambridge University Press

Table 1. Rice blast resistance spectra of *Pi-d2* haplotypes

Test rice lines	*M. oryzae* strains										
	ZB15	08-43-1a	W1-117	W1-79	W1-10	A23	16-2-1e	08-55-1a	08-35-1a	W1-125	15-30-3a
LTH	S	S	S	S	S	S	S	S	S	S	S
*Pi-d2*_UN	S	S	S	S	S	S	MS	S	S	S	MS
*Pi-d2*_NP	S	S	S	S	S	S	MS	S	MS	S	MS
*Pi-d2*_H1	R	R	R	R	MS	MS	R	R	R	R	MS
*Pi-d2*_H2	R	R	R	R	M	S	R	R	MS	MS	MS
*Pi-d2*_H12	R	R	R	R	R	S	R	R	MS	MS	S
*Pi-d2*_H20	R	R	R	R	MS	S	MS	R	MS	S	S
*Pi-d2*_H23	R	R	R	R	R	R	R	R	R	R	MR
*Pi-d2*_H24	R	R	R	R	R	R	R	R	R	R	R

5 scales were rated according to the visual number of lesions at the second youngest leaf, levels 0 and 1 were considered to be resistant (R); levels 2 and 3 represented medium resistance (MR) and medium susceptibility (MS), respectively; and levels 4 and 5 were considered to be susceptible (S).

(HYG) in vector with the forward primer HYG-F: 5′-TGCGC CCAAGCTGCATCAT-3′ and reverse primer HYG-R: 5′-TG AACTCACCGCGACGTCTGT-3′. The following PCR amplification profile was used: initial DNA denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 40 s, annealing at 60°C for 30 s, extension at 72°C for 20 s, and final extension at 72°C for 5 min.

Expression analysis of Pi-d2 *haplotypes*

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to separate RNA from leaf sheath tissue, and cDNA was generated from poly(A) + RNA using a cDNA synthesis kit (Promega, Shanghai). The specific primer pair *Pi-d2* was used in real time quantitative PCR (Pi-d2 D-F: 5′-CACAGGCTTCTTGCCT ACGA-3′ and reverse primer Pi-d2 D-R: 5′-TATGCCAATCC CTTTGCCGT-3′) for 45cycles of amplification. Transcription of the Actin gene was utilised to normalise the cDNA levels using the primer pair 5′-CTGCGGGTATCCATGAGACT-3′ and 5′-GCAATGCCAGGGAACATAGT-3′.

Fungal inoculation

As detailed earlier by Jia *et al.* (Jia *et al.*, 2003), the reactions to rice blast were measured using a modified standard pathogenicity test. First, rice seedlings at the 3rd-4th leaf stage in a plastic sealed enclosure were spray-inoculated with a rice blast spore suspension at $1–5 \times 10^5$ spores/ml; second, the plastic sealed enclosure were sealed to maintain high humidity for 24 h; and finally, the plants were removed from the plastic sealed enclosure and kept in a greenhouse for an additional 6 days to allow for the development of notable disease symptoms. Based on the amount of visible lesions on the second youngest leaf, the reactions were classified as 0–1 for resistant, 2 for moderately resistant, 3 for moderately susceptible, and 4–5 for susceptible.

Computational analysis of DNA and protein sequences

Using DNAMAN (http://www.lynnon.com/) and Clustal X version 2.0, the sequences were aligned. Using version 7.0.1 of BioEdit, sequences were manually modified. The SMART tool (http://smart.embl-heidelberg.de) was utilised to search for protein motifs. The phylogenetic tree of *Pi-d2* orthologs was constructed using MEGA5.0. DnaSP version 5.10 was used to conduct sliding-window analyses, polymorphism tests, and neutral-tests on different sections of *Pi-d2* orthologs.

Results

Sequence characteristics of Pi-d2 *haplotypes*

The analysis of the *Pi-d2* coding sequences of 309 rice varieties, comprising 299 landrace variants from Yunnan region and 10 wild rice varieties, revealed a total of 41 nucleotide changes. There were 28 informative parsimony sites and 13 variable singleton sites detected. In addition to the 25 synonymous mutation locations, 16 substitutional sites were observed.

Alignment of 299 Yunnan-assembled DNA sequences indicated 24 *Pi-d2* haplotypes (H) (online Supplementary Table S3). H1 was compatible with the sequence of *Pi-d2_digu*, which was only observed in one indica variety, Jiugu (I1). H3, which includes 56 japonica types and 69 indica kinds, accounted for 41.67% of the total. H9, containing 88 japonica and 12 indica types, accounted for 33.33% of the total frequency (the second highest frequency, 31.7%). The third category, H2, contains 20 indica and 20 japonica types and accounts for 13.33% of the total. Each of the remaining haplotypes contains no more than ten variants. 14 of all haplotypes were only detected in Japonica varieties, while only 3 were found in indica varieties. However, the Yuanjiang wild rice variety had a unique H24 haplotype.

