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Overexpression of *OsTlp* Improves Resistance of MR 219 Rice (*Oryza sativa* L. ssp. *indica*) to Sheath Blight

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ABSTRACT

Sheath blight, caused by *Rhizoctania solani*, is one of the major diseases affecting global rice production. The lack of resistant rice accessions emphasises the necessity for modern biotechnology approaches to provide an alternative strategy towards selective breeding. Previously, the differential expression of several pathogenesis-related (PR) protein genes revealed the thaumatin-like protein (*OsTlp*) gene, part of the pathogenesis-related PR5 family, uniquely expressed post-infection in the moderately resistant rice cultivar, Tetep, compared to the susceptible check, IR 64. In this study, we isolated the *OsTlp* gene from Tetep and used *Agrobacterium*-mediated transformation to introduce it into the local rice cultivar, MR 219, under a constitutive promoter. We report the performance of several transgenic lines of MR 219, cultivated up to the third generation (T_3) in controlled conditions. Three independent T_3 lines were selected for sheath blight resistance analysis via artificial inoculation with *R. solani* at 45 days after sowing. Disease severity was measured using relative lesion height (RLH) 30 days post-infection. Two promising transgenic lines (PL3-4 and PL6-1) exhibited enhanced tolerance, with average RLH values below 11%, while control plants showed RLH $\geq$ 30%. Additionally, gene expression analysis via quantitative reverse transcription polymerase chain reaction (qRT-PCR) demonstrated that *OsTlp* was overexpressed in the transgenic lines by up to fivefold compared to those expressed in Tetep. These findings underscored the potential of genetic engineering in enhancing rice resilience against sheath blight and addressing future food security challenges.

Keywords: *Agrobacterium*-mediated transformation, rice, sheath blight disease, thaumatin-like protein, transgenic.

INTRODUCTION

Rice (*Oryza sativa* L.) is among the staple crops that serve as a primary food source for half of the world's population, particularly in Asia. To ensure stable rice production for the growing population, disease incidence has to be reduced effectively in addition to providing the best physiological conditions for growth. Among the issues faced in typical rice plantations, sheath blight (ShB) disease, caused by the broad-spectrum, soil-borne pathogenic fungus *Rhizoctonia solani*, has been documented throughout the temperate

and tropical rice growing areas, including Malaysia (Gangopadhyay and Chakrabarti, 1982; Dasgupta, 1992; Sivalingam et al., 2006; Faizal Azizi and Lau, 2022). Historically, ShB was initially reported in Japan over a century ago (Miyake et al., 1910). According to Khaing et al. (2014), ShB can cause a reduction in yield of up to 25% and affect 50% of all countries that produce global rice production. Furthermore, ShB is well-regarded as a significant rice disease in all rice-growing regions globally, especially in areas with high levels of production (Jayaprakashvel and Mathivanan, 2012; Faizal Azizi and Lau, 2022).

In Malaysia, the occurrence of ShB poses a significant problem in the rice cultivation regions, resulting in considerable annual reductions in crop yield (Srinivasachary et al., 2011). Currently, it has become widespread in many rice cultivation areas in Malaysia, leading to reported revenue losses of approximately 50% during severe attacks (Razman, 2017). In nature, *R. solani* survives as either sclerotia or mycelia in plant debris. Currently, the Paddy and Rice Research Centre at MARDI is actively conducting conventional breeding to develop high-quality rice varieties that are resistant to major pests and diseases, aiming to ensure high yields. However, breeding for resistance to ShB faces several setbacks due to the lack of identified resistant donors in cultivated germplasm (Chen et al., 2023). To date, no rice cultivar has been found to be completely resistant to *R. solani*, although varieties and landraces with varying levels of resistance have been reported (Zeng et al., 2011; Hossain et al., 2023). As a result, chemical protection remains the primary method for controlling this disease (Zhao et al., 2008; Chen et al., 2023). Fungicide application to control this disease is neither economical nor environmentally friendly, and methods using biological controls in the field are relatively uncertain (Tan et al., 2010; Khaing et al., 2014).

