Sequences Analysis of Genes Encoding the NB-ARC domain of Blast-Resistant Proteins in New Upland Rice Lines to Detect Broad-Spectrum Blast Resistance

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A B S T R A C T

A total of 19 paddy lineages taken from Bengkulu local paddy varieties were assessed in an open area to find out blast-resistant-gene paddy plants. This study aimed to examine in detail the DNA arrangement producing definite result of heredity units converted into the functional ATPase domain (NB-ARC having in common with APAF 1 - Apoptotic Protease Activating Factor 1, R proteins, and Cell death protein 4) area of blast resistance proteins in new paddy lines from breeding program. Molecular analysis was conducted using six specific primers to detect broad-spectrum blast resistance, and sequence analysis coding was executed to the the functional ATPase area of blast-resistant protein in the lines that were found to have multiple genes. This study identified eight lines of Genotype 7, Genotype 8, Genotype 9, Genotype 11, Genotype 13, Genotype 14, Genotype 15, and Genotype 18 assessed for resistance in the field where the Pi-ta gene was detected; this gene was thought to exhibit broad-spectrum resistance. Conserved domain analysis revealed that eight blast-resistant rice lines were found to encode NB-ARC at sequence lengths between 329 to 873. The sequence analysis on BLASTX revealed 15 gene homologies of the eight rice lines, which was detected by the Pi-ta2 gene, with a similarity level of 81%–99%. Analysis of the gene domain of the NB-ARC domain containing protein, expressed using the CDD program, disclosed that the working part of the enzyme was at 478–487 amino acids and the NBS-LRR class was present at 722–742 amino acids. A comprehensive further study should be performed to confirm the performance and resistance of lines in field trials at various endemic blasts before being released as candidates for new upland rice varieties.

Keywords: blast resistance; landraces; NB-ARC; broad-spectrum; upland rice lines

1. Introduction

Gray leaf spot destructive attack resulted from the paddy blast disease fungus of *Pyricularia grisea* (Cooke) Sacc., constitutes very extremely offensive. In the tropical regions, blast disease becomes generally more important on highland than in low-lying regions of rice cultivation. It is one of the biotic obstacles in the development of upland rice. The rate of yield loss caused by blast disease in endemic areas reaches 50%–100% (X. Wang et al., 2014). In 2007, the blast disease affected 1,285 million ha or 12% of the total area of rice cultivation in Indonesia, and it is predicted to increase in the coming years (Directorate General of Food Crop, 2018).

Chemical control of blast disease increases production costs. Therefore, the application of blast-resistant superior species is much effective and cheap. The development of superior varieties requires a different source of new blast-resistant genes. However, the availability of genetic diversity maintained in germplasm sources is an important factor to support rice breeding programs. The genetic background of superior varieties can be broadened through recommendations for landrace varieties (local) in the action plan of improving the genetic potential of plants (Nickolas, et al., 2018 ; Yadav et al., 2019).

Blast control by fitting together the durable and polygenic resistance paddy varieties is to overcome destructive pathogens that have multiple races and are very dynamic (Wang and Valent, 2017; Sheoran et al., 2021). Therefore, the breeding program to obtain superior rice varieties is

implemented through several stages, namely, determining the parent as the source of resistance genes, crossing between two or more elders, and evaluating the progeny. We have been conducting a breeding program since 2010 with the establishment of a baseline population and recurrent selection using Sriwijaya and Bugis cultivars and IR 7858-1/IR148 lines as donor parents of drought- and blast-resistant genes (Herawati et al., 2017; Herawati et al., 2021).

The resistance evaluation of paddy to blast can be conducted in the field in endemic areas with a natural inoculum or in a screen house with an artificial inoculum (Khan et al., 2014); molecular detection of resistance genes can also be performed in these areas (Deng et al., 2017; Nickolas et al., 2018). Furthermore, field evaluation can be conducted at a further stage to obtain resistant varieties in the field against various racial compositions because the varieties released to farmers will face multiracial environmental conditions (Nickolas et al., 2018). Molecular detection is often conducted and helps breeders in selecting genotypes that have blast-resistant genes before being released as new varieties.

It is necessary to learn more how the molecule works on the pathogen host relationship and strategic interrogation of resistance genes in cultivars, especially by identifying genes of host resistance and not pathogen. Currently, molecular markers are widely used to characterize collections of unrevealed heredity unit banks especially in untapped different allele resources. (Vasudevan et al., 2015; Ning et al., 2020). However, identifying comprehensive description of blast-resistant genes is critical to protect plants from the aggressive race of M. grisea.

The genetically related virulence traits of *Pyricularia oryzae* have been studied by several researchers. There are several genes that have been cloned, namely, Pi37 on chromosome 1 (Lin et al., 2007); Pib on chromosome 2; Pi9 (Qu et al., 2006) and Pid2 (Chen et al., 2006) on chromosome 6; and Pita on chromosome 12 (Bryan et al., 2000). The phosphorus uptake 1 containing other genes such as dirigent-like, fatty acid α-dioxygenase and aspartic proteinase constitutes very determining in producing proteins used in biosynthesis of lignin, which affects the hardness of plant cell walls (Heuer et al., 2009). Yan et al. (2017) used eleven prominent blast-resisting paddy genes, namely Pi-d2, Pi-z, Piz-t, Pi-9, Pi-36, Pi-37, Pi5, Pi-b, Pik-p, Pik-h, and Pi-ta^2 to identify 32 acquisitions of paddy resistance to fungal pathogen of paddy blast fungus using molecular markers.

The most represented group of resistance genes include NBS-LRR class which is the most decisive in the plant defense response, constituting the most considerable family of plant resistance genes (Meyers et al., 1999; Nimchuk et al., 2003). Gene expression occurs found in the recognition of proteins encoded for the immune system in plants. The core nucleotides in the NB-LRR protein are part of the functional ATPase domain due to their existence in APAF-1, protein R, and CED4 protein (Van der Biezen and Jones, 1998). The presence of effectors encoded by pathogens causes intramolecular interactions in NBS-NLR, among others, to produce oligomerization of NLRs due to the substitution of ADP by (d)ATP in the domain of functional ATPase. Induction of oligomerization models is achieved permanent acceptance in animal NLR activation (Danot et al. 2009; Bentham et al., 2017). The functional ATPase which is the main domain for the immune receptors to be self-connected has been revealed in animal NLR structure analysis (Hu et al., 2015). In other words, there has not been much information identified concerning the decisive domain in the oligomerization of plant NLRs.