Nucleotide polymorphism of Pi-d2 *haplotypes*

Pi-d2 encodes a receptor-like kinase protein having an external domain anticipated to be a bulb-type mannose specific binding lectin (B-lectin) and an internal serine-threonine kinase domain. Figure 1 shows the nucleotide diversity distribution in the whole encoding sequences, as shown by a sliding window analysis of 309 *Pi-d2* haplotypes, revealing the following characteristic: Firstly, 5 different variation peaks were found among all varieties, one in the unknown functional region, one in the PAN domain, one in the TM-spanning region, and two in the Serine-threonine kinase

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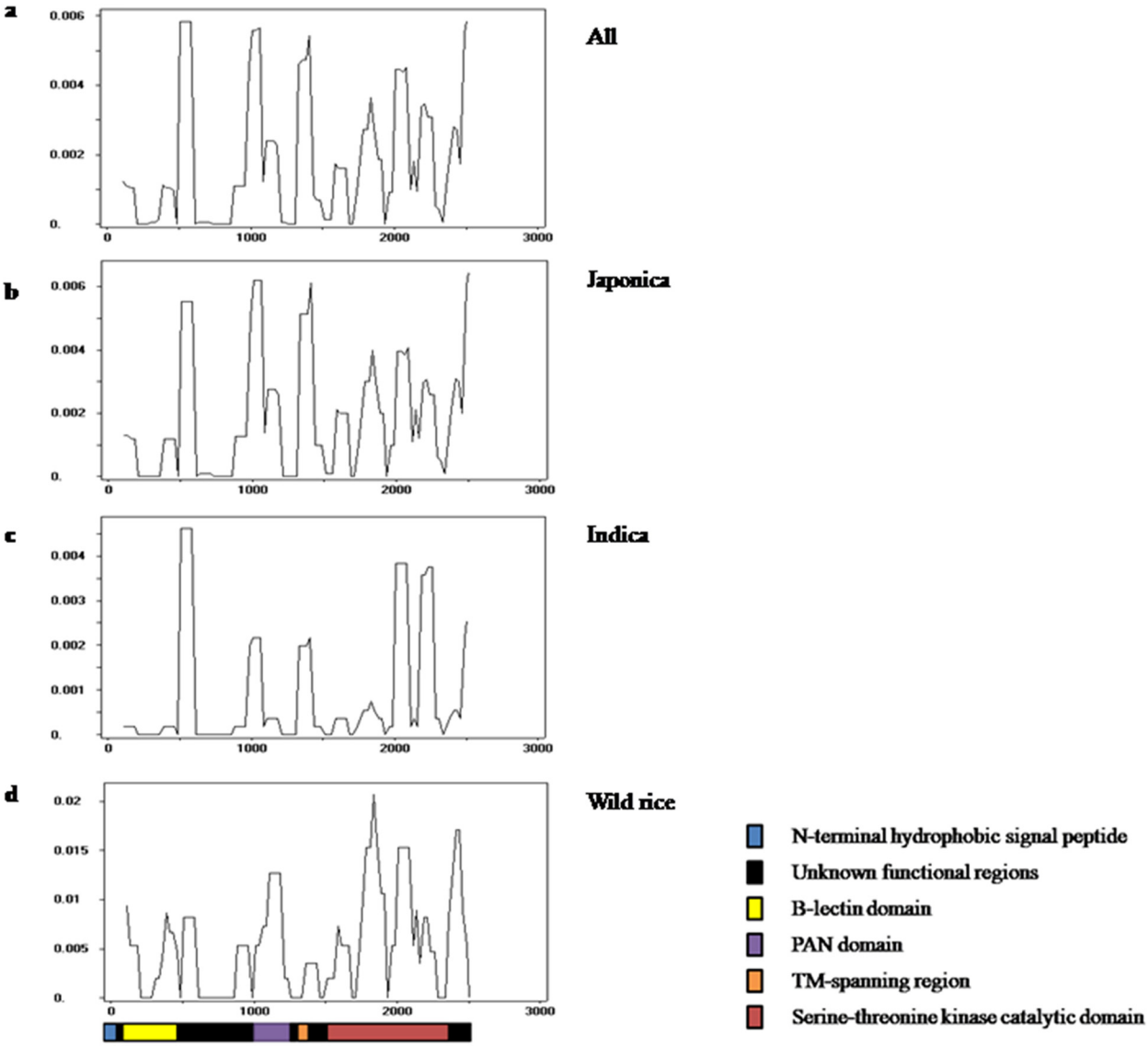

Figure 1. Sliding-window analysis of diversities in *Pi-d2* coding region. The nucleotide diversity (π; *Y*-axis) was generated by DNAsp5.0, and the *X*-axis represents the positions of nucleotides. The blue line stands for the result of cultivated rice varieties; the red line stands for the wild rice, and the black line stands for all rice. The map below the sliding window is the encoding structure of the *Pi-d2* gene, the coloured box denotes the exon region.

catalytic domain; secondly, the two peaks exists in the PAN domain and the TM-spanning region were significantly higher in Japonica, indicating that the diversity of Japonica is greater in these two regions than that of Indica. The PAN domain and the Serine-threonine kinase catalytic domain had greater nucleotide diversity than the other sections, which had lower nucleotide diversity.

