THAI JOURNAL OF
AGRICULTURAL SCIENCE
Vol.58 No.3 July-September 2025
https://li01.tci-thaijo.org/index.php/TJAS

Cloning and characterization of a candidate bacterial leaf blight resistance gene, a serine/threonine protein kinase, in rice

Arisa Laisupannawong[1]
Worrawit Suktrakul[1]
Apinya Longya[1]
Mantira Suksirt[2]
Chatchawan Jantasuriyarat[1,*]

[1] *Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[2] *Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Pathum Thani 12120, Thailand*

*Corresponding author: **fscicwj@ku.ac.th***

Article History

Submission: 4 June 2025
Revised: 12 July 2025
Accepted: 14 July 2025

Keywords

Bacterial leaf blight
Rice
Resistance gene
Genome-wide association study

Abstract

Background and Objective: Bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)*, is a major constraint on rice (*Oryza sativa* L.) production, particularly in Asia, where rice is a dietary staple. Enhancing host resistance through the deployment of resistance genes remains one of the most effective strategies in rice breeding programs. Advances in molecular tools, including marker-assisted selection (MAS), quantitative trait loci (QTL) mapping, and genome-wide association studies (GWAS), have facilitated the identification of at least 46 BLB resistance genes, 11 of which have been functionally characterized. Building on the previous work, 127 putative BLB resistance genes were identified via GWAS. This study aimed to examine the expression and variation of candidate genes to identify genes potentially contributing to BLB resistance.

Methodology: Quantitative PCR (qPCR) was used to evaluate the expression of selected candidate genes in two rice cultivars: IR57514 (a resistant cultivar) and Jao Hom Nin (a susceptible cultivar), following *Xoo* inoculation. One gene, *LOC_Os01g66860*, encoding a serine/threonine protein kinase, was selected for further analysis. A phylogenetic analysis was performed to assess evolutionary conservation across the plant kingdom, and the gene was cloned and sequenced from both cultivars.

Main Results: *LOC_Os01g66860* was significantly upregulated in the resistant cultivar after pathogen challenge. Phylogenetic analysis confirmed its conservation across plant species. Cloning and sequencing revealed one amino acid substitution between the resistant and susceptible cultivars.

Conclusions: These findings suggest that *LOC_Os01g66860* may play a crucial role in BLB resistance. The results provide a foundation for future functional validation and highlight its potential application in rice breeding programs aimed at improving disease resistance. However, a limitation of this study is the need for further functional tests, such as gene knockout or overexpression experiments, to confirm the gene's specific role in resistance.

How to cite : Laisupannawong, A., W. Suktrakul, A. Longya, M. Suksirt and C. Jantasuriyarat. 2025. Cloning and characterization of a candidate bacterial leaf blight resistance gene, a serine/threonine protein kinase, in rice. Thai J. Agric. Sci. 58(3): 223−235.

INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple crop globally, particularly in Asia, where over 90% of the world's estimated 480 million tonnes of annual rice production occurs (Muthayya *et al.*, 2012; 2014). Countries such as China, India, Thailand, Vietnam, and Japan are key producers. With rising global populations, rice consumption has increased significantly in recent decades. However, bacterial leaf blight (BLB), caused by *Xanthomonas oryzae* pv. *oryzae (Xoo)*, remains a serious constraint, capable of causing yield losses of up to 81% (Khush *et al.*, 1989; Srinivasan and Gnanamanickam, 2005).

First reported in Japan in 1884 (Ou, 1985), BLB has since spread across Asia and beyond, including India, Thailand, Sri Lanka, China, and the Philippines (Podishetty, 2014). BLB symptoms include water-soaked lesions along the veins, progressing to yellow, wavy-margined patches that peak during the flowering stage (Mew *et al.*, 1993). Chemical control methods, including antibiotics and pesticides, have proven largely ineffective and raise safety concerns under monsoon conditions (Sundaram *et al.*, 2008; Gnanamanickam, 2009). Therefore, breeding for resistance using specific resistance genes is now the most sustainable and effective approach (Mundt, 2014; Pradhan *et al.*, 2015).

Molecular tools such as marker-assisted selection (MAS), quantitative trait loci (QTL) mapping, and genome-wide association studies (GWAS) have significantly accelerated the identification of BLB resistance loci. To date, at least 46 BLB resistance genes have been identified, with 11 functionally characterized (Fiyaz *et al.*, 2022). Recent GWAS efforts have revealed several novel loci and candidate genes, including both known *Xa* gene regions and entirely new genomic areas (Dilla-Ermita *et al.*, 2017; Korinsak *et al.*, 2021; Shu *et al.*, 2021; Danaisilichaichon *et al.*, 2023).

This study builds upon the work of Korinsak *et al.* (2021), who identified 127 putative BLB resistance genes in rice through GWAS. Based on the hypothesis that candidate genes associated with plant defense or stress responses would exhibit higher expression

levels following *Xoo* inoculation, we aimed to examine the expression and variation of selected genes in IR57514 (a resistant cultivar) and Jao Hom Nin (a susceptible cultivar). Particular focus was given to genes with known or predicted roles in plant defense mechanisms, differential expression between resistant and susceptible cultivars, and potential evolutionary conservation and sequence variation.

Among the 127 candidate genes, *LOC_Os01g66860*, annotated as a serine/threonine protein kinase, was prioritized because it was notably upregulated in the resistant cultivar following *Xoo* infection, in contrast to most other candidate genes, which were downregulated. This differential expression pattern suggests a potential role in the resistance response. These findings support the involvement of *LOC_Os01g66860* in BLB resistance and provide a strong foundation for future functional validation studies, such as gene knockout or overexpression analyses. Furthermore, this underscores its novel potential as a promising candidate for incorporation into rice breeding programs focused on developing enhanced and durable resistance to BLB.

