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RESEARCH ARTICLE **Open Access**

Selection of Rice (*Oryza sativa* L.) T1 for Sweet14 Gene Resistance to Bacterial Leaf Blight Disease

M. Dzaky Syadenta[1], Sri Utami[1,*], Fatin Fathirah[2], Noor Shahira[2], Zulkifli Ahmad Seman[2]

Abstract

Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. oryzae (Xoo), is a major rice disease responsible for significant yield losses in Asia. One effective strategy to control this disease is the development of genetically resistant rice varieties. The xa5 mutant allele, an altered form of the TFIIAγ5 gene, is known to confer broad resistance to BLB. This study aimed to screen T1 generations of rice edited at the TFIIAγ5 gene and assess their resistance to BLB. PCR analysis of the T1 generation revealed the presence of Cas9 gene sequences in some edited lines, indicating inheritance of the transgene from the T0 generation. DNA sequencing of the target site confirmed the presence of small mutations that may alter the function of the TFIIAγ5 gene, rendering it resistant. Disease inoculation assays showed that some T1 lines had significantly shorter lesions than wild-type controls, with lesion length reductions of up to 70%. These results indicate that TFIIAγ5 editing successfully mimics the function of the xa5 allele, conferring resistance to BLB. In conclusion, editing the TFIIAγ5 gene using the CRISPR/Cas9 system is an effective approach to producing BLB-resistant rice and contributes to the development of new, more sustainable, and environmentally friendly varieties.

Keywords: CRISPR/Cas9, *Oryza sativa,* TFIIAγ5, xa5, *Xanthomonas oryzae* pv. oryzae

1. Introduction

Food is a basic human need, and sufficient, nutritious, and safe food can produce high-quality human resources. Grain (rice) is the main source of energy (Alridiwirsah *et al*, 2021). Rice (Oryza sativa L.) is widely grown in Asia, Latin America, and Africa and is a staple food for more than 50% of the world's population. Nearly 90% of rice cultivation in Asia, including China, India, and Pakistan, accounts for 30, 21, and 18%, respectively, while the remaining 30% is contributed by Thailand, Indonesia, Myanmar, and Japan (Barus *et al*., 2023). Several factors, including the conversion of agricultural land to development, global warming, and pest and disease attacks, are causing a decline in rice production in Indonesia. Pest and disease attacks are among the main causes of the decline in rice production, as various plant pest species (OPT) attack rice plants. One of the main diseases in rice plants is HDB (Bacterial Leaf Blight). This disease is caused by the gram-negative bacteria *Xanthomonas oryzae pv. oryzae (Laraswati et al* ., 2023) .

Bacterial leaf blight (BLB) is a major disease affecting rice in rice-producing countries. This disease is caused by the bacterium Xanthomonas oryzae pv. oryzae (Xoo). In Indonesia, crop losses due to BLB infestation can reach 21–36% during the rainy season and 18–28% during the dry season. In 2006, the area affected by BLB exceeded 74,000 hectares, with 16 hectares experiencing complete crop failure. The tropical climate also contributes to the high diversity of pathogen strains found in these regions (Hakim *et al*., 2022).

Xoc and Xoo are closely related bacterial species that infect rice differently. Xoo enters the leaf through hydathodes or wounds, multiplies in the intercellular spaces of the underlying epithelium, and spreads until it reaches the xylem vessels. The bacteria travel through the leaf veins and spread throughout the plant. Water-soaked spots are first observed on the leaf tips and margins. Later, the leaves become chlorotic and necrotic along the veins. (Jiang *et al* ., 2020) . In pathogen-host interactions, *Xoo bacteria* utilize *Transcription Activator-Like Effectors* (TALEs) proteins to

*Correspondence: sriutami@umsu.ac.id
1) Universitas Muhammadiyah Sumatera Utara - Jl. Kapten Muchtar Basri No.3, Glugur Darat II, Kec. Medan Timur, Kota Medan, Sumatera Utara 20238, Indonesia
2) Malaysian Agricultural Research and Development Institute (MARDI) - Ibu Pejabat MARDI, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia

activate susceptibility genes *in* host plants. One important mechanism is the binding of TALEs to cis-elements in the promoters of rice genes, such as SWEET or TFIIAγ5, thereby increasing their expression to support bacterial growth.