There is limited research on the protein structure converted by blast-resistant genes. Among them is the characterization of proteins from Pita and pi54, including the downstream interaction partners of the NBS-LRR class plant proteins (Devanna et al., 2014). The NBS-LRR protein is familiar to be engaged in the plants defense mechanisms. Nevertheless, further activities

performed by this protein and its mechanism of action are unfamiliar. Protein sequences with functions that have not been experimentally confirmed to have been reported. Uncharacterized proteins are potential to be applied as biological markers. A computational approach can be applied to estimate the three-dimensional configuration and use of the selected protein. The model of having similar relative structure is currently widely applied to anticipate the configuration of uncharacterized amino acid (Sharma et al., 2018; Steele et al., 2019).

This research aimed to examine in detail a particular order of DNA to be the primary part of encoding the NB-ARC domain of blast-resistant proteins in new upland rice lines from the breeding program as well as in the detection of paddy blast resistance extensively.

2. Materials and methods

The study was conducted from March 2020 to April 2021. Evaluation of blast resistance was conducted in Aur Gading Village, Kerkap Subdistrict, North Bengkulu District. The analysis of molecules was conducted in the Crop Production Biotechnology Laboratory and the Mathematics and Natural Sciences Biology Laboratory of University of Bengkulu. The materials were 19 lines obtained from the cross breeding of local varieties (landraces) in Bengkulu (Sriwijaya and Bugis) which tolerate substantial dehydration (IR7858 and IR148) and Situ Patenggang and Kencana Bali were obtained as resistant and sensitive checks respectively (Table 1).

Table 1. Selected lines resulting from crossbred for identification of blast resistance

Genotype	Accession number	Pedigree	Genotype	Accession number	Pedigree
G1	BKL1-RS1-1-247-13	Bugis/IR7858-1	G12	BKL2 B-1-262-4	Bugis/IR148
G2	BKL1-RS1-1-248-14	Bugis/IR7858-1	G13	BKL2 B-2-263-5	Bugis/IR148
G3	BKL1-RS1-2-249-15	Bugis/IR7858-1	G14	BKL2 B-2-264-6	Bugis/IR148
G4	BKL2-RS1-1-251-17	Bugis/IR148	G15	BKL3 B-2-266-8	Sriwijaya/IR148
G5	BKL3-RS1-1-253-18	Sriwijaya/IR148	G16	BKL3 B-3-267-9	Sriwijaya/IR148
G6	BKL4-RS1-1-256-21	Sriwijaya/IR7858-1	G17	BKL4 B-1-268-10	Sriwijaya/IR7858-1
G7	BKL4-RS1-2-257-22	Sriwijaya/IR7858-1	G18	BKL4-B2-269-11	Sriwijaya/IR7858-1
G8	BKL4-RS1-3-258-23	Sriwijaya/IR7858-1	G19	BKL4 B-3-270-12	Sriwijaya/IR7858-1
G9	BKL1 B-1-259-1	Bugis/IR7858-1	G20	Kencana Bali	Susceptible Check
G10	BKL1 B-2-260-2	Bugis/IR7858-1	G21	Situ Patenggang	Resistance check
G11	BKL1 B-3-261-3	Bugis /IR7858-1			

2.1. Field evaluation of blast resistance

Field evaluation was conducted in Aur Gading village, an area endemic for blast disease. The seeds were grown in a 2 × 3 m plot, spaced 20cm apart. Fertilization was carried out three times; the first fertilization of the plants was carried out 21–25 days after planting (DAP) at the following doses: 200 kg/ha urea, 150 kg/ha TSP, and 90 kg/ha KCl. The second fertilization was carried out at 60–65 DAP with a 1/3 dose of urea for 200 kg/ha and ½ dose of KCl for 90 kg/ha. The third fertilization was carried out at 70–75 DAP with a 1/3 dose of urea for 200 kg/ha. Blast disease symptoms were observed using a scale built upon the SES (Standard Evaluation System for Paddy (IRRI, 2013) (Table 2). The level of line resistance was determined using the following scales: 0–2, resistant (R); 3, fairly resistant (FR); 4–6, fairly vulnerable; and 7–9, vulnerable (V).

Table 2. Scale of symptom of sudden death of leaf for field assessment based on SES IRRI (2013)

Scale	Symptoms
0	Without taking notice injury
1	Tiny brown spots of pin-point size or larger brown spots with no central spore formation
2	Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin
3	Kind of injury was identical to those in scale 2, yet some important injuries on the upper leaves
4	Distinctive vulnerable blast injury 3 mm or longer, infecting less than 4% of the leaf area
5	Distinctive sudden death of leaf injury contaminating 4-10% of the leaf area
6	Distinctive sudden death of leaf injury contaminating 11-25% of the leaf area
7	Distinctive sudden death of leaf injury contaminating 26-50% of the leaf area
8	Distinctive sudden death of leaf injury contaminating 51-75% of the leaf area and many were no longer alive
9	More than 75% leaf area contaminated

The following formula is used to determine the severity of the disease:

$$DS = \frac{\sum_{i=0}^{n}(nixvi)}{N\ x\ Z}\ x\ 100\%$$

where DS denotes the disease severity; v, score according to symptom criteria in family i; ni, number of families attacked in the i-th score; N, total clumps observed; Z, highest score.

2.2. Analysis of Molecules

2.2.1. DNA extraction, PCR analysis, and DNA amplification

The samples of fresh leaf samples were collected from plants in the field. They were put in plastic and then in a special box filled with ice to maintain their freshness. An amount of 0.1 g of paddy leaves was ground into flour by adding liquid nitrogen, and then, total DNA isolation was conducted according to the Genomic DNA Purification Kit Wizard protocol. The powder was placed in a 2-mL Eppendorf tube, added with 600 µL of Nuclei Lysis Solution, vortexed for 1–3 s, and then heated in a water bath at 65°C for 15 minutes. Then, the sample was added with 3 µL of RNase Solution and then incubated at 37°C for 15 minutes. Afterward, 200 µL of Protein Precipitation Solution was added and centrifuged again for 3 minutes at 13,000 rpm. The supernatant was then taken and put into a 1.5-mL microtube and added with 600 μL of isopropanol at room temperature. The centrifugation was repeated for one minute at room temperature. Afterward, the solution was taken out and let it dry for a quarter of an hour. After all, 100 µL of DNA Rehydration Solution was added and incubated at 65°C for one hour or one night at 4°C. The total isolated DNA was used as a template for gene amplification (Table 3) through polymerase chain reaction (PCR). Situ Patenggang DNA was used for the positive control, whereas Kencana Bali was used for the negative control as a sensitive check. This study used six pairs of primers, that is, Pi-d2, Pup1, Pi-ta2, Pi-37, Pi-z, and Pib. Table 3 shows the specifications of each primer.