Neutral tests were used to undertake additional examination of the different regions of *Pi-d2* haplotypes (online Supplementary Table S5). In japonica and wild rice types, Tajima's *D* values were predominantly negative but not statistically significant, indicating that *Pi-d2* orthologs may experience a slight tendency for positive selection but not balanced selection. However, the Tajima's *D* value was extremely negative for indica strains, particularly in the Serine-threonine kinase catalytic domain, indicating that the indica region may have been subjected to strong positive selection during evolution.

Amino acid sequence analysis of Pi-d2 *haplotypes*

Alignment of *Pi-d2* protein amino acid sequences revealed a high degree of similarity; sequence consistency reached 99.69%. According to the alignment, the most significant amino acid substitution site is located in the TM domain, which is the resistance-determining site (441 I/M). In the remainder of the domain, only a few amino acid substitutions were detected, none of which were found in the region of sequence conservation (Fig. 2). Using the encoded amino acid sequences, a neighbour-joining tree was created to examine the evolutionary relationships of *Pi-d2* haplotypes (Fig. 3). All of the materials were seen to be organised into four large groupings. On branch 1 were grouped five *O.nivara* varieties, one *O.rufipogon* variety, and ten haplotypes from farmed variations. This branch includes the majority of indica strains, including *Pi-d2_Digu*. *Pi-d2_Nipponbare* and eight additional haplotypes of 107 cultivated cultivars were assigned to branch 2. The percentage

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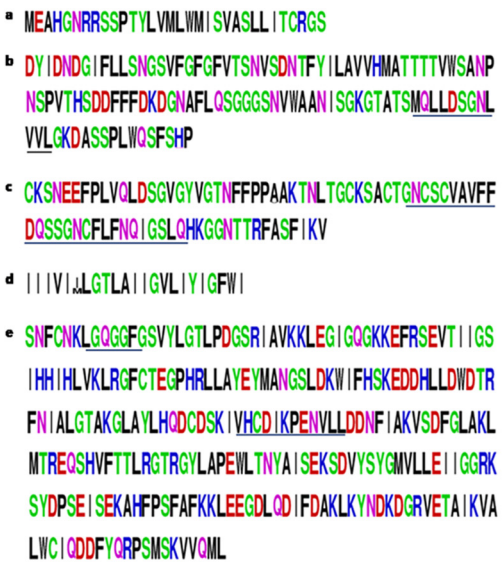

a. N-terminal hydrophobic signal peptide
b. B-lectin domain
c. PAN domain
d. TM-spanning region
e. Serine-threonine kinase catalytic domain

Figure 2. Conservatism analysis of amino acid sequences among *Pi-d2* orthologs. The conservatism analysis of amino acid sequences was produced by the software WebLogo on line. The piled height of amino acid loci showed the degree of conservatism, and the piled height of different amino acids in single locus reflected the degree of correlation with this site. The underline indicated a conservative sequence.

of japonica varieties within this category reached 88.79%. In branch 3, *O.longistaminat*, *Hainan_O.rufipogon*, and one japonica variety were grouped. *Yuanjiang_O.rufipogon* was placed on the same branch as *Dongxiang_O.rufipogon* and three haplotypes from cultivated rice cultivars. These outcomes showed that indica and japonica each had their own dominant haplotype, and that the majority of indica and japonica cultivars differed in the *Pi-d2* coding region.

Blast resistance of Pi-d2 *haplotypes*

Six *Pi-d2* haplotypes, encoding for H1, H2, H11, H20, H23 and H24, containing a single amino acid I at position 441 were chosen for testing their resistance to a collection of rice blast strains. H1 was selected as a positive control since it codes for the same protein as *Pi-d2_Digu*. The coding protein of H2 was the most broadly distributed in Yunnan province, but the other four haplotypes were unique to Yunnan province.

To ensure uniformity, each *Pi-d2* haplotype was placed into the binary vector pUN1301 under the control of the Zea mays ubiquitin 1 (Ubi) gene promoter and then converted into the susceptible rice variety Nipponbare. *Pi-d2* haplotypes from Nipponbare *Pi-d2_Nipponbare* and empty vector UN1301 were also transformed into Nipponbare seedlings as a negative control. The independent primary transgenic lines (T0) were obtained and identified using the CAPS and Hyg transgene markers (Fig. 4). PCR results revealed that every putative transformant was transgene positive. The transgenic lines in which the individual transgenes were expressed at roughly the same levels (online Supplementary Fig. S1) and in which there were no visible side effects on phenotypes were chosen for co-segregation analysis in the T0 to T2 generations using the CAPS marker. In subsequent blast inoculation tests, the homozygous T2 lines of the six *Pi-d2* haplotypes were used.