Conversely, natural or artificial mutations in the *TFIIAγ5 gene* have been identified to confer resistance to BLB. For example, the *xa5 allele*, a mutant form of *TFIIAγ5*, confers broad resistance to various *Xoo strains* by reducing the transcriptional activity required by TALEs (Jiang *et al.*, 2020). However, developing new varieties carrying the *xa5 allele* through conventional breeding is time-consuming and often results in the enhancement of other undesirable agronomic traits.

CRISPR-Cas9 is a powerful gene-editing tool that harnesses the bacterial immune system's ability to target and cleave specific DNA sequences. This system uses short RNA molecules called single-guide RNAs (sgRNAs) to direct the Cas9 nuclease to target DNA sequences, resulting in double-strand breaks. These breaks can then be repaired through cellular repair mechanisms, enabling desired genetic modifications. The simplicity, cost-effectiveness, and high efficiency of CRISPR-Cas9 have made it a game-changer in the field of genetic engineering (N.M. Ansori *et al*, 2021). Through this approach, targeted mutations can be introduced into the TFIIAγ5 gene to mimic the function of the xa5 allele without the need for traditional backcrossing. Several previous studies have reported that site-specific editing of TFIIAγ5 successfully enhanced rice resistance to BLB without affecting plant growth or yield (Oliva *et al.*, 2019). CRISPR-Cas9 has revolutionized genetic editing because it uses RNA for site recognition, making it easier, cheaper, and faster to design than previous methods such as Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), which require periodic protein design and reconstruction. This key advantage has driven the widespread adoption of the CRISPR-Cas9 method in biological and agricultural research.

This study aimed to screen T1 rice lines modified in the TFIIAγ5 gene using the CRISPR/Cas9 system and to assess their resistance to bacterial leaf blight. The results are expected to identify T1 rice lines with potentially stable mutations and provide a foundation for developing next-generation bacterial leaf blight-resistant rice varieties.

2. Material and Methods

2.1. Place and Time

The research was conducted in the MARDI Malaysia genetics laboratory at 40-50m above sea level at coordinates 2.983392, 101.700652.

2.2. Materials and tools

T1 generation seeds of droplet-edited MR219 were produced using the CRISPR/Cas9 gene-editing system targeting the promoter of the SWEET14 gene. The wild-type MR219 variety was used as a control, along with treatments including 100% ethanol, fungicide, water, and a bucket.

2.3. Research Implementation

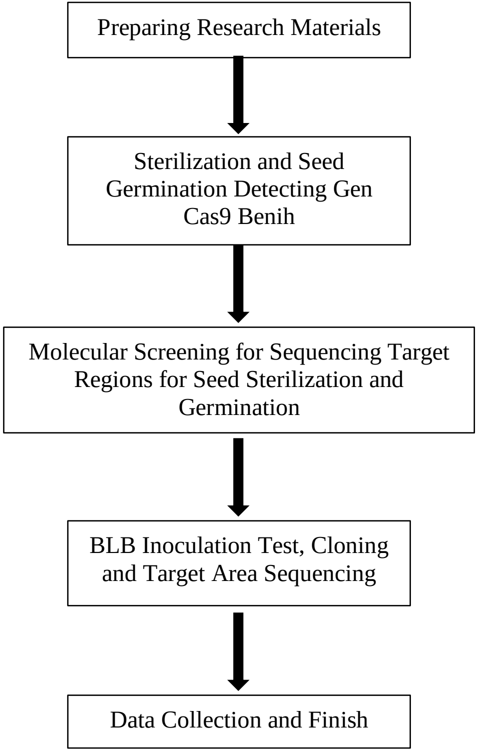


Figure 1. Research flow diagram

2.4. Research methods

This research uses a gene-exploration method to identify genes associated with resistance to leaf blight and bacterial pathogens by analyzing data from multiple samples. Stages include sterilizing and germinating seeds, and molecular screening to detect the presence of the *Cas9 gene*. BLB inoculation test, cloning, and sequencing of target areas.

