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Marker-assisted Breeding and F_3 Progenies Characterization for Improving Local Rice Variety "Tinggong"

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ABSTRACT

Background: Tinggong is an indigenous rice variety in Aceh Province known for its excellent cooking quality and tolerance to adverse climatic conditions such as drought. However, this local rice has a long lifespan ranging from 150-160 days and is susceptible to *Xanthomonas oryzae* pv. oryzae (*Xoo*) causes bacterial leaf blight (BLB).

Methods: To improve the performance of Tinggong, the variety was cross-breed with the isogenic line IRBB27 which confers *Xa-27* and *sd-1* genes. The inheritance of both of these genes and the changes in their agronomic character and the resistance to *Xoo* were analyzed in the F_3 progenies to identify the presence of both *Xa-27* and *sd-1* genes in the F_3 progenies and the implication of their phenotypic characters.

Result: The results showed that 58.33% of progenies inherited both *Xa-27* and *sd-1* genes. Of these, 4 progenies consisting #T5, T6, T29 and T30 are the potential to develop as they show both genotypic and phenotypic improvement. The 4 progenies produce earlier (116-120 days after sowing) with plant height 109-128 cm and more resistance to *Xoo* (lesion length 2.08-2.42 cm), the weight of grains per hill reached 26.24-34.12 g or yield potential (10.40-43.55%) higher compared to their parent Tinggong.

Key words: Bacterial Leaf blight, Indigenous rice variety, Molecular analysis, Oryzae, *sd-1*, *Xa-27*, *Xanthomonas oryzaepv*.

INTRODUCTION

Tinggong is a rice variety cultivated by farmers in the southwest region of Aceh, Indonesia. The variety is adaptive to environmental conditions and has excellent cooking quality. Despite its advantages, characteristics of the plant need to be improved as its height reaches 143-180 cm, harvesting age 5-6 months and susceptibility to BLB disease caused by *Xanthomonas oryzae* pv. oryzae (*Xoo*) (Zakaria *et al*., 2021). Rice plants with a high stem architecture are not capable of supporting the weight of the grains, potentially causing the plants to fall and eventually yield losses. This circumstance is made worse by the occurrence of infectious diseases such as BLB that occurs in all major rice-growing areas in the world, especially in Asia, Northern Australia, Africa and the United States of America (Adhikari *et al*., 1995; Gnanamanickam *et al*., 1999; Sere *et al*., 2005). The disease causes yield losses of up to 70% and is a serious threat to agriculture and food security worldwide (Ke *et al*., 2019; Singh *et al.,* 2009; Verdier *et al*., 2012).

Crossing indigenous rice with introduced types that bestow valuable genes might allow for the enhancement of rice varieties. IRBB27 is an introduced isogenic line that carries both *Xa-27* and *sd-1* genes. *Xa-27* is an important R gene, effective against BLB disease while the *sd-1* gene improves plant architecture (Luo *et al*., 2014; Makino *et al*., 2006; Radhamani *et al*., 2015). The utilization of the resistance gene (R) for rice improvement is the most efficient method for controlling BLB disease (Ikeda *et al.,* 1990; Pradhan *et al.,* 2015). In addition, the *Xa-27* locus provided high resistance to 27 *Xoo* strains (Dossa *et al*., 2015). There are six R-genes including*Xa-1*, *Xa-5*, *Xa-13*, *Xa-21*, *Xa-26* and *Xa-27* that have been obtained through genetic map-based cloning (Luo *et al*., 2012). In Indonesia, there are 11 strains of *Xoo* bacteria with different virulence levels. Two *Xoo* strains that have a high virulence level are strain IV which has a high virulence level in the generative phase and strain VIII which is virulent in the vegetative phase and is dominant in several endemic locations (Suparyono *et al*., 2016).