Six *Pi-d2* haplotype transgenic lines were separately injected with eleven *M. oryzae* strains. As a negative control, LTH exhibited susceptibility to all eleven strains, showing that the inoculation test was successful. *Pi-d2_NP* and UN1301 exhibited susceptibility to ZB15 and three other stains, including w1-117, 08-43-1a and 15-30-301. While exhibiting moderate resistance to w1-79, 16-2-1e, A23, w1-10, 08-55-1a and 08-35-1a, it exhibited resistance to w1-125 and 08-55-1a. All *Pi-d2* haplotype transgenic lines exhibited resistance or moderate resistance to ZB15, as observed (Fig. 5). Nonetheless, various transgenic lines exhibited distinct spectra of resistance to other strains (Table 1). Notable is the fact that *Pi-d2_H23* and *Pi-d2_H24* transgenic lines were resistant to all strains. These outcomes suggest that the resistance of different *Pi-d2* haplotypes transgenic lines has been significantly enhanced; however, there are variations in the resistance and spectrum of the various *Pi-d2* haplotypes. To validate the disease resistance and resistance spectrum of *Pi-d2_H23* and *Pi-d2_H24*, 18 additional *M. oryzae* strains were injected. Unlike the negative control, *Pi-d2_H23* and *Pi-d2_H24* exhibited nearly full resistance to all *M. oryzae* strains (online Supplementary Table S4). These results indicated that these two transgenic lines have a wider spectrum of resistance than the others.

By comparing the protein sequences of several *Pi-d2* haplotypes, it was shown that the broad range of blast resistance was associated with an amino acid substitution at position 363 (online Supplementary Fig. S2), where *Pi-d2_H23* and *Pi-d2_H24* were V (valic acid) and others were A. (alanine). The results of the domain analysis revealed that the substitution site was present in the PAN domain of the extracellular structure of *Pi-d2*, indicating that the PAN domain coding region may have a role in detecting the unique blast strains.

Discussion

The present study showed that the pi value of the wild rice accessions belongs to type II (pi = 0.53%) while the Japonica accessions (pi = 0.191%) and the Indica accessions (pi = 0.085%) belong to type I, which indicated that the diversity of wild rice is larger than that in cultivated rice. This result shows that the encoding sequences of *Pi-d2* gradually tend to unity during the rice domestication. Also *Pi-d2_H24* from wild rice has a broader range of blast resistance than others. Plant disease resistance is connected with the expression pattern of R genes or genes involved in pathogenesis (PR genes) (Hayashi and Yoshida, 2009; Amaranatha *et al.*, 2013; Li *et al.*, 2017). Although our study did not consider the effect of the endogenous expression pattern of *Pi-d2* on the