Table 3. PCR primary characteristics to identify blast resistance

No.	Target gene	Primer Sequence (Forward/Reverse)	Chromosome number	Expected size (bp)	Anneling Temperature (°C)	Reference
1	Pib	F: GACTCGGTCGACCAATTCGCC R: ATCAGGCCAGGCCAGATTTG	2	388	54°C	(Yan et al., 2017)
2	Pi-37	F: TCTTGAGGGTCCCAGTGTAC R: CGAACAGTGGCTGGTATCTC	1	1149	58°C	(Yan et al., 2017)
3	Pi-d2	F: TTGGCTATCATAGGCGTCC R: ATTTGAAGGCGTTTGCGTAGA	6	1058	57°C	(Yan et al., 2017)
4	Pi-ta2	F: AGCAGGTTATAAGCTAGGCC R: CTACCAACAAGTTCATCAAA	12	1042	59°C	(Yan et al., 2017)
5	Pik	F: CGTGCTGTCGCCTGAATCTG R: CACGAACAAGAGTGTGTCGG	11	226	55 °C	(Hayashi, et al., 2006)
6	Pik-m	F:GCAGATGCATCAGCCAGTGAGTT R:GTGCAGGACCGGCACGCAG	11	171	55 °C	(Hayashi et al., 2006)

The amplification process was conducted at a pre-denaturation temperature of 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for one minute, heating and allowing it to cool slowly for 2 minutes, prolonging the process for 2 minutes at 72°C and being extended for 10 minutes at the same temperature. The PCR products were strengthened by electrophoresis in 1% agarose gel in the TBE buffer. The electrophoretic gel was dipped in 1% EtBr for 10 minutes and washed with ddH2O for 5 minutes. It was visible to the eye under an ultraviolet transilluminator to see the distribution of the DNA bands. The presence of bands in the tested strains on each primer with a certain size indicated the presence of a blast-resistant gene.

3.1.2. Sequencing and analysis data

DNA sequencing was performed on rice lines that were detected to have several multigenic resistance genes (pyramiding genes) using BigDye® Terminator First Base Services (PT. Genetics Sains Indonesia). The sequence data obtained were edited using BioEdit version 7.2 and aligned using ClustalX and Mega X version 10.2.6. The amino acids were analyzed using BLASTX (Basic Local Alignment Search Tool X) from NCBI (National Centre for Biotechnology Information) at www.ncbi.nlm.nih.gov/BLAST/. The phylogenetic construction uses the MEGA X program with the neighbor-joining method with 1000× bootstrap test. The domains and active site proteins were analyzed using the Conserved Domain Database accessed from the NCBI website and scanned using the ScanProsite. Prediction three-dimensional structure of protein in silico using the SWISS-MODEL program.

3. Results and discussion

3.1 Results

3.1.1. Field evaluation of blast resistance

Symptoms of blast began to appear on the leaf margins of the plants 7 weeks after planting (WAP; Figure 1a), and rectangular brown spots appeared at 10 WAP (Figure 2b). No blast attack by several line numbers was observed until the end of the observation (Figure 2c).

Figure 1. Blast attack in the field (a. blast symptom on the edge of the leaf; b. blast attack has led to the formation of a rectangular brown spot; c. resistant line)

Table 4. Field blast observations based on IRRI SES, severity level, and plant resistance criteria

Genotype	Accession	The lowest score	The highest score	Severity level (%)	Criteria
G1	BKL1-RS1-1-247-13	0	2	11	R
G2	BKL1-RS1-1-248-14	0	3	20	MR
G3	BKL1-RS1-2-249-15	0	3	29	MR

G4	BKL2-RS1-1-251-17	0	2	17	R
G5	BKL3-RS1-1-253-18	2	3	32	MR
G6	BKL4-RS1-1-256-21	0	3	30	MR
G7	BKL4-RS1-2-257-22	0	1	4	R
G8	BKL4-RS1-3-258-23	0	2	8	R
G9	BKL1 B-1-259-1	0	1	8	R
G10	BKL1 B-2-260-2	3	5	38	MS
G11	BKL1 B-3-261-3	0	2	13	R
G12	BKL2 B-1-262-4	0	2	19	R
G13	BKL2 B-2-263-5	0	2	19	R
G14	BKL2 B-2-264-6	0	2	9	R
G15	BKL3 B-2-266-8	0	2	9	R
G16	BKL3 B-3-267-9	2	4	37	MS
G17	BKL4 B-1-268-10	4	4	49	MS
G18	BKL4-B2-269-11	0	1	4	R
G19	BKL4 B-3-270-12	0	1	4	R
G20	Kencana Bali	5	6	62	S
G21	Situ Patenggang	0	1	3	R

*Scale 0-2 = resistance (R), scale 3 = moderate resistance (MR), scale 4-5 = moderate susceptibility (MS), and scale 6-7=susceptible (S); 8-9=highly susceptible (HS)

Field blast attacks are not too severe, with the highest symptom in Kencana Bali reaching only a score of 6, indicating that the blast attack has not demonstrated the severity level that causes death to all plants. In Situ Patenggang, a large portion of the population was affected by the blast disease at a score of 1 (Table 4). The highest attack was on the tested lines, namely, G10, G16, and G17, at a score of 4–5, indicating moderate susceptibility. Several line numbers that were tested showed resistant criteria with an attack score of 0–1 in the G7, G9, G18, and G19 lines.

All the evaluated lines showed a severity level below the susceptible variety of Kencana Bali, which reached 62% (Figure 2); however, some lines in the population obtained a score of 4–5. The evaluated lines had the highest severity level, ranging from 30% to 49%, namely, G10, G16, and G17 (Table 4; Figure 2), with scores between 4 and 5, which indicate moderate susceptibility based on the SES IRRI (Table 4). Situ Patenggung obtained a score of 1. Some lines had the same score as Situ Patenggang, which had a score of 1 and a severity level of <20%, with a scale of 0–2 for resistance criteria, namely, lines of Genotype1, Genotype4, Genotype7, Genotype8, Genotype9, Genotype11, Genotype12, Genotype13, Genotype14, Genotype15, Genotype18, and Genotype19 (Figure 2; Table 4).