MATERIALS AND METHODS

Plant Materials

Two rice (*Oryza sativa* L.) cultivars with contrasting responses to BLB were used in this study: Jao Hom Nin (JHN), a susceptible cultivar, and IR57514, a resistant cultivar. Both cultivars were obtained from the Rice Department, Ministry of Agriculture and Cooperatives, Thailand. Seeds of both cultivars were surface-sterilized and soaked in distilled water for 2–3 days to promote uniform imbibition. Subsequently, the seeds were placed on moist germination paper and maintained under controlled conditions for 7 days until the emergence of radicles and green shoots. The germinated seedlings were then transplanted into pots containing sterilized clay soil and grown under greenhouse conditions for 45 days prior to pathogen inoculation.

Bacterial Materials

The *Xanthomonas oryzae* pv. *oryzae (Xoo)* strain CR2-4, obtained from the Department of Plant Pathology, Faculty of Agriculture, Kamphaeng Saen Campus, Kasetsart University, was used for inoculation in this study. The bacterial strain was cultured on peptone sucrose agar (PSA) medium, consisting of 10 g peptone, 1 g glutamic acid, and 10 g sucrose per liter of distilled water, supplemented with 20 g agar. Cultures were incubated at 28 °C for 48–72 hours. Bacterial cells were then harvested by gently scraping all the colonies on the surface and suspending them in sterile 15% (v/v) glycerol solution. The bacterial suspension was adjusted to an optical density (OD_{600}) of 1.0 using a spectrophotometer. The inoculation was performed using the standard leaf-clipping method as described by Kauffman *et al.* (1973). Surgical scissors were used to clip the leaves. In the greenhouse, the inoculation involved dipping the scissors into the bacterial suspension, grasping the leaves in the pot with one hand, and simultaneously clipping off the top 1–3 inches of each leaf.

DNA Extraction

Genomic DNA was extracted from rice leaf tissue using the standard cetyltrimethylammonium bromide (CTAB) method. Approximately 0.3–0.5 g of fresh leaf tissue was collected and ground into a fine powder in liquid nitrogen to disrupt cell walls and facilitate DNA release. The homogenized tissue was mixed with 700 µL of preheated CTAB extraction buffer (containing 2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, and 1.4 M NaCl) supplemented with 2 µL of ß-mercaptoethanol. The mixture was incubated at 65 °C for 1 hour with occasional gentle mixing. Following incubation, the samples were centrifuged at 13,000 rpm for 30 seconds to remove debris. The supernatant was transferred to a fresh

microcentrifuge tube and extracted with an equal volume (500 µL) of chloroform:isoamyl alcohol (24:1, v/v) by gentle inversion. The mixture was centrifuged at 13,000 rpm for 15 minutes at 4 °C. The upper aqueous phase was carefully transferred to a new tube, and DNA was precipitated by adding an equal volume of isopropanol (500 µL), followed by incubation at −20 °C overnight. The DNA was pelleted by centrifugation at 13,000 rpm for 10 minutes at 4 °C. The pellet was washed sequentially with 500 µL of 95% ethanol and 70% ethanol, each followed by centrifugation under the same conditions. After the final wash, residual ethanol was removed, and the DNA pellet was air-dried for 10–30 minutes. The purified DNA was finally resuspended in an appropriate volume of ultrapure water and stored at −20 °C until use.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the top 5 cm of rice leaves using an RNA extraction kit (Vivantis, USA) according to the manufacturer's protocol. Samples were collected four days post-inoculation, as disease symptoms typically begin to appear around 4 to 5 days after inoculation, and *R* gene expression is expected to peak around 3 days post-inoculation, as reported by Gu *et al.* (2005). RNA quality and quantity were assessed using agarose gel electrophoresis and a Nanodrop (Thermo Scientific, USA). One microgram of RNA from each sample was then reverse transcribed into cDNA using the Reverse Transcription Kit (Promega, USA) as per the manufacturer's instructions. The reaction mix included 1 µg RNA, 4 µL of 10X buffer, 2.4 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTP mix, 0.5 µL of 10 µM oligo (dT), 1 unit of reverse transcriptase, and deionized water to a final volume of 20 µL. The reaction was incubated at 42 °C for 1 hour, followed by heat inactivation at 85 °C for 10 minutes.

Selection of Candidate Resistance Genes

From the 127 genes previously reported by Korinsak *et al.* (2021), a total of 93 candidate genes with known or putative functional annotations related to plant defense or stress responses—such as serine/threonine protein kinases or receptor-like protein kinases—were selected for further analysis (Supplementary Table S1A). Genes lacking annotations or classified as hypothetical or uncharacterized proteins were excluded from subsequent analysis. Gene-specific primers (Supplementary Table S1B) were designed using Primer3 software (Untergasser *et al.*, 2012). Primer specificity and amplification efficiency were tested via conventional PCR, conducted twice. Only genes that consistently produced successful amplification were retained for downstream expression analysis using quantitative real-time PCR (qPCR), which was performed in three independent replicates. Genes that exhibited distinct expression profiles—particularly those with higher expression after inoculation in the resistant cultivar compared to the susceptible one—were prioritized for further analysis, including phylogenetic evaluation and gene cloning, as candidate genes potentially involved in BLB resistance.

Gene Expression Analysis Using Quantitative PCR

IR57514 and Jao Hom Nin rice plants were inoculated simultaneously under identical conditions, with two plants per cultivar included in each biological replicate. Each biological replicate was evaluated using three technical replicates. Disease symptoms were assessed following the standard protocol described by IRRI (1996).