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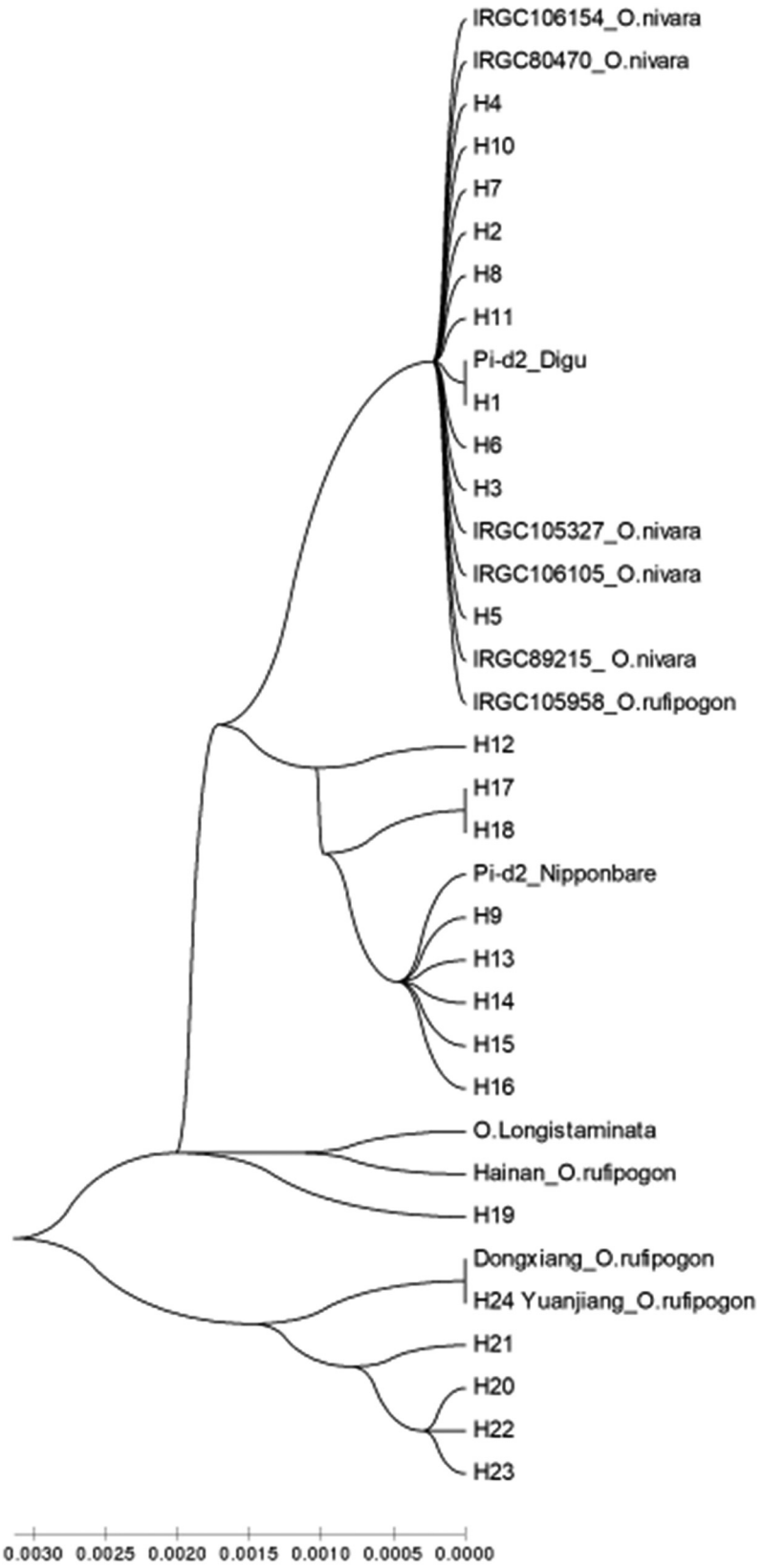

Figure 3. Phylogenetic analysis of *Pi-d2* orthologs.

disease resistance, the phenotypic determination results of transgenic plants with different *Pi-d2* haplotypes in the same genetic background can also have a great reference value (Lv *et al.*, 2013).

Our study show that there are two polymorphic loci in the TM domain, excluding the resistance determinant site (441 I/M), with the other site found at the 437th I/F, which is unique to one variety. To determine the biological significance of this polymorphism

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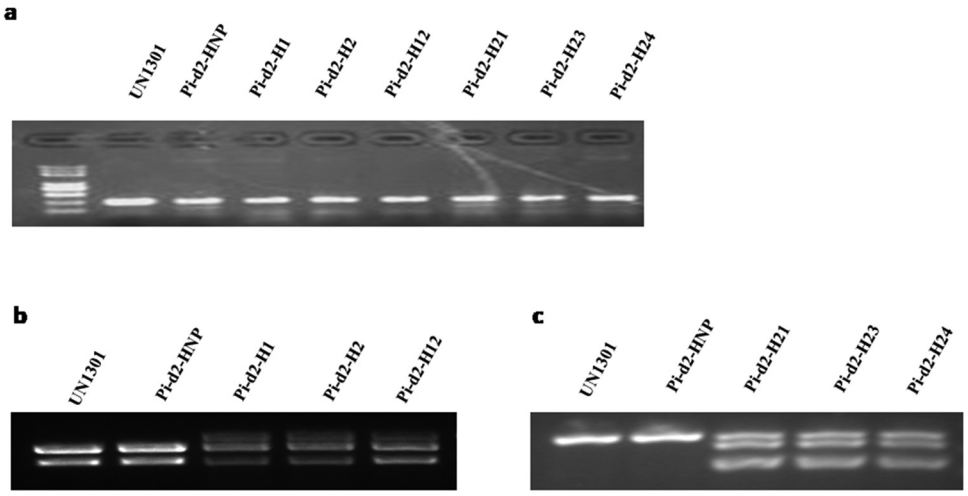

Figure 4. Detection of the DNA to the transformants. (a) HYG marker was used in the DNA detection. (b) (c) CAPS marker was used to confirm the positive transformants precisely and the restriction enzyme cutting site (Fau1) exit in *Pi-d2*_H1/ *Pi-d2*_H2/ *Pi-d2*_H12 and (Xmn1) exist in *Pi-d2*_H20/ *Pi-d2*_H23/ *Pi-d2*_H24.

location, additional research is required. The majority of plant disease-resistant proteins are unstable. Similar to XA21, PI-D2's juxtamembrane domain contains a putative proteolytic motif (Ding *et al.*, 2009). It has been suggested that this motif contributed to the protein's instability (Xu *et al.*, 2006). All *Pi-d2* haplotypes were found to be conserved in this motif range, indicating that the proteins coded by these *Pi-d2* haplotypes remain unstable.