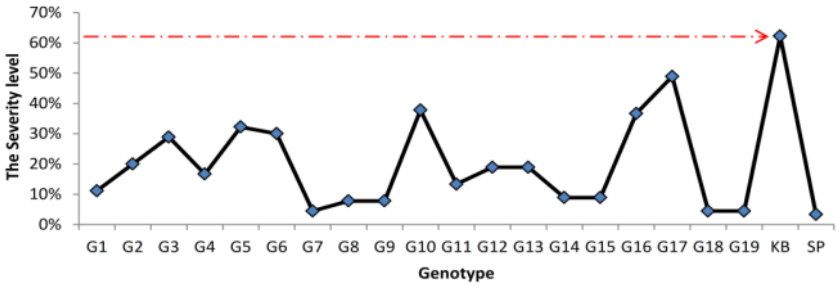


Figure 2. The severity level of blast attacks in the field in 19 genotypes and Kencana Bali as sensitive and Situ Patenggang as resistant check

3. 1. 2. Resistance gene detection through molecular analysis

Identifying the existing blast-resistant genes was performed on 19 inbred lines resulting from single crosses of Bengkulu landrace varieties with six specific primers of blast-resistant genes (Table 4). The results indicated that six primers could detect the existence of the genes Pi-d2, Pi-ta2, Pi-37, Pik, Pik-m, and Pib (Figure 3). The genes Pi37, Pib, Pid2, Pik-m, and Pik were detected in almost 100% of the tested lines expressed at 1149, 388, 1058, 171, and 226 bp, respectively. While the Pi-ta2 gene was detected by 40% in size 1042 bp (Figure 3; Table 5).

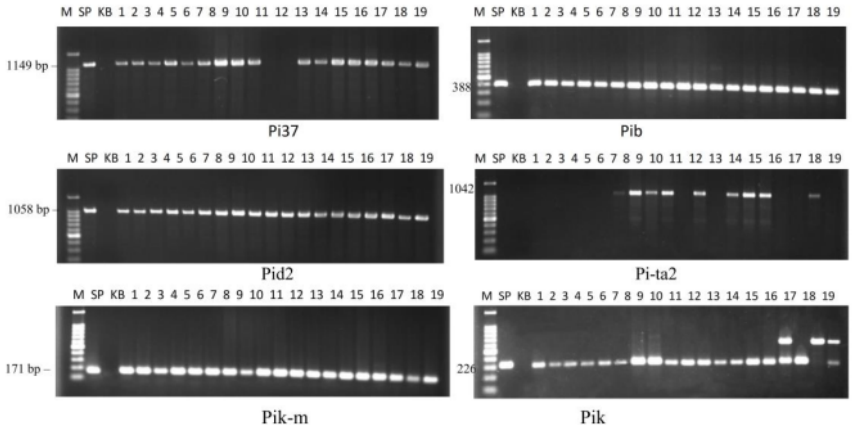


Figure 3. Results of the PCR resistance blast gene amplification using specific primers Pi37, Pib, Pi-d2, Pi-ta2, Pik-m, and Pik (M = Marker 100 bp, SP = Situ Patenggang, KB = Kencana Bali; 1–19 = line number evaluated)

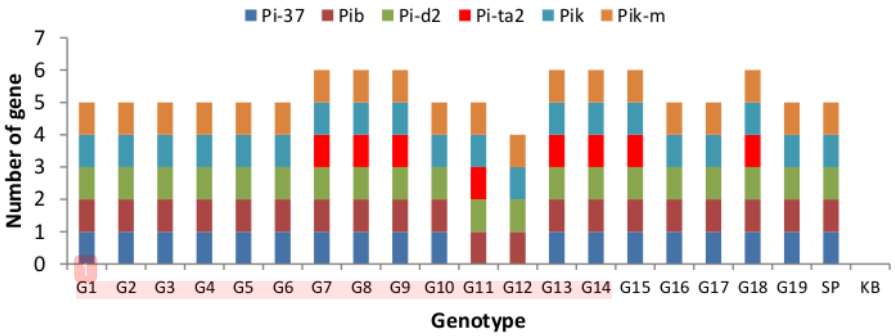


Figure 4. The presence of blast resistant genes in rice lines; red color indicates Pi-ta2 gene which is an atypical protein required for broad-spectrum disease resistance

In the detection *via* PCR, eight lines were found to possess all the blast-resistant genes, namely, Genotype7, Genotype8, Genotype9, Genotype13, Genotype14, Genotype15, and Genotype18 (Figure 4). Situ Patenggang as a resistant check has five genes, while Kencana Bali as a susceptible check does not have any resistance genes at all. The Pi-ta2 gene is an atypical resistance gene encoding a protein required for broad-spectrum blast resistance mediated by the NLR R gene. Several lines were found to carry the Pi-ta2 gene, namely, Genotype7, Genotype8, Genotype9, Genotype11, Genotype13, Genotype14, Genotype15, and Genotype18. Sequencing analysis was conducted to determine the genes encoding the NB-ARC domain of blast-resistant proteins in new upland rice lines to detect broad-spectrum blast resistance.

3.1.3. Sequences analysis of genes encoding the NB-ARC domain of blast-resistant proteins

The Basic Local Alignment Search Tool X (BLASTX) program was used to confirm amino acid sequence homology of this study with amino acid sequences of the NCBI (Table 5). The highest similarity between amino acid sequences for the Pi-ta2 gene detected in eight rice lines was found between the NBS-LRR resistance protein-partial [*Oryza sativa* Indica Group], which was 96%–99% similarity typically encode proteins with NLR domains. Other homologies found were NB-ARC domain containing protein-expressed [*Oryza sativa* Japonica Group] with 96.88% similarity and blast resistance protein Pi-ta variant 12 [*Oryza barthii*], blast resistance protein Pi-ta variant 14 [Oryza nivara], and NBS-LRR-partial [*Oryza sativa* Indica Group] has a similarity of 95.3%, 93.2%, and 81.25%, respectively. A higher similarity indicates a more accurate gene sequence where two DNA fragments can be determined as homologous, in which 70% of the base sequence or 25% of the amino acid sequence are identical (order of at least 100 base pairs in size).