Pathotype IV and pathotype V were Two representative strains of Xoo pathotypes prevalent in South China (Zeng *et al*., 2002). Specifically, pathotype V and IV can overcome the resistance conferred by *Xa4* and *Xa21* (Zeng *et al*., 2002; Zhang, 2009), respectively. Moreover, two strains of *Xoo* from Indonesia were virulent to cultivars containing the bacterial blight resistance gene *Xa5*. In addition, the varieties of containing *Xa21* and *Xa5* genes were resistant to almost Asian Xoo strains. Indonesian rice cultivar Pandan Wangi"

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was susceptible to Xoo strain III and VIII and very susceptible to strain IV (Hadiwiyono *et al.*, 2021).

The use of the *sd-1* gene results in a short plant phenotype and improves plant architecture, increasing plants' ability to assimilate substances into seeds (Vikram *et al.*, 2016). Luo *et al.* (2014) employed *the sd-1*gene to improve other Aceh local rice, called Siputeh, to shorten the harvesting age from 5.5 months to 3.5 months.

We have studied crossbreed between Tinggong and IRBB27 (Tg/IRBB27) in F_2 and learned that 73.91% of plants with 115 cm height, 27.27% inherited resistance to *Xoo* strain IV. From these progenies, 21.73% of plants inherited both *sd-1* and *Xa-27* genes. Only 20% of these progenies have the potential to be developed as they can be harvested between 110-122 days after sowing and seed weight per hill up to 32 g or an estimated yield of 8 tons ha^{-1}.

The purpose of this study was to analyse the variation in genotypic and phenotypic levels of the characters F_3 progenies from cross-breeding between Tinggong and IRBB27. Through this research, it is hoped that prospective progenies can be obtained, those that inherit both the Xa-27 gene and the sd-1 gene and have a phenotypic character resistant to BLB and better agronomic characteristics compared to their parent 'Tinggong'.

MATERIALS AND METHODS

Research implementation and genetical resources

The research was carried out in the experimental farm and Plant Breeding Laboratory, Faculty of Agriculture, Universitas Syiah Kuala, Darussalam Banda Aceh from March to July 2018. This study utilized 48 plants of F_3 progeny of the cross between Tg/IRBB27 as planting material. The plants were selected from F_2 Tg/IRBB27 that inherited *sd-1* and *Xa-27* as well as showed good agronomic characters (harvesting time 110-120 DAS, plant heigh 125-135 cm with production per hill 43.55 g (estimation yield 8 ton ha^{-1}).

Plants cultivation technique

The seeds were soaked for 24 hours for the imbibition, then germinated on straw paper for 48 hours. The germinated seeds were then sown on the sowing media consisting of soil and manure mixed with a ratio of 2:1. The seeds were transplanted after 15 days to a 5 kg pot containing soil and manure in a ratio of 3:1 (v/v). As much as 2.6 g pot^{-1} of basic fertilizer, consisting of nitrogen, phosphorus and potassium (15-15-15) and 0.6 g pot^{-1} urea were applied one day before planting. At the age of 15, 30 and 45 days after planting, urea was applied as top dressing at a dose of 0.6 g pot^{-1}.

Bacterial inoculation and disease scoring

The bacterial suspension of *Xoo* used in this study is Pathotype IV obtained from the Muara Rice Research Centre, Bogor, Indonesia. Reisolation was carried out at the Plant Diseases Laboratory, Faculty of Agriculture, Universitas Syiah Kuala. The bacterial suspension was grown on Natrium Agar (NA) media at 28°C for two days. The composition of the media was peptone 10 $g.L^{-1}$, sucrose 10 $g.L^{-1}$, glutamic acid 1 $g.L^{-1}$, bakto-agar 16 $g.L^{-1}$ and pH 7.0. Bacterial cells were mixed in distilled water at an optical density of 0.5 (OD_{600}) (Luo *et al.*, 2012). Inoculation of *Xoo* was carried out 14 days after planting (DAP) by applying the leaf-clipping method (Kauffman, 1973).The tip of the leaves was cut (starting from the 3rd leaf in each clump of 5 leaves) with scissors that have been dipped in *Xoo* suspension. Inoculation was carried out in the afternoon to avoid scorching heat and high evaporation.

The length of the lesion was observed 3 weeks after inoculation. The length of the lesion was measured from the tip of the cut leaf to the base of the leaf indicating *Xoo* infection. Disease scoring was measured as described by Gu *et al.* (2004).