We show that, there are five polymorphic loci in the serine-threonine kinase catalytic domain. Specifically, at site 686H/R, nearly all rice varieties (R) differ from *Pid2-digu* (H). The significantly low value of Tajima'*D* in the Serine-threonine kinase catalytic domain of indica strains indicates that this region of indica may have been subjected to a strong positive selection during evolution. Although these polymorphism sites are not located in the kinase's ATP-binding region or serine-threonine specificity signature sequence region. However, these polymorphic sites may lead to conformational changes in the serine-threonine kinase domain, thereby altering the PI-D2 intracellular domain's ability to bind to substrates. And additional research is required to confirm this conclusion.

Figure 5. Phenotyping of rice lines with *Magnaporthe oryzae* isolates. Disease reaction of transgenic plants inoculated with *M. oryzae* ZB15, W1-10, A23, 15-30-3a, 08-35-1a and W1-125. Columns: 1, UN1301 (empty vector control); 2, *Pi-d2*_NP; 3, *Pi-d2*_H1; 4, *Pi-d2*_H2; 5, *Pi-d2*_H12; 6, *Pi-d2*_H20, 7, *Pi-d2*_H23; 8, *Pi-d2*_H24.

https://doi.org/10.1017/S1479262124000248 Published online by Cambridge University Press

Therefore, it was hypothesised that the extracellular domain of PI-D2 could bind to hydrophobic macromolecules, such as plant hormones or proteins, with greater probability (Chen *et al.*, 2006). Among 309 rice varieties, our findings revealed two polymorphism loci in the b-lectin domain (including 299 landrace and 10 common wild rice lines). Nonetheless, these two loci are not within the core sequence of the b-lectin domain, indicating that this domain has been conserved throughout the evolution of rice. Interestingly, our investigation also suggested that resistance differences across *Pi-d2* alleles are associated with the change of amino acids at position 363, with *Pi-d2_H23* and *Pi-d2_H24* containing valine and the others containing alanine (alanine).

Over all, in this study, we investigated the nucleotide polymorphism and evolution patterns of the *Pi-d2* gene among 299 rice landraces of Yunnan province and six wild rice relatives, and 50 polymorphic sites and 24 haplotypes of *Pi-d2* were found. Unlike the results of the previous studies, the functional site of Pi-d2 did not show strict differentiation between Indica subgroub and Japonica subgroub (Xie *et al.*, 2022), which provides new clues on the origin and evolution of *Pi-d2*. Furthermore, we observed Tajima's *D* value was −1.87012 with statistical significance for Indica subgroub, which suggested that the rice blast resistant gene *Pi-d2*, especially Kinase domain of it indeed, may have undergone positive selection in Indica subgroub. *Pi-d2* acts as a haploid gene and originated in early stage of Poales evolution (Vaid *et al.*, 2012; Xie *et al.*, 2022). All of functional/non-functional haplotypes of *Pi-d2* are present in the genome under strong directional selection during rice evolution, suggesting that *Pi-d2* may palys other biological role besides pathogen recognition. Therefore, studying the mechanism of intracellular signalling mediated by kinase domain of *Pi-d2* is likely to further reveal its biological function. In addition, we compared the blast resistance spectra of six *Pi-d2* haplotypes carrying the functional site under *Nipponbare*'s genetic background. Intriguingly, the data revealed that *Pi-d2_H23* and *Pi-d2_H24* have a broader range of blast resistance than others, including the *Pi-d2* haplotype of Digu, which indicated that this two haplotype are beneficial for improving the breeding of rice blast resistance.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1479262124000248

Data availability statement. The data that supports the findings of this study are available in the supplementary material of this article. These data are available from the corresponding author upon reasonable request.

Acknowledgements. We thank Professor Cailin Lei from Chinese Academy of Agricultural Sciences for providing *M. oryzae* strains ZB15. This research was supported by the China National Natural Sciences Foundation (#32260523/#31860369) and Agricultural Cooperative Research Programs of Yunnan of China (#202301BD070001-218/ #202101BD070001-002). The authors would like to express their gratitude to EditSprings (https://www.editsprings.cn) for the expert linguistic services provided.

Author contributions. Y.S. and M.X. conceived and designed the experiment and revised the paper. Y.Y. and J.M. conducted the main experiments, such as the RNA and DNA template preparation, gene cloning, vector construction, genetic transformation, material planting. J.L. performed the resistance to rice blast species identification of transgenic plants. J.T. provided assistance during paper revision. C.L. provided assistance during the planting of materials and genetic transformation. All authors have read and approved the final paper.

Ethical standards. This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study. Neither the entire paper nor any part of its content has been published or has been accepted elsewhere. It is not being submitted to any other journal. All authors agree that there have no conflict of interest.

References

Amaranatha RM, Gambhire VB and Rajeswar RT (2013) Allele mining in crop improvement. *International Journal of Development Research* **4**, 300–305.