Table 5. Analysis of gene homology of the eight rice lines detected by the Pi-ta2 gene using BLASTX

Genotype	Homology	E-value	Identify	Accession number

	NBS-LRR, partial [Oryza sativa Indica Group]	2E-123	96.91%	CCF78549.1
	NBS-LRR resistance protein, partial [Oryza sativa Indica Group]	2E-119	96.88%	AFH54050.1
	NBS-LRR resistance protein, partial [Oryza sativa]	3E-119	99.90%	AFH58009.1
	NBS-LRR resistance protein, partial [Oryza sativa]	4E-119	99.88%	AFH54039.1
G7	NBS-LRR resistance protein, partial [Oryza sativa Japonica Group]	4E-119	96.88%	AFF54048.1
G8	NBS-LRR resistance protein, partial [Oryza sativa Indica Group]	4E-119	96.88%	AFF54047.1
G9	NB-ARC domain containing protein, expressed [Oryza sativa Japonica Group]	6E-119	96.88%	ABA97435.1
G11	blast resistance protein Pi-ta variant 10 [Oryza sativa Japonica Group]	6E-119	96.88%	ACI49447.1
G13	blast resistance protein Pi-ta variant 5 [Oryza sativa Japonica Group]	7E-119	96.88%	ACI49442.1
G14	Pi-ta protein [Oryza sativa Japonica Group]	8E-119	96.88%	AAK00132.1
G15	NBS-LRR, partial [Oryza sativa Indica Group]	1E-118	96.35%	CCD33216.1
G18	Pi-ta variant KPt.1 [Oryza sativa tropical japonica subgroup]	3E-118	96.88%	ACV87221.1
	blast resistance protein Pi-ta variant 12 [Oryza barthii]	1E-116	95.31%	ACI49449.1
	blast resistance protein Pi-ta variant 14 [Oryza nivara]	2E-114	93.23%	ACI49451.1
	NBS-LRR, partial [Oryza sativa Indica Group]	7E-92	81.25%	CCD21829.1

The expected E-value describes the statistically calculated probability value of the sequence similarity between the NLR domains of rice lines obtained from Gene Bank (www.ncbi.nlm.nih.gov). Phylogenetic analysis applying the neighbor-joining method revealed that the detected rice lines carrying the Pi-ta2 gene formed the same three groups. Figure 5 demonstrates that there were three large clusters, namely cluster I showing 97% similarity in the CCF78549.1 (NBS-LRR, partial [Oryza sativa Indica Group]) assessment with e-value 2E-123. Cluster II has 98% similarity with the accession groups AFH58009.1, CCD33216.1, AFH54048.1, AFH54050.1, AFH54047.1, AFH54039.1, ACV87221.1, and CCD21829.1, while the cluster III has a similarity of 44% with the accession groups ABA97435.1, ACI49447.1, AAK00132.1, ACI49442.1, ACI49449, ACI49451.1. BLAST analysis of the amino acid coding gene for the NB-ARC domain containing protein was significant.

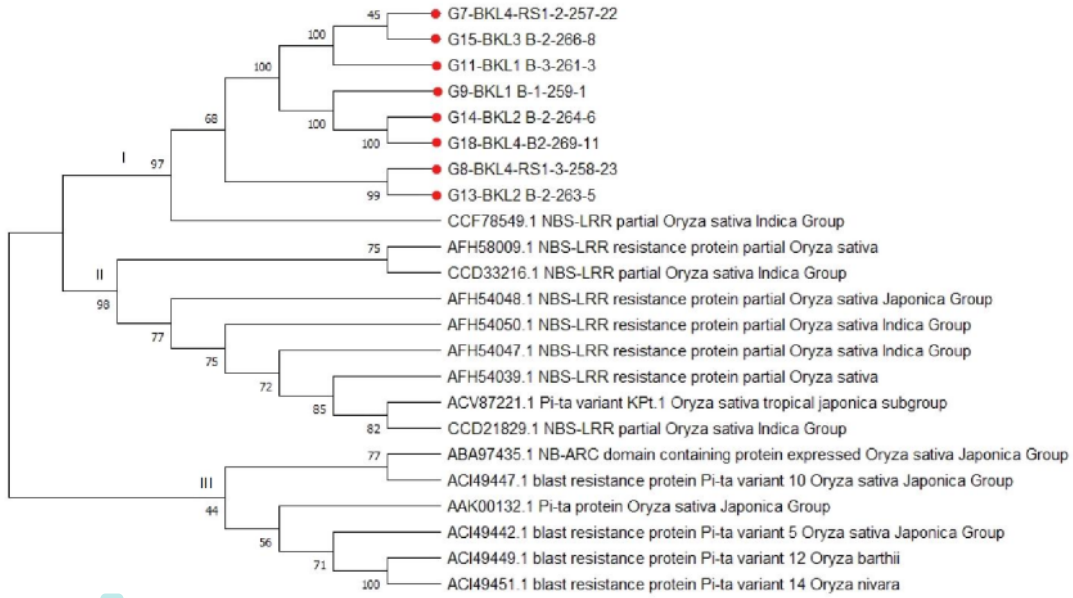


Figure 5. Phylogenetic tree based on the comparison of amino acid sequences from the eight rice lines detected by the Pi-ta2 gene using the neighbor-joining method with 1000× bootstrap replications.

The conserved domain in eight blast-resistant rice lines detected the location of NA-ARC at sequence lengths between 329 and 873 and was very significant at the lowest E-value of 8.45e-11 (Figure 6). The results of the alignment analysis indicated that the query sequences were highly similar to 15 subjects that were homologous to the data in the gene bank (a); the results of the putative conserved domain detected the query gene (G15) containing the NA-ARC superfamily (b); The NBS-LRR protein has been identified as a key defense resistance mechanism in plants (c) (Figure 7).