Complementary DNA (cDNA) synthesized from BLB-inoculated rice samples was used for gene expression analysis. Each quantitative real-time PCR reaction was carried out in a final volume of 20 µL, containing 1 µL of cDNA template, 1X QPCR Green Master Mix LRox (Biotech Rabbit, Germany), and 0.2 µM of each gene-specific primer. Amplification

was conducted using a MasterCycler Realplex4 Thermal Cycler (Eppendorf, Germany) with the following cycling conditions: an initial denaturation at 95 °C for 2 minutes, followed by 30 cycles of denaturation at 95 °C for 10 seconds, annealing at 56 °C for 10 seconds, and extension at 72 °C for 20 seconds. Each reaction was performed in triplicate to ensure the reliability and reproducibility of the data. The relative expression levels of candidate genes were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$), with normalization to the *Actin* gene (NM_001402243.1) as an internal control. Primer sequences used for amplification were: *OsActin_F* (5′-GACCTTGCTGGGCGTGAT-3′) and *OsActin_R* (5′-GTCATAGTCCAGGGCGATGT-3′) for the *Actin* gene, and *BLB57_F* (5′-ACTGCGACCTAAAGC-CAAAA-3′) and *BLB57_R* (5′-ATTGTGTGGCCAGAT-TCCTC-3′) for *LOC_Os01g66860*.

The comparative Ct method ($2^{-\Delta\Delta Ct}$) was used to quantify relative gene expression levels before and after inoculation in both the susceptible line (Jao Hom Nin) and the resistant line (IR57514). Statistical analyses were performed using R version 4.2.3 (RStudio Cloud environment). Visualization and data manipulation were carried out using the tidyverse and ggpubr packages, while statistical tests, including ANOVA and Tukey's HSD, were conducted with the rstatix and agricolae packages, respectively.

Phylogenetic Analysis

A total of 18 gene sequences from different plant species were included in the phylogenetic analysis (Supplementary Table S2), representing 8 monocotyledonous and 10 dicotyledonous species. The monocot-derived sequences were obtained from *Brachypodium distachyon*, *Zea mays* (maize), *Sorghum bicolor* (sorghum), *Setaria italica* (foxtail millet), *Triticum aestivum* (wheat), *Lolium perenne* (perennial ryegrass), *Hordeum vulgare* (barley), and *Oryza sativa* ssp. *japonica* (rice). The dicot-derived sequences corresponded to *Solanum lycopersicum* (tomato), *Medicago truncatula* (barrel clover), *Glycine max* (soybean), *Ricinus communis* (castor bean), *Manihot esculenta* (cassava), *Cucumis sativus* (cucumber), *Eutrema salsugineum* (saltwater cress), *Brassica rapa* (Chinese cabbage), *Arabidopsis thaliana* (thale cress), and *Gossypium raimondii* (cotton). The coding sequence (CDS) of *LOC_Os01g66860* from *Oryza sativa* was retrieved from the NCBI database and used as a query in BLAST searches to identify homologous protein sequences from the selected species. The resulting protein sequences were aligned using the MUSCLE algorithm implemented in AliView (Larsson, 2014). A phylogenetic tree was generated using the Maximum Likelihood (ML) method in MEGA X (Kumar *et al.*, 2018), with 100 bootstrap replicates to assess branch support.

The Candidate Gene Cloning and Sequence Analysis

Genomic DNA extracted from *Oryza sativa* cultivars Jao Hom Nin and IR57514 was used to amplify the full-length coding sequence of *LOC_Os01g66860*, spanning from the start to the stop codon. For Jao Hom Nin, PCR amplification was performed using i-Star Max Solution II (iNtRON, Korea) in a 20 µL reaction mixture containing 9.5 µL sterile distilled water, 12.5 µL of 2× PCR Master Mix, 1.0 µL each of 10 µM gene-specific primers *Os01_F* (5′-ATGGACACCGGGATGAGG-3′) and *Os01_R* (5′-TCACATTGCGACATGGACTC-3′), and 1.0 µL of genomic DNA (50 ng/µL). PCR cycling conditions included an initial denaturation at 94 °C for 1 minute; 30 cycles of denaturation at 94 °C for 10 seconds, annealing at 58 °C for 35 seconds, and extension at 72 °C for 3 minutes; followed by a final extension at 72 °C for 5 minutes.

For IR57514, the same gene was amplified using Phusion Plus DNA Polymerase (Thermo Scientific, USA). The 10 µL PCR reaction contained 3.5 µL sterile distilled water, 2.0 µL of 5× Phusion

Plus Buffer, 0.2 µL of 10 mM dNTP mix, 0.6 µL each of 5 µM *Os01_F* and *Os01_R* primers, 2.0 µL of 5× Phusion GC Enhancer, 0.1 µL of Phusion Plus DNA Polymerase (2 U/µL), and 1.0 µL genomic DNA. PCR was carried out under the following conditions: initial denaturation at 98 °C for 30 seconds; 30–35 cycles of denaturation at 98 °C for 10 seconds, annealing at 58–60 °C for 10–35 seconds, and extension at 72 °C for 1.5 minutes; with a final extension at 72 °C for 5 minutes. Amplified products were visualized on a 1% agarose gel. Bands of the expected size were excised, and PCR conditions were optimized as needed for re-amplification.