Arunakumari K, Durgarani CV, Satturu V, Sarikonda KR, Chittoor PDR, Vutukuri B, Laha GS, Nelli APK, Gattu S, Jamal M, Prasadbabu A, Hajira S and Sundaram RM (2016) Marker-assisted pyramiding of genes conferring resistance against bacterial blight and blast diseases into Indian rice variety MTU1010. *Rice Science* **23**, 306–316.

Barre A, Herve C, Lescure B and Rouge P (2002) Lectin receptor Kinases in plants. *Critical Reviews in Plant Sciences* **21**, 379–399.

Bellande K, Bono J, Savelli B, Jamet E and Canut H (2017) Plant lectins and lectin receptor-like kinases: how do they sense the outside? *International Journal of Molecular Sciences* **18**, 1164.

Chen XW, Li SG, Xu JC, Zhai WX, Ling ZZ, Ma BT, Wang YP, Wang WM, Gao G, Ma YQ, Shang JJ, Zhao XF, Zhou KD and Zhu LH (2004) Identification of two blast resistance genes in a rice variety, Digu. *Journal of Phytopathology* **152**, 77–85.

Chen XW, Shang JJ, Chen DX, Lei CL, Zou Y, Zhai WX, Liu GZ, Xu JC, Lin ZZ, Cao G, Ma BT, Wang YP, Zhao XF, Li SG and Zhu LH (2006) A B-lectin receptor kinase gene conferring rice blast resistance. *Plant Journal* **46**, 794–804.

Chen DX, Chen XW, Ma BT, Wang YP, Zhu LH and Li SG (2010) Genetic transformation of rice with pi-d2 gene enhances resistance to rice blast fungus *Magnaporthe oryzae*. *Rice Science* **17**, 19–27.

Clark SE, Williams RW and Meyerowitz EM (1997) The *CLAVATA*1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* **89**, 575–585.

Dai YT, Jia YL, Correll J, Wang XY and Wang YL (2010) Diversification evolution of the avirulence gene *AVR-Pita*1 in field isolates of *Magnaporthe oryzae*. *Fungal Genetics and Biology* **47**, 973–980.

Dean R, Kan JALV, Pretorius ZA, Hammond-Kosack KE, Pietro AD, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J and Foster GD (2012) The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology* **13**, 414–430.

Deng Y, Zhu X, Shen Y and He Z (2006) Genetic characterization and fine mapping of the blast resistance locus Pigm(t) tightly linked to *Pi2* and *Pi9* in a broadspectrum resistant Chinese variety. *Theoretical and Applied Genetics* **113**, 705–713.

Ding XD, Richter T, Chen M, Fujii H, Seo YS, Xie MT, Zheng XW, Kanrar S, Stevenson RA, Dardic C, Li Y, Jiang H, Zhang Y, Yu FH, Bartley LE, Chern MS, Bart R, Chen XH, Zhu LH, Farmerie WG, Gribskov M, Zhu JK, Fromm ME, Ronald PC and Song WY (2009) A rice kinase-protein interaction map. *Plant Physiology* **149**, 1478–1492.

Dodds PN and Rathjen JP (2010) Plant immunity: towards an integrated view of plantpathogen interactions. *Nature Reviews Genetics* **11**, 539–548.

Gomez-Gomez L, Bauer Z and Boller T (2001) Both the extra cellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in Arabidopsis. *Plant Cell* **13**, 1155–1163.

Hayashi K and Yoshida H (2009) Refunctionalization of the ancient rice blast disease resistance gene *Pit* by the recruitment of a retrotransposon as a promoter. *Plant Journal* **57**, 413–425.

Hiei Y, Ohta S, Komari T and Kumashiro T (1994) Effificient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant Journal* **6**, 271–282.

Hua LX, Wu JZ, Chen CX, Wu WH, He XY, Lin F, Wang L, Ashikawa I, Matsumoto T, Wang L and Pan QH (2012) The isolation of Pi1, an allele at the Pik locus which confers broad spectrum resistance to rice blast. *Theoretical and Applied Genetics* **125**, 1047–1055.

Jia YL, Valent B and Lee FN (2003) Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. *Plant Disease* **87**, 129–133.

Jiang HC, Li Z, Liu J, Shen ZK, Gao GJ, Zhang QL and He YQ (2019) Development and evaluation of improved lines with broad-spectrum resistance to rice blast using nine resistance genes. *Rice* **12**, 29.

Li JB, Sun YD, Liu H, Wang YY, Jia YL and Xu MH (2015) Natural variation of rice blast resistance gene *Pi-d*2. *Genetics and Molecular Research* **14**, 1235–1249.