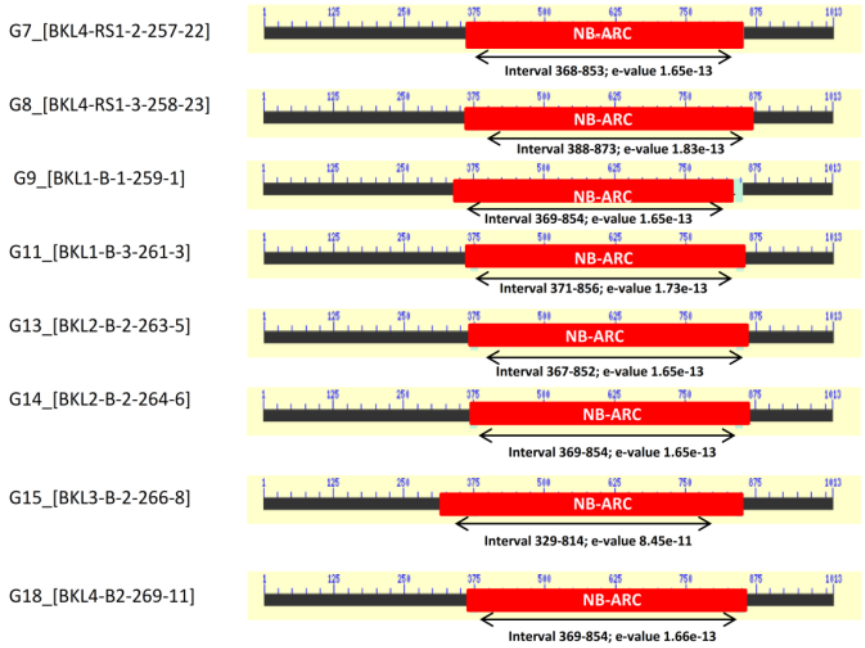


Figure 6. Conserved domain detected amino acid coding gene for the NB-ARC domain containing protein was significant in eight rice lines blast resistance

Figure 7. Query sequence with 15 subjects homologous to the data in the gene bank (a); the results of the putative conserved domain detected by the query gene (G15) containing the NA-ARC superfamily (b); The NBS-LRR protein has been identified as a key defense resistance mechanism in plants (c).

Analysis of the gene domain of the protein-contained NB-ARC was mentioned [*Oryza sativa* Japonica Group] (ABA97435.1) using the CDD program accessed from the NCBI website, disclosing that the enzyme active site was at 478–487 amino acids and the NBS-LRR existed at 722–742 amino acids (Figure 8).

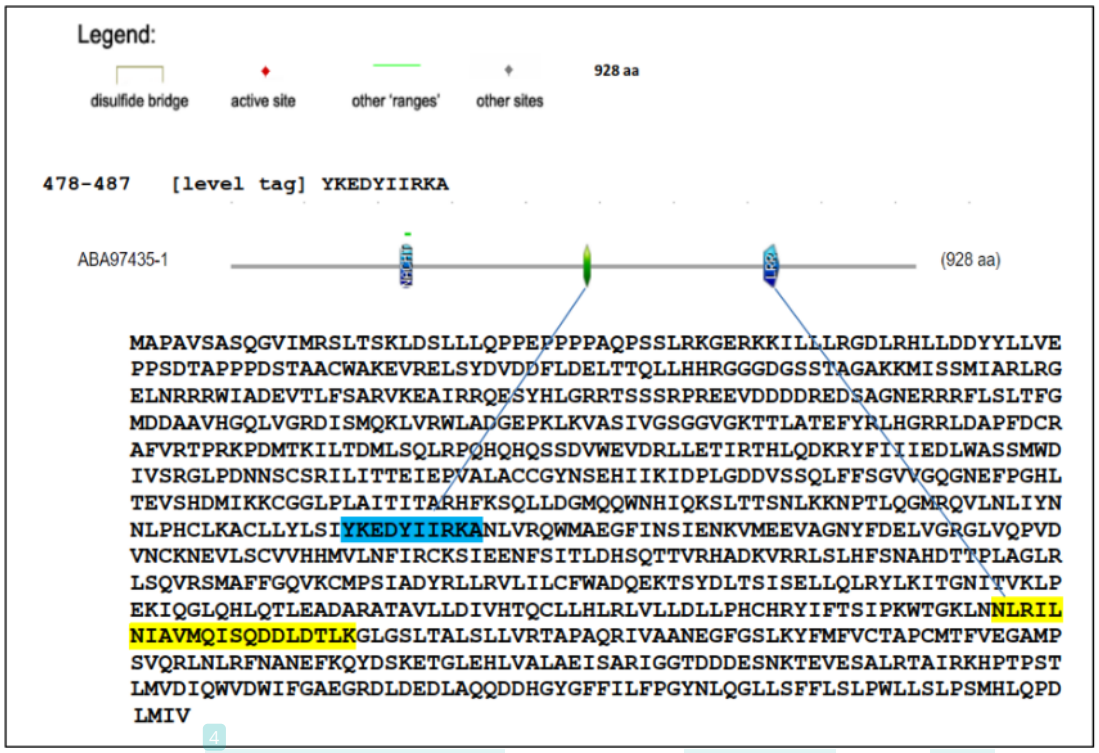


Figure 8. Domain analysis using Scan Prosite; the position of the active site domain of the protein-containing NB-ARC is shown in blue letters; yellow letters are amino acids encoding leucine-rich repeat domain resistance (LRR) genes that are decisive in plant defense responses.

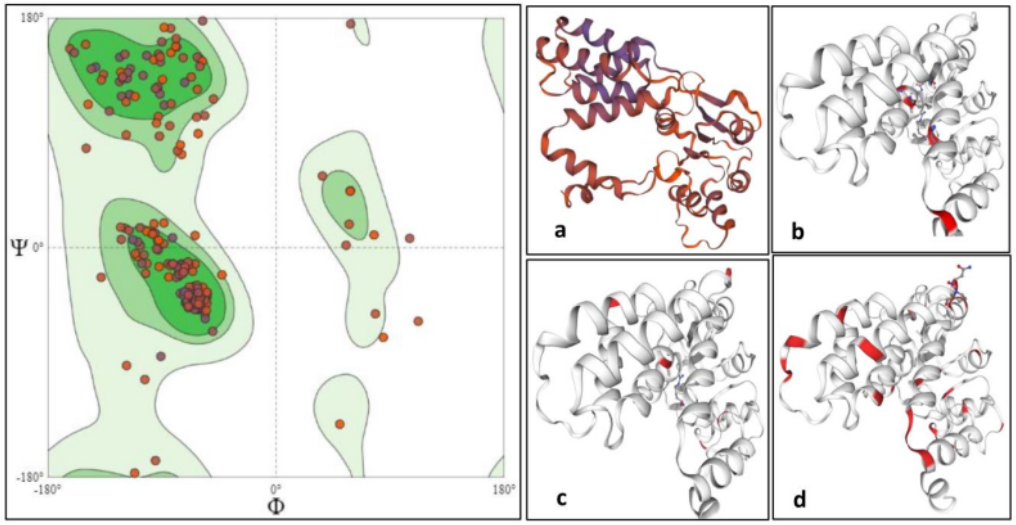
The NBS-LRR protein has been identified as a key defense mechanism in plants. However, this protein use and process has not yet been described in detail. There are many protein sequences whose function has not been confirmed by research. This uncharacterized protein has the potential to be used as a marker in biological research. Homology from the database is an appropriate way to predict the uncharacterized model of protein structure. Modeling the target protein structure using Swiss-Model program. Determination of the template was determined by entering the target amino acid sequence of the G13-BKL2 B-2-263-5 line as a representative model. The template used to build the target protein model was 6j5w.1.B (Disease resistance RPP13-like protein 4 Ligand-triggered allosteric ADP release primes a plant NLR complex). The three-dimensional structure of the protein obtained all 303 amino acid residues (Figure 9 (2)a). The distribution of the residues of the target protein structure using the 6j5w.1.B template in Ramachandran Plots is shown in Figure 9(1). The quality of the protein structure is good or bad depending on the few or many non-glycine residues in the preferred and refused regions in the Ramachandran Plots.