2.5. Sterilization and Germination of Seeds

Seeds are sterilized before germination. The seeds are first soaked in 100% ethanol (v/v) for 10 minutes, then stirred gently, and then soaked a second time in the fungicide solution for 10 minutes. At each step, the seeds are rinsed 2-3 times using sterile water. The seeds are allowed to dry for 10 minutes before being soaked in sterile water and placed under light for germination. After 7 days, the seeds will be transferred to the transgenic greenhouse for an acclimation process for 7 days before being transferred into containers.

2.6. Molecular Screening to Detect the Presence of the Cas9 Gene

Genomic DNA was extracted from young leaves of T1 generation rice plants using a plant extraction kit and then used for PCR analysis. PCR detected the presence of the Cas9 gene in the progeny with primers specific to Cas9. The PCR products were then separated on a 1% agarose gel to identify the T1 generation carrying the Cas9 gene. Plants producing thicker Cas9 PCR bands were selected for inoculation with the BLB pathogen to assess their susceptibility to BLB disease.

2.7. BLB Inoculation Test, Cloning and Target Area Sequencing

To assess resistance to BLB, control rice seedlings T1 and MR219 were grown in a greenhouse until the vegetative stage (45–50 days). Rice leaves were inoculated using the leaf cutting method with the local virulent Xoo strain MXO-1536 (density of 108 CFU/mL). A total of 4–5 leaves were used for inoculation. The infection rate was determined 21 days post-inoculation by measuring lesion length relative to leaf length. Plants with a resistance percentage <50% were subjected to targeted modification before cloning into the pGEM-T Easy vector for sequencing analysis. The sequencing results were analyzed by comparing them with the wild-type sequence, focusing on a 100 bp region to the left and right of the gRNA.

This procedure is performed under aseptic conditions using *a Laminar Flow Hood*. After rinsing, the seeds were allowed to dry for 10 minutes and then soaked in sterile distilled water and transferred to a growth chamber under controlled lighting conditions. The seedlings were left for 7 days to germinate before being transferred to the transgenic (controlled) greenhouse, where they underwent acclimatization for another 7 days. After completing the initial acclimatization phase, the seedlings are transferred into a suitable final planting container (bucket) for further growth. The data collected and analyzed included the percentage of germination after 7 days, the percentage of survival after the 7-day acclimatization phase, and initial growth data to assess the success of adaptation. The data analysis software used is Microsoft Excel.

3. Results and Discussion

3.1. Confirmation of the Presence of the Cas9 Gene in the T1 Generation

Genomic DNA was extracted from the 7th leaf of T1-generation rice seedlings and used as a template for PCR analysis with primers specific to the Cas9 gene. 1% agarose gel electrophoresis analysis showed that four T1 progenies produced an amplification band measuring ±900 bp corresponding to the Cas9 gene (+ve control). The presence of the Cas9 gene confirmed that the progeny still carried the transgenic element, whereas progeny lacking the band were considered Cas9-free (Cas9–ve). Cas9-positive progeny are the best candidates for mutation analysis because the remaining Cas9 gene in the genome can still carry out gene mutation.

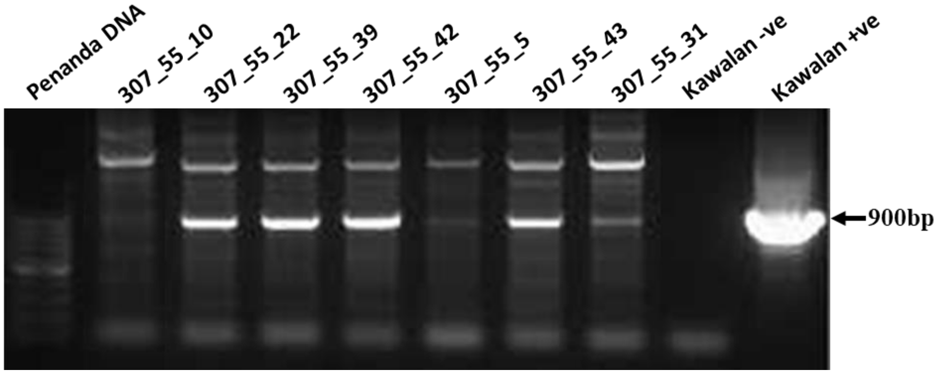


Figure 1. Molecular Screening Targeting the Cas9 Gene to Know for Sure the T1 Generation Carrying the Cas9 Gene