Molecular analysis of *sd-1* and *Xa-27* gene presence in F_3 progenies

DNA was extracted from 0.2 g young leaves of each sample of rice plants at the age of 14 DAP. The leaves were cut into small pieces and placed into a 2 ml microtube containing one stainless steel bead. The samples were set in a microtube box and stored at -86°C for 3-5 hours. After the sample was crushed by shaking the tube until the leaf sample was crushed, each micro centrifugation tube contained the sample was filled with 300 µl of TPE buffer. The samples were then incubated in a water bath at a temperature of 65°C for 20 minutes. After incubation, the samples were centrifuged at 13,000 gravity for 10 minutes. 100 µl of supernatant DNA was taken and transferred into a fresh 1.5 ml microtube. The PCR template was constructed by mixing 1% DNA supernatant with distilled water that has been prepared in a 200 µl microtube (Koeda and Fujiwara, 2019).

PCR-based molecular markers and PCR conditions

The molecular marker for gene *Xa-27* is the codominant SSR marker M964, located at 0.964 kb (F: 5-TGT GCA ATG CAG GAT TTC AGT TACT-3; R: 5-TTT CAC CTG CAT AAT GCA AAA GCT AA -3) (Gu *et al.*, 2004). The molecular marker for the *sd-1* locus is a co-dominant STS (sequence-tagged site) marker derived from the *sd-1* gene. (F: 5-CAC GCA CGG GTT CTT CCA GGT G-3 ; R: 5-AGG AGA ATA GGA GAT GGT TTA CC-3) (Spielmeyer *et al.*, 2002; Srivastava *et al.*, 2019).

PCR amplification (My Cycler™ thermal cycler) for *the Xa-27* gene was performed according to (Gu *et al.*, 2004) with initial denaturation of DNA at 94°C for 2 minutes. The next denaturation was 35 cycles for PCR amplification, consisting of 94°C for 1 minute, 37.0°C for 45 seconds and 72°C for 1 minute and 30 seconds. Final extension at 72°C for 5 minutes. Whereas PCR amplification for the *sd-1* gene was conducted with initial denaturation of DNA at 94°C for 5 minutes. The subsequent denaturation was 35 cycles for PCR amplification, consisting of 94°C for 1 minute, 55°C

for 1 minute and 72°C for 2 minutes. Final extension at 72°C for 7 minutes (Spielmeyer *et al*., 2002; Srivastava *et al*., 2019). The amplified product was resolved on 1.5% agarose in 0.5xTAE buffer. For identification of amplification of *Xa-27* gene, initially5 µl of PCR product was used for gel electrophoresis. Electrophoresis was performed for 35 minutes for primary *Xa-27* and 30 minutes for primary *sd-1*. The gel was visualized under ultraviolet light using UV Transilluminator.

Agronomic character analysis

The variables used for agronomic character analysis consist of harvesting time, plant height at harvesting time, the weight of 1,000 grains, the weight of grain per hill and production estimation per ha based on the population of 250,000 hills ha^{-1}.

RESULTS AND DISCUSSION

Molecular analysis of *sd-1* and *Xa-27* genes presence in F_3 progenies of Tg/IRBB27

The performance of F_3 individual progenies based on the presence of *Xa-27* and *sd-1* genes by molecular analysis for 48 individual plants is shown in Fig 1.

Molecular analysis showed that 48 plants of F_3 progenies analysed for the presence of the *Xa-27* gene, 30 plants (62.50%) inherited the *Xa-27* gene through DNA band formation at 0.96 kb. Meanwhile, based on the analysis of the presence of the *sd-1* gene based on the co-dominant *sd-1* STS marker, it showed that of the 48 plants analysed only 32 plants (66.67%) inherited the *sd-1* gene through the formation of DNA bands at 0.85 kb. Fig 1 also shows that 48 individual plants derived from Tg/IRBB27, there were only 28 (58.33%) plants that inherited both the *Xa-27* gene and the *sd-1* gene.