Li WT, Zhu ZW, Chern MS, Yin JJ, Yang C, Ran L, Cheng MP, He M, Wang K, Wang J, Zhou XG, Zhu XB, Chen ZX, Wang JC, Zhao W, Ma BT, Qin P, Chen WL, Wang YP, Liu JL, Wang WM, Wu XJ, Li P, Wang JR, Zhu LH, Li SG and Chen XW (2017) A natural allele of a transcription factor in rice confers broad-spectrum blast resistance. *Cell* **170**, 114–126.

Lv QM, Xu X, Shang JJ, Jiang GH, Pang ZQ, Zhou ZZ, Wang J, Liu Y, Li T, Li XB, Xu JC, Chen ZK, Zhao XF, Li SG and Zhu LH (2013) Functional analysis of *Pid*3-*A*4, an ortholog of rice blast resistance gene *Pid*3 revealed by allele mining in common wild rice. *Phytopathology* **103**, 594–599.

Ma J, Lei CL, Xu XT, Hao K, Wang JL, Cheng ZJ, Ma XD, Ma J, Zhou KN, Zhang X, Guo XP, Wu FQ, Lin QB, Wang CM, Zhai HQ, Wang HY and Wan JM (2015) *Pi*64, encoding a novel CC-NBS-LRR protein, confers resistance to leaf and neck blast in rice. *Molecular Plant-microbe Interactions* **28**, 558–568.

Nasrallah ME (2000) Arabidopsis species hybrids-emerging model systems for the analysis of species differences. *Journal of Plant Growth Regulation* **19**, 326–333.

Peng YJ, Wersch RV and Zhan YL (2018) Convergent and divergent signaling in PAMP-triggered immunity and effector-triggered immunity. *Molecular Plant-microbe Interactions* **31**, 403–409.

Selisana SM, Yanoria MJ, Quime B, Chaipanya C, Lu G, Opulencia R, Wnag GL, Mitchell T, Correll J, Talbot NJ, Leung H and Zhou B (2017) Avirulence (AVR) gene-based diagnosis complements existing pathogen surveillance tools for effective deployment of resistance (R) genes against rice blast disease. *Phytopathology* **107**, 711–720.

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF and Li WH (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* **16**, 1220–1234.

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C and Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa*21. *Science (New York, N.Y.)* **270**, 1804–1806.

Sun X, Cao Y, Yang Z, Xu C, Li X, Wang S and Zhang Q (2004) *Xa*26, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein. *Plant Journal* **37**, 517–527.

Vaid N, Pandey PK and Tuteja N (2012) Genome-wide analysis of lectin receptor-like kinase family from Arabidopsis and rice. *Plant Molecular Biology* **80**, 365–388.

Wang L, Xu XK, Lin F and Pan QH (2009) Characterization of rice blast resistance genes in the *Pik* cluster and fine mapping of the *Pik-p* locus. *Phytopathology* **99**, 900–905.

Woolhouse MEJ, Webster JP, Domingo E, Charlesworth B and Levin BR (2002) Biological and biomedical implications of the co-evolution of pathogens and their hosts. *Nature Genetics* **32**, 569–577.

Wu YY, Xiao N, Chen Y, Yu L, Pan CH, Li YH, Zhang XX, Huang NS, Ji HJ, Chen XJ and Li AH (2019) Comprehensive evaluation of resistance effects of pyramiding lines with different broad-spectrum resistance genes against *Magnaporthe oryzae* in rice (*Oryza sativa L.*). *Rice* **12**, 11.

Xie PF, Liu J, Lu RS, Zhang YM and Sun XQ (2022) Molecular evolution of the *Pi-d*2 gene conferring resistance to rice blast in *Oryza*. *Frontiers in Genetics* **13**, 991900.

Xu WH, Wang Y, Liu G, Chen X, Tinjuangjun P, Pi LY and Song WY (2006) The autophosphorylated Ser686, Thr688 and Ser689 residues within a putative protease cleavage motif in the juxtamembrane domain of XA21 are implicated in the stability control of the rice receptor-like kinase. *Plant Journal* **45**, 740–751.

Zeng YW, Zhang HL, Li ZC, Shen SQ, Sun JL, Wang MX, Liao DQ, Liu X, Wang X K, Xiao FH and Wen GS (2007) Evaluation of genetic diversity of rice landraces (*Oryza sativa* L.) in Yunnan, China. *Breeding Science* **57**, 91–99.

Zhou B, Qu S, Liu G, Dolan M, Sakai H, Lu G, Bellizzi M and Wang GL (2006) The eight amino-acid differences within three leucine-rich repeats between Pi2 and Piz-t resistance proteins determine the resistance specificity to *Magnaporthe grisea*. *Molecular Plant Microbe Interactions* **19**, 1216–1228.

Zhu YY, Chen HR, Fan JH, Wang YY, Li Y, Chen JB, Fan JX, Yang SY, Hu LP, Leung H, Mew TW, Teng PS, Wang ZH and Mundt CC (2000) Genetic diversity and disease control in rice. *Nature* **406**, 718–722.

https://doi.org/10.1017/S1479262124000248 Published online by Cambridge University Press