3.2. Analysis of Resistance to BLB Disease

Disease resistance was assessed by inoculating leaf clippings with a suspension of the Xoo strain MXO-1536. The assessment time was 14 days after inoculation. The length of the lesions formed in the edited T1 sample was significantly greater than that of the wild-type control MR219, as shown in Figure 2. This finding shows that the mutation in the SWEET14 promoter has successfully increased resistance to BLB. This finding is in accordance with the results of studies by Blanvillain-Baufumé *et al.* (2017) and Oliva *et al*. (2019), which stated that editing the TAL effector binding site in the SWEET14 promoter causes a decrease in gene expression and an increase in resistance to Xoo. Therefore, the promoter mutation detected in the T1 generation is a potential candidate for further agronomic evaluation in the T2 generation.

3.3. Mutations in the TFIIAγ5 Target Gene

DNA sequence analysis of the target site region of the TFIIAγ5 gene in the T1 generation revealed multiple small nucleotide substitutions. These mutations were detected near the Cas9 cleavage site, defined by the guide RNA (gRNA) sequence and the protospacer adjacent motif (PAM). These results confirmed that the alterations were successfully made on target, as designed, using the CRISPR/Cas9 system.

This type of mutation is thought to alter the structure or activity of the TFIIAγ5 protein, mimicking the function of the xa5 allele, which is known to reduce the bacterium

Xanthomonas oryzae pv. oryzae (Xoo) to activate susceptibility genes in its host (Jiang *et al*., 2020).

Sequence alignment results between the transformed T1 plants and the wild-type control showed clear differences in the gRNA site region. Figure 3 shows the type and location of mutations detected for each T1 line. In most cases, mutations occurred within positions 124-132 bp of the coding region of the TFIIAγ5 gene. Although the detected mutations were small, their impact on protein function was significant because they involved codon changes within a conserved domain of TFIIAγ5, which is essential for transcriptional activity (Yuan et al., 2009). The presence of these mutations in the T1 generation suggests a high probability that they were stably inherited from the T0 generation, consistent with a report by Li et al. (2021), who found that CRISPR/Cas9 gene editing in rice resulted in consistent mutation inheritance through the T2 generation.

Similar findings by Duy *et al. (2020):* all T1 individuals derived from T0 plants previously genotyped as homozygous had the same allele as their parent, indicating stable inheritance of the mutation to the next generation.