Purified PCR products were obtained using the QIAquick PCR Purification Kit (Qiagen, Germany), then cloned into the pGEM-T Easy vector (Promega, USA). The ligation reaction contained 2.5 µL of 2× Rapid Ligation Buffer, 0.5 µL vector, 1.5 µL purified PCR product, and 0.5 µL T4 DNA Ligase. The ligation mix was transformed into *Escherichia coli* strain DH5α via heat shock, and positive clones were screened by colony PCR using gene-specific primers under the previously optimized conditions. Confirmed clones were submitted for Sanger sequencing. Resulting sequences were aligned against the *LOC_Os01g66860* reference CDS from the Rice Genome Annotation Project. The confirmed DNA sequences were translated into corresponding amino acid sequences using the ExPASy translation tool (Gasteiger *et al.*, 2003). Protein sequence alignments were performed using AliView (Larsson, 2014) to identify amino acid polymorphisms between the two cultivars.

RESULTS AND DISCUSSION

Identification and Expression Analysis of Candidate Resistance Genes

To identify candidate genes associated with BLB resistance, a total of 93 primer pairs, along with a control primer targeting the *Actin* gene, were used for PCR amplification. Initial testing of primer specificity was performed using genomic DNA extracted from the BLB-resistant rice cultivar IR57514 (Supplementary Figure 3A). Of the 93 primer pairs tested, 52 successfully amplified the target DNA fragments and were selected for further analysis (Supplementary Figure 3B).

These 52 genes were subsequently evaluated for transcriptional activity through preliminary reverse transcription PCR (RT-PCR). Based on expression patterns observed during this screening, 13 primer pairs showing notable expression differences were selected for quantitative real-time RT-PCR analysis (Supplementary Figure 3C).

Quantitative expression analysis revealed that most of the selected genes were downregulated following inoculation with *Xanthomonas oryzae* pv. *oryzae (Xoo)*. However, only genes that exhibited consistent upregulation in both cultivars post-inoculation were selected for statistical analysis using ANOVA followed by Tukey's HSD test. One gene showed a notable increase in expression in response to *Xoo* infection. In Jao Hom Nin (JHN), a statistically significant difference was observed between the pre-inoculation sample (JHN_Mock; fold change = 1.07 ± 0.47) and post-inoculation samples (JHN_1 = 24.43 ± 1.04 and JHN_2 = 23.61 ± 9.46), with a P-value of 0.005. In the resistant line IR57514, comparisons between the pre-inoculation sample (IR57514_Mock = 4.58 ± 1.69) and post-inoculation samples (IR57514_1 = 11.32 ± 3.97 and IR57514_2 = 12.78 ± 4.25) yielded P-values of 0.08 and 0.04, respectively. Sequence analysis identified this gene as *LOC_Os01g66860*, which encodes a serine/threonine protein kinase (Figure 1). The consistent upregulation of *LOC_Os01g66860* following *Xoo* inoculation suggests its potential role in the rice defense response against BLB.

Functional annotation of the identified gene indicates that it encodes a serine/threonine protein kinase, which is a key component of signal transduction pathways involved in plant immune responses. However, *in silico* transcriptome analysis using the RiceMetaSysB:

Bacterial Blight RG database revealed that *LOC_Os01g66860* was downregulated at 72 hours post-inoculation (hpi) with the *Xoo* strain PXO99 in the Japonica cultivars Osaba1-1 (logFC = –2.018) and Nipponbare (logFC = –1.220). This finding contrasts with our quantitative PCR (qPCR) results, which showed upregulation of *LOC_Os01g66860* following inoculation using the indica rice cultivars JHN and IR57514 with the *Xoo* strain CR2-4. This discrepancy may be due to differences in experimental conditions, including the timing of RNA extraction—our study sampled at 4 days post-inoculation—as well as the use of different bacterial strains and rice cultivars. These factors suggest that the expression of *LOC_Os01g66860* is both time- and cultivar-dependent, and may also be influenced by pathogen strain variation, highlighting the complexity of the rice immune response to BLB. A similar observation was reported by He *et al.* (2006), who found that a leucine-rich repeat receptor kinase (LRK) gene cluster, particularly the *LRK2* gene, was highly expressed in *Oryza sativa* ssp. *indica* var. 93-11, but was undetectable by RT-PCR in *Oryza sativa* ssp. *japonica* cv. Nipponbare.

Notably, expression divergence may be driven by underlying genetic polymorphisms. Single nucleotide polymorphisms (SNPs) in regulatory regions—such as promoters or enhancers—can disrupt transcription factor binding sites, leading to differential gene expression. Likewise, SNPs within coding regions,