To further understand rice-pathogen interactions, studies on the plant responses to biotic and abiotic stresses have been reported in terms of quantitative and qualitative resistance. Quantitative resistance is a complex trait influenced by multiple genes — often associated with quantitative trait loci (QTLs) — and studies have resorted to gene co-expression network (GCN) analysis to garner a deeper understanding of genotype-phenotype correlations (Zainal-Abidin et al., 2022). Genetic and molecular evidence has suggested that quantitative resistance can be race-nonspecific and even species-nonspecific pathogen, implying that the genetic mechanisms underlying quantitative resistance are broad and can confer protection against a range of pathogenic races or species (Kou and Wang, 2012; Pilet-Nayel et al., 2017). The defence mechanisms observed in this type of resistance can be attributed to a variety of factors, including morphological and ecological characteristics of the host plant (Jwa et al., 2006). It is worth noting that the genetic basis of quantitative resistance to *R. solani* is complex, involving the participation of several genes in conferring sheath blight resistance in rice, and this is known as co-expressed genes (Chen et al., 2023; Hossain et al., 2023).

In addition to quantitative resistance, plants produce pathogenesis-related (PR) proteins that act as antimicrobial agents against invading pathogens. Generally, PR proteins are produced in response to effector genes produced by invading pathogens and play important roles in plant defence (Abu Bakar et al., 2017; Juri et al., 2020). There have been multiple reports indicating that the overexpression of PR protein genes, such as thaumatin-like protein (PR5), chitinase (PR3), and β-glucanase (PR2) genes in rice, lead to enhanced resistance against *R. solani* in susceptible rice (Sridevi et al., 2008; Naseri et al., 2012; Richa et al., 2017). Our previous study showed significant differences in the expression profiles of the rice thaumatin-like protein gene (*OsTlp*) between resistant Tetep and susceptible IR64 (*Oryza sativa* ssp. *indica*) when challenged with *R. solani* (Ahmad Redzuan et al., 2015). Tetep is a well-studied Vietnamese *indica* rice landrace known for its resistance to sheath blight and blast diseases (Suharti et al., 2016; Biswas et al., 2023). Therefore, this gene was hypothesised to be involved in the defence system, which allowed Tetep to tolerate the ShB infection. The antifungal activities of PR5 have also been reported previously (Datta et al., 1999; Singh et al., 2013; Sharma et al., 2022), suggesting that the gene encoding the protein is an excellent one to be used in the genetic engineering of crops.

In this study, our goal was to utilise genetic engineering to overexpress the *OsTlp* gene, which was isolated from Tetep, in MR 219 rice (a ShB-susceptible Malaysian cultivar). The entire process involved isolating and characterising the *OsTlp* coding DNA region (CDS), developing an *OsTlp* gene cassette driven by a strong promoter, and using the *Agrobacterium*-mediated transformation method to produce transgenic rice. After obtaining the transgenic lines, the plants were exposed to *R. solani* in a controlled and contained

environment (specifically at the Transgenic Glasshouse, MARDI), to assess the level of resistance. Disease resistance screening was conducted from the first generation (T_1) up to the third generation (T_3) of transgenic plants. Conducting multi-generational screening of transgenic plants is important to confirm consistent performance before proceeding with experiments in confined or open environments to confirm the desired traits. A total of three potential transgenic rice lines (T_3) (identified from the screening of T_2 lines with 20 replicates for each line) were planted prior to this work. Therefore, in this study, we used the standard method developed by the Paddy and Rice Research Centre at MARDI for sheath blight screening. In this paper, we highlight the screening results for the T_3 generation transgenic rice against *R. solani.*

MATERIALS AND METHODS

Isolation and Characterisation of Thaumatin-Like Protein (*OsTlp*) Gene

The genomic DNA was extracted from the Vietnamese *indica* landrace Tetep using the DNeasy Plant Mini Kit (Qiagen, Germany). Based on the gene sequence determined by Ahmad Redzuan et al. (2015), gene-specific primers, *OsTlp Forward* (5'-CCATGGATGGCGTCTCCGGCC-3') and *OsTlp Reverse* (5'-CACGTGTTATGGGCAGAAGACGACTTGG-3') containing *Nco*I (CCATGG) and *Pml*I (CACGTG) restriction enzyme (RE) sites (underlined), were used to simultaneously isolate and introduce restriction enzyme sites to the *OsTlp* gene. PCR reactions were conducted in a 25 mL reaction mixture comprising 1× PCR buffer, 1.0 mM MgCl2, 0.2 mM of each dNTP, 0.5 mM of each primer, 0.5 unit (U) of Taq DNA polymerase (Qiagen GmbH, Germany), and 100 ng of template DNA. The PCR conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s, and extension at 72 °C for 50 s. A final extension step was performed at 72 °C for 10 min. The amplicon was then purified and sent for sequencing.