Table 6. MolProbity assessment on the protein structure model of the G13- BKL2 B-2-263-5 line

No.	MolProbity assessment	MolProbity result
1	MolProbity Score	1.78
2	Clash Score	2.29

3	Preferred Area	90.30%
4	Outliers Area	2.24% (b102 pro, b149 asn, b80 cys, b54 arg, b101 ile, b257 asn)
5	Rotamer Outliers	2.04% (b270 ile, b76 arg, b39 asn, b222 leu, b224 ile)
6	C-Beta Deviation	5 (b257 asn, b101 ile, b222 leu, b184 asp, b291 asp)
7	Bad Angles	26/3022 (b257 asn-b258 pro), (b101 ile-b102 pro), b127 phe, b180 asp, b257 asn, b131 glu, b140 asp, b253 asn, b108 phe, (b162 glu-b163 pro), (b274 leu-b275 pro), b82 his, (b202 phe-b203 pro), b276 his, b39 asn, b244 his, b93 his, b230 his, b211 his, (b100 ala-b101 ile), b205 his, b129 ile, b99 his, b175 his, b63 tyr)

The MolProbity assessment showed that the Ramachandran favored value was 90.30%, clash score was 2.29. The MolProbity value of 1.78 describes the resolution of the predicted structure with a clash score of 2.29 (Table 6). The clash score was equivalent in the opposite manner to the percentile value. The structure obtained from the MolProbity value shows that a number of amino acids undergo conformation in small amounts (Table 6).



(1) (2)

Figure 9. (1) Ramachandran Plots of protein G13-BKL2 B-2-263-5 with template 6j5w.1.B; (2) Protein Structure Model (a); Rotamer Outliers (b); C-Beta Deviation (c); and Bad Angles (d). Bad Angles, rotamer outliers, and Cß deviations are indicated by the red areas in the structure.

Rotamer outliers was 2.04% and there were five remains of amino acid in the conceded area. They were referred to as Rotamer Outliers were b270 ile, b76 arg, b39 asn, b222 leu, b224 ile (Figure 9 (2)b). Molprobity showed that there were five regions in the protein structure, including the Cß-deviation region, namely b257 asn, b101 ile, b222 leu, b184 asp, b291 asp (Figure 9 (2)c). The protein structure built based on the analysis of ramachandran plots has 3022 angles and 26 of them fall into the Bad Angles category, namely (b257 asn-b258 pro), (b101 ile-b102 pro), b127 phe, b180 asp, b257 asn, b131 glu, b140 asp, b253 asn, b108 phe, (b162 glu-b163 pro), (b274 leu-b275 pro), b82 his, (b202 phe-b203 pro), b276 his, b39 asn, b244 his, b93 his, b230 his, b211 his , (b100 ala-b101 ile), b205 his, b129 ile, b99 his, b175 his, b63 tyr (Figure 9 (2)d). Bad Angles, rotamer outliers, and Cß deviations are indicated by the red areas in the structure.

3.2. Discussion

The intensity of the effect of blast on the field was not optimal, as indicated by the Kencana Bali variety as a sensitive check at score 6, which has not yet caused plant mortality. The indication of initial blast in the field at the edge of the leaves was observed at 7 WAP, whereas brown spots have appeared at 10 WAP. The blast fungus sporulation in the field depends on the presence of a natural inoculum at the time of assessment, whereas it depends on the weather (Mousanejad et al., 2009; Peng et al., 2019). Weather conditions, degree of heat, and humidity are the main environmental factors influencing fungi virulence, including spore formatted, contaminated, and injured growing process (Mousanejad et al., 2009; Calvero et al., 2010). At the time of the experiment, the air humidity was low because the rate of rainfall was not high. It could be seen from the severity of the field blast attacks at Kencana Bali, which only reached 62%. All lines had a severity level below the Kencana Bali, although G17 reached a severity level of 49%.

In the field assessment conducted in this study, several line numbers had blast attacks that were less than 20% at a scale of 0–2, namely, Genotype1, Genotype4, Genotype7, Genotype8, Genotype9, Genotype11, Genotype12, Genotype13, Genotype14, Genotype15, Genotype18, and Genotype19. The results of the blast-resistant gene detection revealed that the lines had 5–6 resistance genes, that is, Pi-d2, Pi-ta2, Pi-37, Pik, Pik-m, and Pib, which is very consistent in the field blast observations with scores of 0–2. Generally, varieties with single genes only outgrow the emerging lethal races (Bonman et al., 1992; Koide et al., 2013). The several major resistance genes in plants will confer broad-spectrum resistance for a long duration (Xiao et al., 2015; Xiao et al., 2016; Zhao et al., 2018; Orasen et al., 2020). The results of this study also indicated that plants have different resistances, confirming that the ability of plants to overcome blast attacks is also different. Molecular detection *via* PCR revealed that not all tested lines had the Pi-ta2 gene, which is very consistent with the field blast observations, where the lines detected by the Pi-ta gene had a severity level of less than 10% and scores of 0–2, namely, Genotype7, Genotype8, Genotype9, Genotype11, Genotype13, Genotype14, Genotype15, and Genotype18. Resistance genes like Pi1, Pi5, Piz-5, Pita, and Pi-gm were improved to be the best plant varieties using marker assisted selection (Sharma et al., 2012; Deng et al., 2017).