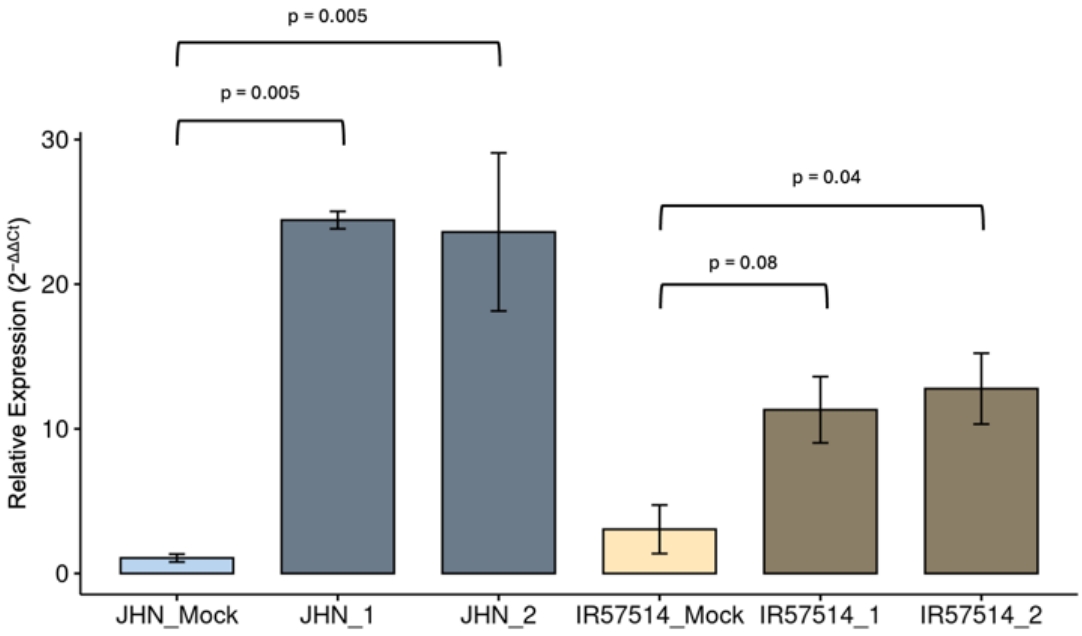

Figure 1 Relative expression of *LOC_Os01g66860* in rice cultivars IR57514 and Jao Hom Nin (JHN) before and after inoculation with *Xanthomonas oryzae* pv. *oryzae (Xoo)*. Gene expression was quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The x-axis represents sample groups, and the y-axis indicates relative expression levels ($2^{-\Delta\Delta Ct}$). Sample groups include JHN_Mock (uninoculated control), and JHN_1 and JHN_2 (post-inoculation replicates), IR57514_Mock (uninoculated control), IR57514_1 and IR57514_2 (post-inoculation replicates). Statistical significance was determined using Tukey's Honestly Significant Difference (HSD) test; P-values (P) are indicated.

particularly nonsynonymous SNPs (nsSNPs), may alter amino acid residues and affect protein structure, stability, or localization, thereby influencing downstream signaling dynamics even when transcript levels are unchanged. SNPs represent the most common type of genetic variation in plant genomes and are increasingly accessible through advances in genome sequencing. For instance, a recent study on the rice *GPAT* gene family using data from the 3000 Rice Genomes Project identified 98 nsSNPs across 26 genes, with 13 predicted to have deleterious effects on protein structure. This highlights the functional significance of coding-region SNPs in shaping phenotypic variation and supports the use of *in silico* tools to prioritize candidate variants for experimental validation in rice and other crop species (Safder *et al.*, 2021). These differences underscore the importance of considering both genetic and regulatory variation when interpreting gene expression data across different platforms. Further functional analysis, including promoter activity assays, would be valuable in elucidating the regulatory mechanisms governing *LOC_Os01g66860* and its potential utility in breeding for BLB resistance.

Phylogenetic Analysis of The *LOC_Os01g66860* Gene

Notably, *Oryza sativa* ssp. *japonica* clustered closely with *Zea mays*, *Sorghum bicolor*, and *Setaria italica* within the monocot group, reflecting their evolutionary relatedness and suggesting conservation of the *LOC_Os01g66860* protein sequence among these species (Figure 2). This phylogenetic pattern supports the hypothesis that *LOC_Os01g66860* is a conserved gene across the plant kingdom, with potential functional significance in monocot and dicot lineages. The close relationship observed among monocot species may indicate shared mechanisms of pathogen recognition and defense mediated by this gene, warranting further comparative functional studies.

The evolutionary dynamics of leucine-rich repeat receptor-like kinase (*LRR-RLK*) genes exhibit patterns similar to those observed for the *LOC_Os01g66860* gene. Extensive studies on *LRR-RLKs*, particularly in flowering plants, have provided valuable insights into their evolutionary history. Liu *et al.* (2017) reported that the core structural features of *LRR-RLK* genes were established early in the evolution of land plants and have since been conserved across subfamilies and diverse plant lineages. Despite this conservation, significant divergence exists between different subfamilies. Members within the same subfamily typically share similar protein motif compositions, whereas distinctly different motif patterns characterize different subfamilies. Supporting these findings, Dufayard *et al.* (2017) performed a comprehensive analysis of 101 orthologous gene groups (OGs) of *LRR-RLKs* across monocot and dicot species. Their study demonstrated that orthologous genes have been largely preserved throughout angiosperm evolution in both lineages. By examining 20 subgroup-specific phylogenetic trees, they identified key bifurcation points separating monocots and dicots and observed that orthologous relationships were conserved across nearly all species analyzed. Together, these studies highlight the evolutionary conservation and divergence patterns of *LRR-RLK* genes, reflecting their important functional roles across plant taxa.

The Gene Construct and Amino Acid Analysis of *LOC_Os01g66860* from The IR57514 and Jao Hom Nin Rice Cultivars

To elucidate the gene structure of *LOC_Os01g66860*, the full-length gene was cloned from genomic DNA of the rice cultivars Jao Hom Nin (susceptible) and IR57514 (resistant) using primers *Os01_F* and *Os01_R*, which amplify the sequence spanning from the start to the stop codon. The gene

comprises 11 exons and 10 introns, with a total length of 2,887 base pairs (Figure 3). DNA sequencing and alignment revealed a single nucleotide polymorphism in exon 6: cytosine (C) was present at position 1574 in Jao Hom Nin, whereas thymine (T) occupied the same position in IR57514 (Figure 4).

Further analysis of the 500 amino acid-long protein revealed several annotated functional domains based on NCBI region annotations: an ANKYR region spanning amino acids 68..169, two ankyrin repeats located at 109..140 and 142..167, respectively, and a protein kinase catalytic domain from 207..456. Sequence alignment of the deduced amino acids, retrieved via the ExPASy web tool and aligned using AliView, revealed a single amino acid substitution at position 287, with leucine (L) in IR57514 replaced by proline (P) in Jao Hom Nin (Figure 5). These differences at both nucleotide and protein levels may contribute to the differing resistance phenotypes observed between these cultivars.