The CDS of the gene was characterised using a set of bioinformatics tools, including ClustalW (BioEdit, Raleigh, NC, USA) and the nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The ExPASy translate tool (SIB Bioinformatics Resource Portal, Lausanne, Switzerland) was employed to convert the *OsTlp* nucleotide sequence into its corresponding amino acid sequence. The AlphaFold Protein Structure Database (AlphaFold DB) (Jumper et al., 2021) was used to search for matching amino acids and predict the 3D structure of the protein. Additionally, the amino acid sequence was submitted to Plant-mSubP (https://bioinfo.usu.edu/Plant-mSubP/) for predicting the subcellular localisation of the protein using hybrid prediction modules of pseudo amino acid composition, N-Center-C terminal amino acid composition, and dipeptide composition (PseAAC-NCC-DIPEP). STRING (https://string-db.org/) was then employed to study the interaction network between *OsTlp* and other associated proteins in rice.

Gene Cassette Development

The isolated CDS of *OsTlp* was cloned into the pGEM-T Easy vector (Promega, USA) following the manufacturer's protocol, and the plasmid was transformed into *Escherichia coli* TOP10 competent cells. Subsequently, bacterial colony PCR was performed to screen for successful transformants. The plasmid DNA was purified from a positive clone and digested with restriction enzymes *Nco*I and *Pme*I (New England Biolabs (NEB), New York) to release and generate *OsTlp* with overhangs. The released insert was then sub-cloned in a sense orientation into linearised plant binary vector pCAMBIA 1305.2 (digested with the same restriction enzymes, *Nco*I and *Pme*I). This vector also contained the *hpt*II (hygromycin phosphotransferase) gene as a selectable marker in the T-DNA region. The gene was driven by the 35S promoter from the *Cauliflower mosaic virus* (CaMV 35S) and terminated by the *nopaline synthase* terminator (*nos*-T). Finally, the orientation and the reading frame of all transgenes inside the vector were

confirmed through sequencing analysis. The validated vector was then mobilised into *Agrobacterium tumefaciens* strain EHA105 before proceeding to *Agrobacterium*-mediated transformation.

Embryogenic callus induction and *Agrobacterium*-mediated transformation

Mature seeds of MR 219 rice were obtained from the National Rice Genebank, MARDI Seberang Perai, Penang, Malaysia. The mature seeds were dehusked and surface-sterilised with 80% (v/v) ethanol for 1 min, followed by shaking in 1% (v/v) Clorox bleach for 30 min, and then in 70% (v/v) sodium hypochlorite (NaOCl) for 30 min. The seeds were rinsed thoroughly with sterile distilled water five times (1 min each) to remove traces of NaOCl. They were then dried on sterile filter papers in a petri dish and cultured on callus induction media. The callus induction media consisted of Gamborg's B5 basal medium, supplemented with 0.1 g/L arginine, 0.1 g/L L-glutamine, 0.1 g/L asparagine, 10 g/L maltose, 10 mg/L 1-naphthaleneacetic acid (NAA), 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified with 4 g/L Gelrite (Sigma). After 2 to 3 weeks, developing calli became visible on the scutellum of the mature seeds. These calli were subcultured onto fresh induction medium for further proliferation.

One-month-old embryogenic calli were subjected to *Agrobacterium*-mediated transformation using *A. tumefaciens* strain EHA105 carrying the pCAMBIA-PR5 plasmid (Figure 2). For *Agrobacterium* infection, the density of *Agrobacterium* used was OD_{600} 0.3. Embryogenic rice calli were immersed in a bacterial suspension for 30 min, blotted on sterile filter paper to remove excess suspension, and then transferred to co-cultivation media. The calli were co-cultivated for 48 h on co-cultivation medium consisting of Gamborg's B5 basal medium, containing 0.1 g/L arginine, 0.1 g/L L-glutamine, 0.1 g/L asparagine, 10 g/L maltose, 10 g/L glucose, 10 mg/L NAA, 1 mg/L 2,4-D, 0.2 µM acetosyringone, and 4 g/L gelrite. After 48 h of co-cultivation, the calli, which exhibited an overgrowth of *Agrobacterium*, were washed 3 - 4 times with sterile distilled water containing 250 mg/L cefotaxime and 250 mg/L carbenicillin. They were then dried on sterile filter paper and transferred onto the first selection medium consisting of MS medium (Murashige and Skoog, 1962), supplemented with 50 mg/L hygromycin. Selections of the transformed tissues were carried out for 3 months on selection media, subcultured every 2 weeks. A total of 6000 calli have been transformed with the *OsTlp* gene cassette (pCAMBIA-PR5), and 500 embryogenic calli were transformed with the empty vector pCAMBIA 1305.2 as a control.