Therefore, further studies were conducted by sequencing the eight lines to determine the expression of genes encoding the NB-ARC domain of blast-resistant proteins. Conserved domain analysis revealed that eight blast-resistant rice lines were found to encode NB-ARC at sequence lengths between 329 to 873 and very significant at the lowest E-value of 8.45e−11. The BLASTX program was used to determine amino acid sequence homology obtained from this study with

amino acid sequences derived from NCBI. Sequence analysis on BLASTX revealed that there were 15 gene homologies of the eight rice lines detected by the Pi-ta2 gene. The highest similarity between amino acid sequences for the Pi-ta2 gene detected in eight rice lines was observed between the NBS-LRR resistance protein-partial [*Oryza sativa* Indica Group], with a similarity level of 81%–99%. The result is consistent with the phylogenetic tree, which demonstrates that the groups of homologous genes were closely related to the sequences of eight lines. In addition, the Pi-ta protein [*Oryza sativa* Japonica Group], blast resistance protein Pi-ta variant 5 [*Oryza sativa* Japonica Group], and NBS-LRR partial [*Oryza sativa* Indica Group] have the lowest E values, which are between 7e−119 and 8e−119. The BLAST analysis for the amino acid coding gene for the NB-ARC domain containing protein was significant. The E-value of the BLAST analysis was considered to be significant if the in-between value was 1×10^{-10} or lower (Altschul et al., 1990).

The phylogeny also demonstrated that the sequences of eight lines had the three cluster. This study also revealed that the alignment analysis of the query sequences had a very high similarity with 15 subjects that were homologous to the data in the gene bank. The putative conserved domain detected the query gene (G15) containing the NA-ARC superfamily. Furthermore, Furthermore, the NBS-LRR resistance gene has been expressed in the largest group of plant resistance genes as a key role in plant defense responses. The immune system in plants occurs through the role of encoded proteins, it is revealed these genes are conserved better than others. Plants encode resistance proteins (R) through pathogen gene-to-gene recognition by inducing defense hypersensitivity responses to protect against pathogen infection (Gururani et al., 2012). The putative central NBS and a carboxy-terminal LRR are protein codes for rice blast-resistant genes. These NBS-LRR protein consists of two main levels: the first level has a homologous and identical N-terminal domain to the mammalian Toll-interleukin-1-receptor (TIR) domain, while the second level encodes amino-terminal coiled-coil motif (CC-NBS-LRR) (DeYoung and Innes, 2006; Ma et al., 2015). The immunity is triggered by interactions with fungal effectors, either directly or indirectly, encoding the nod-like receptor (NLR) protein family as the role of the blast R gene (Zhao et al. 2018; Wang et al., 2020).

The analysis of the gene domain of the NB-ARC using the CDD program accessible on the NCBI website, showed that the active site of the enzyme was in the 478-487 amino acid sequence and the NBS-LRR is present at 722–742 amino acids. R protein is between APAF-1 and CED-4 on the plant-conserved ARC domain. Nucleotide bond formation with P-loop and Walker motifs, ARC1 contains a four helical bundle and ARC2 has a winged helical fold together constructing three subdomains of the NB-ARC domain (Leipe et al., 2004; Takken et al., 2006). Signal initiation is triggered by the ARC domain which translates the modulated elicitor from terminal C (Rairdan and Moffett, 2006). The NB-ARC domain is a type of STAND protein (multi-domain signal transduction ATPase) lead to cell death (Leipe et al., 2004; Danot et al., 2009; Wang et al. 2020).

This study used a target protein model of the G13-BKL2 B-2-263-5 line. The stability of the protein model structure was obtained using the MolProbity Result in the Ramachandran Plot analysis that the non-glycine residues in the outlier area were only around 2.24% (b102 pro, b149 asn, b80 cys, b54 arg, b101 ile, b257 asn) and the number of amino acids remains in the favored region was 90.30%. The MolProbity value obtained was 1.78, which describes the resolution of the predicted structure with a clash score of 2.29. The class chore value is inversely equivalent to the percentile value. The higher the value on the clash score is, the lower the percentile value will be. The structure obtained from the MolProbity value showed that several amino acids undergo conformation in small numbers. Evaluation of the value of rotamer outliers on the structure explains that the allowed area has a category of 0.3 % or 2.0%. This category makes it possible to

determine the conformation of a number of amino acids at the edges of the rotamer distribution, close to the outliers region (Hintze et al., 2016). Analysis of the model in the Ramachandran plots explains that the Cβ deviation sensitive to incompatibility between the side chain and the back chain is caused by the conformation of a number of mismatched amino acids (Lovell et al., 2003; Sharma et al., 2018; Chandrakanth et al., 2020). The structure of the model has a difference with the structure of the template when you see some angular shapes that were built, the difference is shown by the red color in the image. The target structure obtained was close to the actual model, this is influenced by the percentage of similarity between the target and the template. The three-dimensional structure of the target structural protein indicates a steadfast quality of the protein structure. The stability of the structure of the target protein obtained can be used as a basis for studying the development of cloning designs for blast resistance genes in rice.

We confirmed the presence of the gene which encoded the NB-ARC domain of blast resistance proteins in eight new rice lines from the breeding program through the development of landrace varieties. The study also revealed that these lines was also detected to have polygenic genes with broad-spectrum blast resistance potential. Further studies on the performance and resistance of lines in local blast race isolates and field trials at various endemic blast will also provide valuable information as candidates for new rice varieties.

4. Conclusion

In this study, eight rice accessions were found to have complete resistance to blast based on the field evaluation. The analysis of molecular showed that the line has numerous genes, where the Pi-ta2 gene is the target of the DNA sequencing for the Analysis of Genes Encoding the NB-ARC domain of blast resistance proteins at sequence lengths between 329 to 873. Protein structural studies confirmed the predicted structural similarity between the target protein model was 6j5w.1.B (Disease resistance RPP13-like protein 4 Ligand-triggered allosteric ADP release primes a plant NLR complex). This study proved that resistance gene introgression in landrace parents could be a source of blast-resistant genes to develop new varieties. The eight lines, namely, Genotype7, Genotype8, Genotype9, Genotype11, Genotype13, Genotype15, and Genotype18, analyzed in this study had polygenic resistance and potential as candidate genes to overcome blast pathogens that have multiple races and are dynamic. Comprehensive further study should be performed to confirm the performance and resistance of lines in field trials at various endemic blasts before being released as candidates for new rice varieties.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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