This observation aligns with previous findings reported by Iyer and McCouch (2004) regarding the *xa5* gene, a recessively inherited gene conferring resistance to *Xoo*. The *xa5* gene encodes the γ subunit

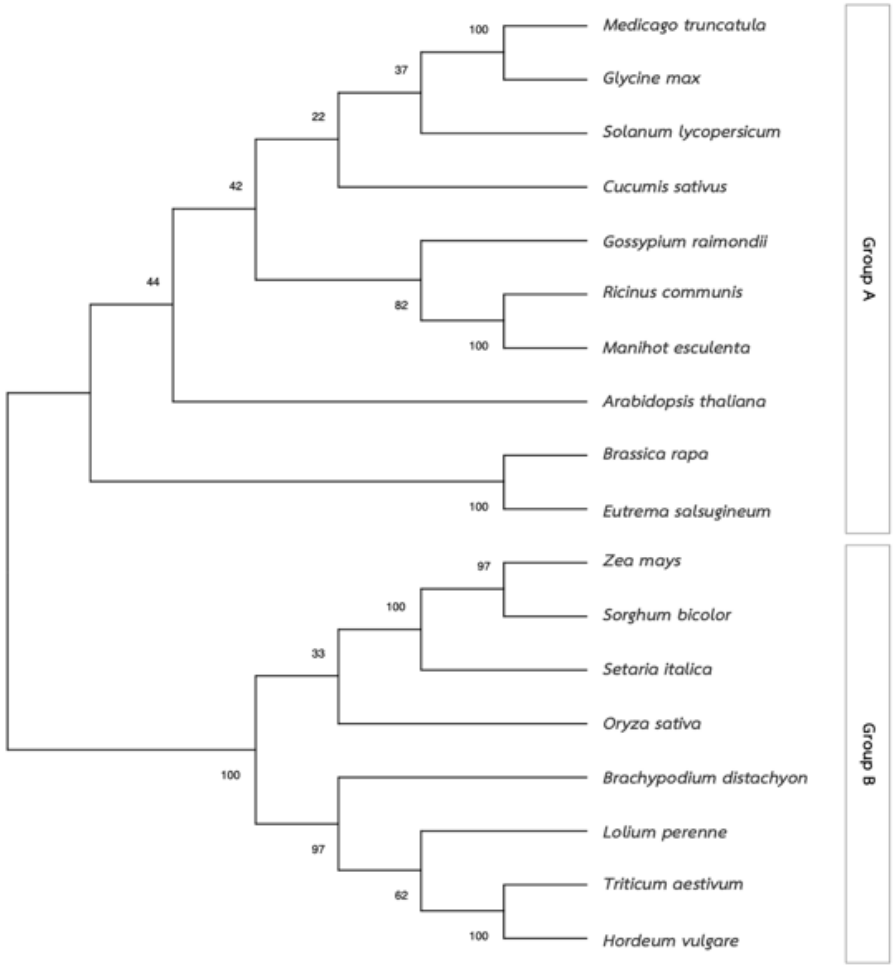

Figure 2 Phylogenetic analysis of the *LOC_Os01g66860* gene across 18 plant species, constructed using the Maximum Likelihood method in MEGA X based on amino acid sequences. The species analyzed and their accession numbers are listed in Supplementary Table S2.

of transcription factor IIA (*TFIIAγ*), a general transcription factor in eukaryotes that had not been traditionally linked to plant defense mechanisms. Sequence comparisons between resistant and susceptible rice isolines revealed two nucleotide substitutions in the *TFIIAγ* gene, resulting in an amino acid change that differentiates the resistance phenotypes. This genetic variation was consistently observed across multiple resistant and susceptible Aus-Boro rice lines. Similar to *xa5*, the variation identified in *LOC_Os01g66860* may represent a novel mechanism contributing to bacterial leaf blight resistance in rice.

However, further studies involving functional validation, such as gene knockout or overexpression assays, would be instrumental in conclusively determining the specific role of the gene in conferring resistance.