Regeneration of Transformed Calli and Acclimatisation of Plantlets

After the selection process, the putatively transformed calli were transferred to regeneration media consisting of MS medium supplemented with 1 mg/L kinetin, 2 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L NAA. For the regeneration phase, the microcalli were first incubated at 25 ± 2 °C in the dark for 7 days. Subsequently, they were cultured on fresh regeneration medium (RM) and subjected to alternating dark and light conditions (1600 lx, 12 h of light per day, followed by 12 h of darkness) at 25 ± 2 °C for 8 months. During this time, the calli were subcultured monthly on fresh RM until the shoots emerged.

For root development, 3 - 4 cm regenerated shoots were transferred to MS media without plant growth regulators and incubated under the same alternating dark and light conditions for 1 month. Once rooted, the plantlets were transferred to vermiculite supplemented with MS liquid and grown for another month before being transplanted into soil for further growth. Finally, the plantlets were moved to the transgenic glasshouse for hardening. The temperature inside the glasshouse was approximately 35 - 37 °C, with approximately 90% light transmission and a relative humidity between 80% and 90%. The survival rates of the plantlets were recorded 4 weeks after being transferred to this transgenic glasshouse.

Polymerase Chain Reaction Analysis of Transgenic Plants

To verify the presence of the T-DNA in the regenerated putatively transformed rice plants (T_0) and subsequent generations (T_1 - T_3), polymerase chain reaction (PCR) analysis was carried out using the gene-specific primers (Table 1) to detect both transgenes (*OsTlp* and *hpt*II genes). The total genomic DNA was

extracted from young rice leaves using the DNeasy Plant Mini Kit (Qiagen, Germany). Approximately 50 ng of each extracted genomic DNA sample was used for PCR analysis. PCR reactions were carried out in a 25 mL reaction mixture containing 1× PCR buffer, 1.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM of each primer, 0.5 unit (U) of Taq DNA polymerase (Qiagen GmbH, Germany), and 100 ng of template DNA. The PCR conditions were the same as those used for gene isolation, except that the annealing temperature (Ta) was set to 62 °C.

Table 1. The primers used for PCR analysis of transformed MR 219 rice plants. These two sets of primers amplify *OsTlp* (with the respective RE junctions underlined) and *hpt*II transgenes

Primer	Sequence (5'-3')	Length of primer	Size of amplicon
OsTlp Forward	ACTCTTGACCATGGCGTCTCC	21	552
OsTlp Reverse	CAATTCACACGTGTTATGGGC	21	552
*hpt*II *Forward*	GTCGGTTTCCACTATCGG	18	984
*hpt*II *Reverse*	CTGAACTCACCGCGACGTCTGT	22	984

Expression Analysis of the *OsTlp* Gene by Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Fresh leaves of each potential T_3 transgenic line were collected and ground to a fine powder with a mortar and pestle in liquid nitrogen, and 100 mg of the material was used for RNA isolation. The total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. Purity and concentration of RNA samples were measured using a Nanodrop spectrophotometer, and integrity was checked on agarose gel electrophoresis. RNA samples with a 260/280 ratio between 1.9 and 2.1 were used for subsequent experimentation. The removal of genomic DNA contamination and first strand cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions in a total volume of 20 µL containing 1 µg total RNA. Specific primer pairs for gene of interest (*OsTlp*) and housekeeping genes (*OsGADPH*, *OsNABP*, and *OsEF1a*) were designed using Beacon Designer 8 software, with melting temperatures (Tm) of 58 - 62 °C and a GC content of 40 - 60% (Table 2). All primer pairs were synthesised by a commercial supplier (Apical Scientific Sdn. Bhd.) and were used to amplify the target gene by PCR and analysed by electrophoresis on 1.5% (w/v) agarose gels. SYBR Green (QuantiTect SYBR Green RT-PCR Kit, Qiagen, Germany), was conducted following the manufacturer's recommended protocol. RNA template (500 ng) was incubated at 55 °C for 30 min for cDNA synthesis, 2 min at 95 °C for denaturation, followed by PCR amplification with the subsequent conditions which were initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 40 s; and final extension for 5 min at 72 °C.