The resistance of Tinggong/IRBB27 to *X. oryzae* and their agronomic characters

The symptom of BLB indicated by lesion length on the leaves of 48 F_3 progenies is shown in Table 1. The table showed that among all of the progenies 14 plants showed resistant reactions (29.17%) including #T5-8, T13-16, T18, T27, T29-30, T43 and T46. The table also showed that 19 plants (39.58%) showed moderately resistant reaction (MR), 4 plants (8.33%) showed moderately susceptible reaction (MS) and 11 Plant (22.91%) showed susceptible reaction (S) at the third week after inoculation. The resistance of the F_3 progenies genotype tested with *Xoo* strains IV after inoculation shows a positive response when viewed from the level of lesion length caused by *Xoo* disease inoculation (Fig 2). Plant resistance to BLB symptoms was evaluated quantitatively, such as disease index, lesion area, or lesion length (Bhattacharjee *et al*., 2020; Jerish *et al*., 2022; Ogawa, 1993; Sharma *et al*., 2020).

BLB intensity is highly dependent on the virulence level of *X. oryzae*, environmental conditions and climatic conditions in the area. Environmental conditions with lots of weeds and high humidity can stimulate the development of virulent *Xanthomonas*. High humidity conditions are usually often found in plants with close spacing with continuous watering conditions (Chen *et al*., 2020; Jiang *et al*., 2020).The mechanism of virulent bacteria in plants is that

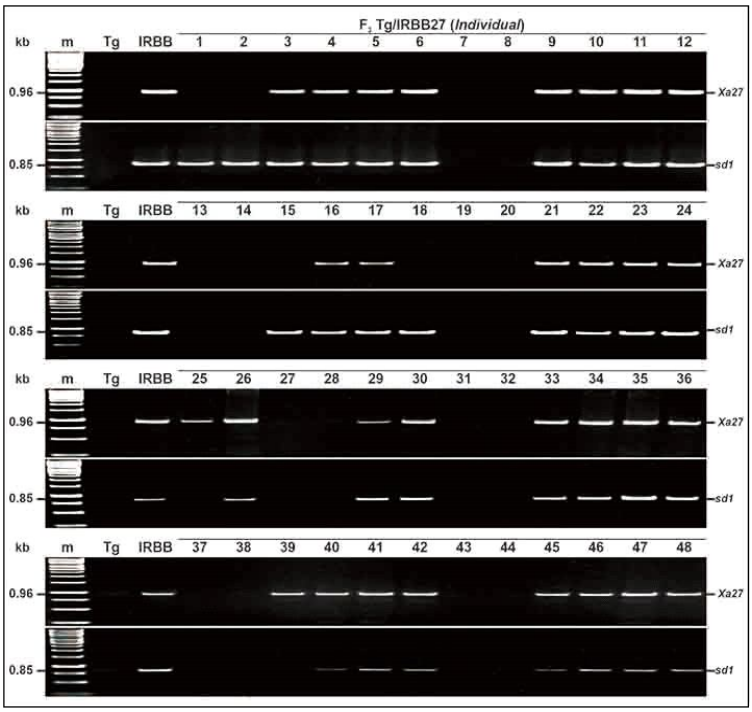

Fig 1: Genetic analysis of *Xa-27* dan*sd-1* gene from Tinggong, IRBB27 and F_3 Progenis Tg/IRBB27.

Table 1: Disease reaction of paternal lines and F_3 Progenis Tg/IRBB27 to different Xoo strains IV.