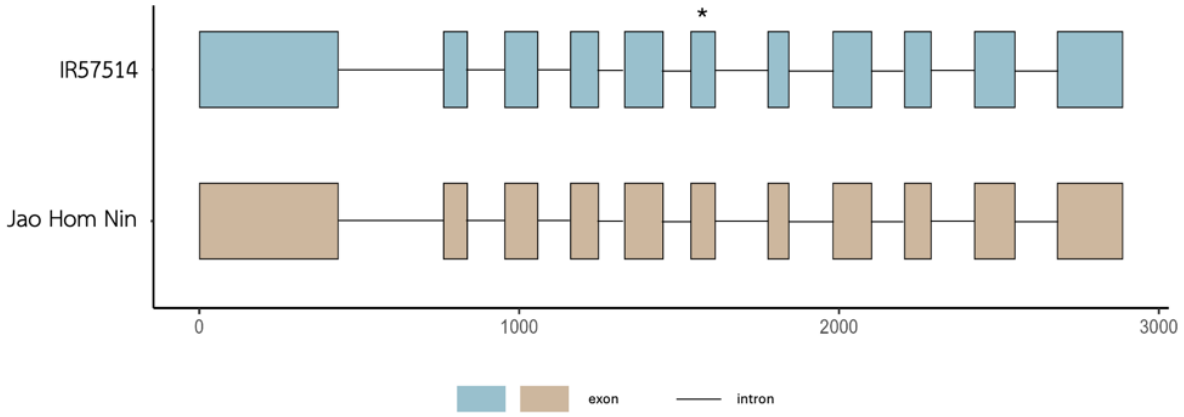

Figure 3 Gene structure of *LOC_Os01g66860* in the rice cultivars Jao Hom Nin and IR57514. The x-axis indicates sequence length (bp), and the y-axis lists the cultivars. Exons are shown in brown for Jao Hom Nin and blue for IR57514; introns are represented by black lines. The asterisk (*) denotes the mutation site at exon 6. The full-length *LOC_Os01g66860* gene was cloned from genomic DNA of both the susceptible cultivar Jao Hom Nin and the resistant cultivar IR57514. The gene comprises 11 exons and 10 introns, with a total length of 2,887 base pairs.

```
                                                  *
IR57514             GGTGATTTATCAAGCTGTATTCCGAGGAAAGGAAAGCTACATGGTCAGAAGGTTCTAAAATATGGCCTTGAGATCGC   1614
Jao Hom Nin         GGTGATTTATCAAGCTGTATTCCGAGGAAAGGAAAGCCACATGGTCAGAAGGTTCTAAAATATGGCCTTGAGATCGC   1614
```

Figure 4 Alignment of exon 6 sequences of *LOC_Os01g66860* from IR57514 and Jao Hom Nin. The asterisk (*) marks the mutation site. DNA sequencing and alignment revealed a single nucleotide polymorphism in exon 6: cytosine (C) was present at position 1574 in Jao Hom Nin, whereas thymine (T) occupied the same position in IR57514.

```
                                                    *
 IR57514             TQNIPMMIISEYLPNGDLSSCIPRKGKLHGQKVLKYGLEIA      300
 Jao Hom Nin         TQNIPMMIISEYLPNGDLSSCIPRKGKPHGQKVLKYGLEIA      300
```

Figure 5 Amino acid alignment highlighting a point mutation (*) in the coding sequence of *LOC_Os01g66860*, showing a single amino acid substitution at position 287, with leucine (L) in IR57514 replaced by proline (P) in Jao Hom Nin

CONCLUSIONS

This study identified *LOC_Os01g66860* as encoding a serine/threonine protein kinase that is upregulated following bacterial leaf blight (BLB) inoculation in both the susceptible cultivar Jao Hom Nin (JHN) and the resistant cultivar IR57514. Phylogenetic analysis revealed that orthologs of this gene are conserved across major plant lineages, highlighting its potential evolutionary importance. A notable base substitution between the two cultivars led to a single amino acid change at position 287 within the 500-residue protein sequence, which may contribute to their contrasting resistance responses. Given its differential expression and sequence variation correlated with resistance, *LOC_Os01g66860* represents a promising candidate for developing molecular markers. These markers could facilitate marker-assisted selection (MAS) or be integrated into genomic selection models to accelerate breeding of BLB-resistant rice cultivars. However, further functional characterization is required to confirm its role in plant immune responses and determine its utility as a reliable resistance gene in breeding applications.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest related to this research.

ACKNOWLEDGEMENTS

This research was financially supported by the Faculty of Science, Kasetsart University, Thailand. Arisa Laisupannawong received financial support through a fellowship from the Development and Promotion of Science and Technology Talents Project (DPST).

REFERENCES

Danaisilichaichon, C., P. Vejchasarn, S. Patarapuwadol, A. Tondelli, G. Valè, T. Toojinda and C. Jantasuriyarat. 2023. Genome-wide association study using genotyping by sequencing for bacterial leaf blight resistance loci in local Thai indica rice. Agronomy. 13(5): 1286. https://doi.org/10.3390/agronomy13051286.

Dilla-Ermita, C.J., E. Tandayu, V.M. Juanillas, J. Detras, D.N. Lozada, M.S. Dwiyanti, C. Vera Cruz, E.G.N. Mbanjo, E. Ardales, M.G. Diaz, M. Mendioro, M.J. Thomson and T. Kretzschmar. 2017. Genome-wide association analysis tracks bacterial leaf blight resistance loci in rice diverse germplasm. Rice. 10: 8. https://doi.org/10.1186/s12284-017-0147-4.

Dufayard, J.F., M. Bettembourg, I. Fischer, G. Droc, E. Guiderdoni, C. Périn, N. Chantret and A. Diévart. 2017. New insights on leucine-rich repeats receptor-like kinase orthologous relationships in angiosperms. Front. Plant Sci. 8: 381. https://doi.org/10.3389/fpls.2017.00381.

Fiyaz, R.A., D. Shivani, K. Chaithanya, K. Mounika, M. Chiranjeevi, G.S. Laha, B.C. Viraktamath, L.V. Subba Rao and R.M. Sundaram. 2022. Genetic improvement of rice for bacterial blight resistance: Present status and future prospects. Rice Sci. 29(2): 118–132. https://doi.org/10.1016/j.rsci.2021.08.002.

Gasteiger, E., A. Gattiker, C. Hoogland, I. Ivanyi, R.D. Appel and A. Bairoch. 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 31(13): 3784–3788. https://doi.org/10.1093/nar/gkg563.

Gnanamanickam, S.S. 2009. Biological control of bacterial blight of rice, pp. 67–78. *In:* S.S. Gnanamanickam, (Ed.), Biological Control of Rice Diseases. Progress in Biological Control, Vol. 8. Springer, Dordrecht, the Netherlands.

Gu, K., B. Yang, D. Tian, L. Wu, D. Wang, C. Sreekala, F. Yang, Z. Chu, G.L. Wang, F.F. White and Z. Yin. 2005. *R* gene expression induced by a type-III effector triggers disease resistance in rice. Nature. 435: 1122–1125. https://doi.org/10.1038/nature03630.