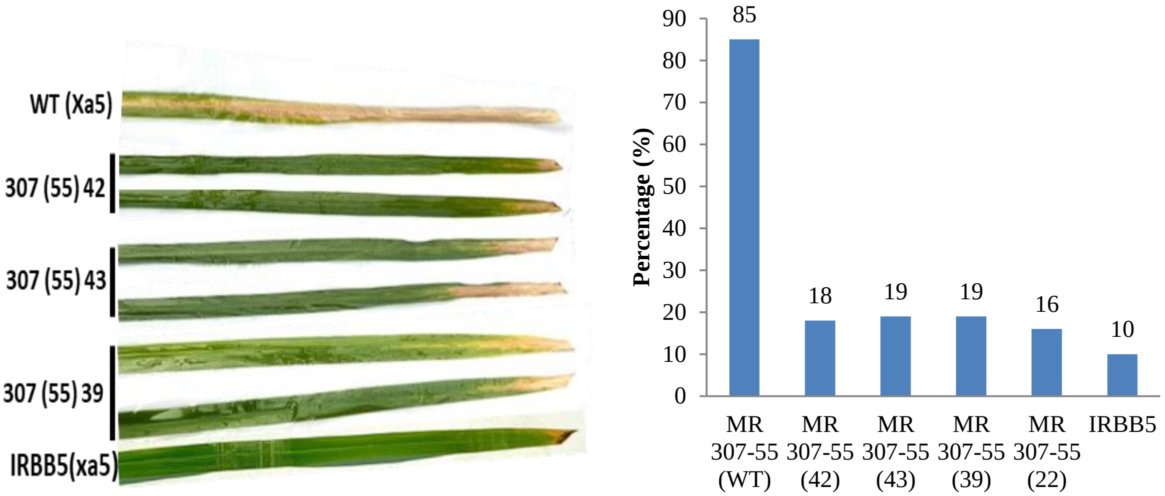
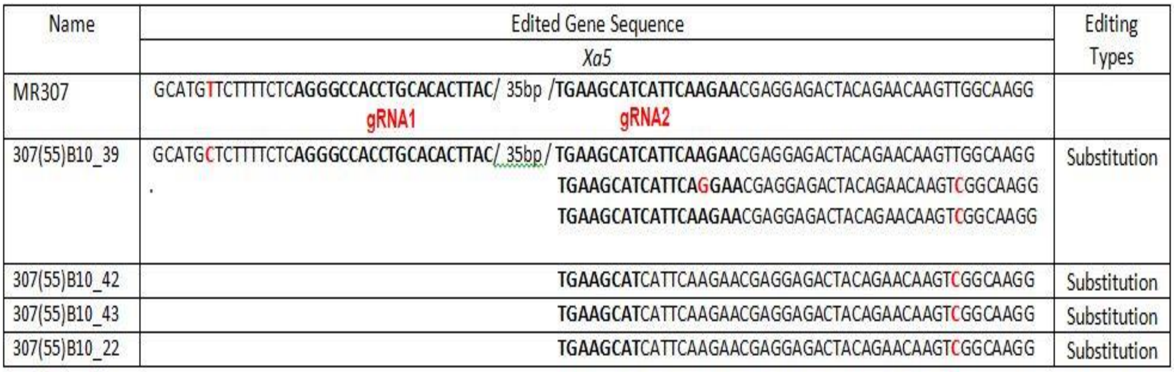


Figure 2. The percentage of T1 Generation Infection is changed compared to the Wild Type Control MR219. There is a Length Variation After 21 Days Post-Inoculation compared to the Wild Type (WT) and Positive Control (IRBB5).



Name	Edited Gene Sequence *Xa5*	Editing Types
MR307	GCATGTTCTTTTCTC**AGGGCCACCTGCACACTTAC**/ 35bp /**TGAAGCATCATTCAAGAA**CGAGGAGACTACAGAACAAGTTGGCAAGG gRNA1 gRNA2	
307(55)B10_39	GCATGCTCTTTTCTC**AGGGCCACCTGCACACTTAC**/ 35bp/ **TGAAGCATCATTCAAGAA**CGAGGAGACTACAGAACAAGTTGGCAAGG **TGAAGCATCATTCAGGAA**CGAGGAGACTACAGAACAAGTCGGCAAGG **TGAAGCATCATTCAAGAA**CGAGGAGACTACAGAACAAGTCGGCAAGG	Substitution
307(55)B10_42	**TGAAGCAT**CATTCAAGAACGAGGAGACTACAGAACAAGTCGGCAAGG	Substitution
307(55)B10_43	**TGAAGCAT**CATTCAAGAACGAGGAGACTACAGAACAAGTCGGCAAGG	Substitution
307(55)B10_22	**TGAAGCAT**CATTCAAGAACGAGGAGACTACAGAACAAGTCGGCAAGG	Substitution

Figure 3. Sequence Alignment Between Transformed T1 Plants and Wild Control MR219.

4. Conclusion

Overall, these results indicate that the CRISPR/Cas9 system employed in this study successfully induced mutations in the target gene TFIIAγ5. These mutations have the potential to alter the protein's function, conferring resistance to bacterial leaf blight, consistent with the mechanism of the xa5 allele found in rice varieties naturally resistant to this disease. In summary, CRISPR/Cas9 targeting of the TFIIAγ5 gene facilitates the development of more efficient, resilient, and profitable super-crops, thereby enhancing both farmer income and the sustainability of agricultural practices.

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