Line		Xoo strain IV	Harvesting time (DAS)	Plant height (cm)	Productive panicles per plant	Panicle length (cm)	1000-grain weight	Weight of filled grains per plant (g)	Estimated yield (t/ha)
Tinggong		5.27±0.98 MR	163	143.0	13.2	23.7	16.9	23.8	5.94
IRBB27		2.11±0.18 R	110	86.7	8.2	19.7	22.3	22.1	5.53
F_3 Tg/IRBB27	1	3.56±0.88 MR	104	114	7	22.10	22.0	13.42	3.36
	2	4.48±1.56 MR	104	113	6	25.30	23.7	27.27	6.82
	3	5.96±2.24 MR	109	112	8	23.30	22.6	28.23	7.06
	4	7.08±3.30 MS	118	119	9	24.80	20.7	31.23	7.81
	5	2.25±0.64 R	116	116	8	19.50	23.8	26.85	6.71
	6	2.25±0.64 R	120	128	7	23.60	20.9	28.15	7.04
	7	2.86±1.09 R	109	101	7	22.30	17.0	9.62	2.41
	8	2.04±0.32 R	118	103	9	21.30	18.6	8.48	2.12
	9	17.16±5.66 S	116	127	7	25.00	19.9	13.50	3.38
	10	12.04±3.94 S	109	116	6	22.60	19.5	10.26	2.57
	11	4.16±0.93 MR	112	119	9	16.90	22.4	15.27	3.82
	12	4.38±1.51 MR	117	121	8	22.90	21.7	14.07	3.52
	13	1.44±0.49 R	105	106	5	22.30	21.8	11.57	2.89
	14	2.48±0.46 R	109	99	7	21.50	19.6	15.97	3.99
	15	2.76±0.34 R	105	105	11	21.04	21.8	38.78	9.70
	16	1.32±0.42 R	105	94	6	19.08	20.6	9.87	2.47
	17	4.04±1.24 MR	105	94	12	20.40	22.1	38.54	9.64
	18	1.68±0.41 R	105	103	6	19.30	23.0	17.20	4.30
	19	15.36±6.16 S	116	128	8	24.30	25.4	29.19	7.30
	20	9.18±4.06 S	118	110	8	23.50	21.8	17.22	4.31
	21	6.50±2.94 MS	109	119	10	23.90	23.1	37.12	9.28
	22	5.03±1.78 MR	126	117	6	28.00	19.9	11.37	2.84
	23	4.82±1.35 MR	116	134	6	20.80	24.9	14.94	3.74
	24	5.16±0.75 MR	129	128	7	27.50	20.3	29.25	7.31
	25	15.16±8.13 S	118	97	5	23.50	19.1	5.42	1.36
	26	5.46±0.80 MR	119	121	10	25.60	20.3	18.00	4.50
	27	2.04±0.05 R	116	126	5	23.20	20.6	12.59	3.15
	28	12.90±4.49 S	118	119	10	26.00	20.8	16.63	4.16
	29	2.08±0.34 R	116	109	9	24.30	22.2	34.12	8.53
	30	2.42±1.43 R	116	111	8	24.70	22.2	26.24	6.56
	31	6.53±0.17 MS	126	124	8	22.70	16.4	12.96	3.24
	32	5.00±0.56 MR	118	125	10	25.50	19.1	24.02	6.01
	33	4.76±0.57 MR	123	117	10	20.70	18.5	19.62	4.91
	34	5.68±0.82 MR	123	133	9	26.40	18.5	19.62	4.91
	35	4.44±1.77 MR	117	124	9	21.10	20.5	26.03	6.51
	36	5.10±2.14 MR	117	127	8	23.30	19.9	25.24	6.31
	37	6.32±2.79 MS	105	120	11	23.30	26.1	25.97	6.49
	38	9.58±1.92 S	109	119	11	24.10	22.8	26.87	6.72
	39	10.83±3.15 S	112	125	10	20.60	25.5	33.18	8.30
	40	10.12±3.10 S	118	115	8	26.06	24.8	22.96	5.74
	41	14.96±3.77 S	116	111	12	22.10	24.4	37.41	9.35
	42	11.42±3.75 S	118	107	7	22.10	24.1	19.64	4.91
	43	2.08±0.42 R	108	109	8	23.00	20.2	21.30	5.33
	44	5.56±0.26 MR	122	116	9	26.40	19.2	33.18	8.30
	45	5.42±1.86 MR	125	135	10	24.40	17.0	13.66	3.42
	46	2.47±0.26 R	128	90	6	20.60	16.7	1.81	0.45
	47	5.62±0.41 MR	118	111	9	23.02	21.1	19.95	4.99
	48	5.54±0.95 MR	104	123	8	18.30	22.2	23.05	5.76

[a]R, resistant (Lesion length =3.0 cm); MR, moderately resistant (3.0 cm < Lesion length =6.0 cm); MS, moderately susceptible (6.0 cm < Lesion length =9.0 cm); S, susceptible (Lesion length >9.0 cm).