He, G., X. Luo, F. Tian, K. Li, Z. Zhu, W. Su, X. Qian, Y. Fu, X. Wang, C. Sun and J. Yang. 2006. Haplotype variation in structure and expression of a gene cluster associated with a quantitative trait locus for improved yield in rice. Genome Res. 16(5): 618–626. https://doi.org/10.1101/gr.4814006.

IRRI (International Rice Research Institute). 1996. Standard Evaluation System for Rice. 4th Edition. International Rice Research Institute, Los Baños, Philippines.

Iyer, A.S. and S.R. McCouch. 2004. The rice bacterial blight resistance gene *xa5* encodes a novel form of disease resistance. Mol. Plant Microbe Interact. 17: 1348–1354. https://doi.org/10.1094/mpmi.2004.17.12.1348.

Kauffman, H.E., A.P.K. Reddy, S.P.Y. Hsieh and S.D. Merca. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. Plant Dis. Rep. 57(6): 537–541.

Khush, G.S., D.J. Mackill and G.S. Sidhu. 1989. Breeding rice for resistance to bacterial blight, pp. 207–217. *In:* Bacterial Blight of Rice. International Rice Research Institute, Manila, Philippines.

Korinsak, S., C.T. Darwell, S. Wanchana, L. Praphaisal, S. Korinsak, B. Thunnom, S. Patarapuwadol and T. Toojinda. 2021. Identification of bacterial blight resistance loci in rice (*Oryza sativa* L.) against diverse *Xoo* Thai strains by genome-wide association study. Plants. 10(3): 518. https://doi.org/10.3390/plants10030518.

Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35(6): 1547–1549. https://doi.org/10.1093/molbev/msy096.

Larsson, A. 2014. AliView: A fast and lightweight alignment viewer and editor for large datasets. Bioinformatics. 30(22): 3276–3278. https://doi.org/10.1093/bioinformatics/btu531.

Liu, P.L., L. Du, Y. Huang, S.M. Gao and M. Yu. 2017. Origin and diversification of leucine-rich repeat receptor-like protein kinase (*LRR-RLK*) genes in plants. BMC Evol. Biol. 17: 47. https://doi.org/10.1186/s12862-017-0891-5

Mew, T.W., A.M. Alvarez, J. Leach and J. Swings. 1993. Focus on bacterial blight of rice. Plant Dis. 77(1): 5–12.

Mundt, C.C. 2014. Durable resistance: A key to sustainable management of pathogens and pests. Infect. Genet. Evol. 27: 446–455. https://doi.org/10.1016/j.meegid.2014.01.011.

Muthayya, S., J. Hall, J. Bagriansky, J. Sugimoto, D. Gundry, D. Matthias, S. Prigge, P. Hindle, R. Moench-Pfanner and G. Maberly. 2012. Rice fortification: An emerging opportunity to contribute to the elimination of vitamin and mineral deficiency worldwide. Food Nutr. Bull. 33(4): 296–307. https://doi.org/10.1177/156482651203300410.

Muthayya, S., J.D. Sugimoto, S. Montgomery and G.F. Maberly. 2014. An overview of global rice production, supply, trade, and consumption. Ann. N. Y. Acad. Sci. 1324(1): 7–14. https://doi.org/10.1111/nyas.12540.

Ou, S.H. 1985. Rice Diseases. 2nd Edition. Commonwealth Mycological Institute, Surrey, UK.

Podishetty, N.K. 2014. Bacterial leaf blight resistance gene, *Xa33* in rice: Identification and genetic characterization of new bacterial leaf blight (BLB) resistance gene in rice. Scholars' Press.

Pradhan, S.K., D.K. Nayak, S. Mohanty, L. Behera, S.R. Barik, E. Pandit, S. Lenka and S. Anandan. 2015. Pyramiding of three bacterial blight resistance genes for broad-spectrum resistance in deepwater rice variety, Jalmagna. Rice. 8: 19. https://doi.org/10.1186/s12284-015-0051-8.

Safder, I., G. Shao, Z. Sheng, P. Hu and S. Tang. 2021. Identification of SNPs in rice *GPAT* genes and *in silico* analysis of their functional impact on *GPAT* proteins. Not. Bot. Horti Agrobot. Cluj-Napoca. 49(3): 12346. https://doi.org/10.15835/nbha49312346.

Shu, X., A. Wang, B. Jiang, Y. Jiang, X. Xiang, X. Yi, S. Li, Q. Deng, S. Wang, J. Zhu, Y. Liang, H. Liu, T. Zou, L. Wang, P. Li and A. Zheng. 2021. Genome-wide association study and transcriptome analysis discover new genes for bacterial leaf blight resistance in rice (*Oryza sativa* L.). BMC Plant Biol. 21: 255. https://doi.org/10.1186/s12870-021-03041-2.

Srinivasan, B. and S.S. Gnanamanickam. 2005. Identification of a new source of resistance in wild rice, *Oryza rufipogon* to bacterial blight of rice caused by Indian strains of *Xanthomonas oryzae* pv. *oryzae. Curr.* Sci. 88(8): 1229–1231.

Sundaram, R.M., M.R. Vishnupriya, S.K. Biradar, G.S. Laha, G.A. Reddy, N.S. Rani, N.P. Sarma and R.V. Sonti. 2008. Marker assisted introgression of bacterial blight resistance in Samba Mahsuri, an elite indica rice variety. Euphytica. 160: 411–422. https://doi.org/10.1007/s10681-007-9564-6.

Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm and S.G. Rozen. 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40(15): e115. https://doi.org/10.1093/nar/gks596.