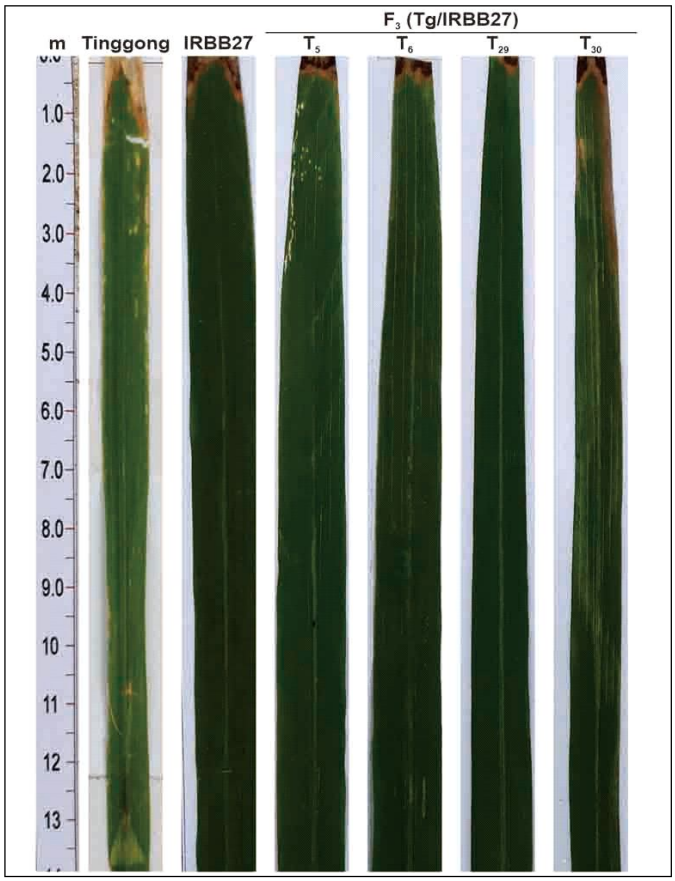

Fig 2: Evaluation of rice lines for disease resistance to Xoo strains IV. The images of inoculated leaves were taken 21 days after inoculation.

bacterial cells enter plant tissues through pores or leaf stomata. After entering the plant tissue, the bacteria then multiply or grow, then attack the vascular system of the plant. The liquid containing bacteria eventually comes out onto the leaf surface, which then forms a lesion.

Moreover, Table 1 also showed that the whole F_3 pprogenies had harvesting time from 104-129 DAS with plants height ranging from 90-135 cm or their harvesting time and plant height were 36.07% faster and 20.70% shorter compared to their female parents Tinggong respectively. The table also showed that the whole progenies had productive panicles ranging from 5-12, with panicle lengths ranging from 16.90-28.00 cm. The 1,000 grains weight and the weight of filled grains per hill of the progenies ranged from 1.81-38.78 and 26.24-34.12 respectively.

The age of the rice plant is the main factor for farmers in the selection of rice varieties to be cultivated. Rice plant age criteria based on Committee Standards (1980) early 110-125 DAS, moderate 126-145 and deep >145 DAS. In addition to improving plant architecture, the *sd-1* gene can indirectly shorten the lifespan of rice plants. The *sd-1* gene has been used by (Devi *et al.*, 2019; Luo *et al.*, 2014) to improve the architecture of rice plants and the lifespan of TS4 with its harvesting time and plant height shorter compare to its parent Siputeh.

CONCLUSION

Research results showed that 62.50% of F_3 progenies inherited the *Xa-27* gene, 66.67% inherited the *sd-1* gene and 58.33% inherited both the *Xa-27* and *sd-1* genes. Based on the existing both of these genes, resistance to strain IV of BLB diseases and agronomic performance, there were four F_3 progenies consisting #T5, T6, T29 and T30 plants were prospective to be developed further based on the criteria for early maturing age ranging from 116-120 DAS, short plant height of 109-128 cm and estimated yield per ha of 6.56-8.53 tons ha^{-1}.

Conflict of interest: None.

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