Screening of Transgenic Plants Against *Rhizoctonia solani*

The screening (artificial infection) and evaluation of the transgenic MR 219 lines against *R. solani* were conducted in a controlled and enclosed environment at Wing 1, Transgenic Glasshouse, MARDI Headquarters, in compliance with the Biosafety Act Regulation 2007. Furthermore, two control groups were included: MR 219 wild type (WT) and MR 219 transformed with the empty pCAMBIA1305.2 vector (VC). Pure mycelia from virulent *R. solani* was cultured on potato dextrose agar (PDA) (Oxoid, United Kingdom) at 22 - 24 °C, inoculated to rice straws, and incubated for 7 days at 28 °C. Then, five rice straws (approximately 10 cm each) were tied around the base of each transgenic and control plant. This artificial infection was carried out on rice plants 45 days after sowing. The infected plants were left in a surrounding

area that was maintained at 80 - 90% humidity. Plants were grown at 35 - 37 °C under natural light in standard greenhouse conditions.

Table 2. Primer sequences and amplicon sizes of *OsTlp* and reference genes used for qRT-PCR experiment

Gene name	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)
Pathogenesis-related thaumatin-like protein 5 (*PR5-tlp*)	*qTlp*	F: CAGCCAGGACTTCTACGA R: TGTGTCTTGGTGTTGTCTTC	144
Glyceraldehyde 3-phosphate dehydrogenase (*OsGAPDH*)	*OsGAPDH*	F: AAGCCAGCATCCTATGATCAGATT R: CGTAACCCAGAATACCCTTGAGTTT	79
Nucleic acid binding protein (*OsNABP*)	*OsNABP*	F: GGAATGTGGACGGTGACACT R: TCAAAATAGAGTCCAGTAGATTTGTCA	100
Elongation factor 1-alpha (*OsEF1a*)	*OsEF1a*	F: TGGTATGGTGGTGACCTTTG R: GTACCCACGCTTCAGATCCT	151

To evaluate ShB resistance or susceptibility in rice cultivars, the lesion length and the degree of disease severity in each sheath of inoculated plants were recorded following the Standard Evaluation System (SES) for rice (IRRI, 2002). The degree of resistance to ShB disease was assessed after 30 days post-infection, which included the height of each infection's lesions as well as visual records of ShB severity. The total lesion spread and RLH (%) were used to compare the relative resistance and susceptibility of transgenic and non-transgenic lines to *R. solani*. The degree or score of resistance was assigned as follows: 0 = no lesion, 1 = a lesion that appears less than 20%, 3 = a lesion that appears between 20 - 30%, 5 = a lesion that appears between 31 - 45%, 7 = a lesion that appears 46 - 65%, and 9 = a lesion that appears more than 65% and results in leaf death. The analysis of variance of the susceptibility index and the lesion length caused by *R. solani* was performed using SAS (version 6.12).

RESULTS AND DISCUSSION

Sequence Characterisation and *In Silico* Analysis of *OsTlp*

The *OsTlp* CDS isolated from Tetep is 534 bp long and is located on chromosome 12 (Figure 1). It harbours three single nucleotide polymorphisms (SNPs) when compared to the sequences from Zhong (accession no. CP141108.1) and Nipponbare (Accession no. NM_001423635.1); yet these variations are silent, causing no changes at the amino acid level. It was further demonstrated that the encoded amino acid sequence exhibits 100% sequence similarity to the thaumatin-like protein found in *japonica* rice (Uniprot accession no. P31110), and it has a 79% probability of being an extracellular and secreted protein. Previously, Zhang et al. (2018) confirmed that the wheat homolog, TaLr35PR5 protein, was secreted into the apoplast region, which supported our prediction on the protein localisation prediction for OsTlp. Structure reconstruction using AlphaFold (https://alphafold.ebi.ac.uk/) visualised that OsTlp contains the three conserved TLP domains namely Domain I which is a β-barrel protein core, Domain II which is an α-helix loop, and Domain III which is a β-hairpin region (Figure 1C). This was similar to the findings reported by Zhang et al. (2018) and Faillace et al. (2021).

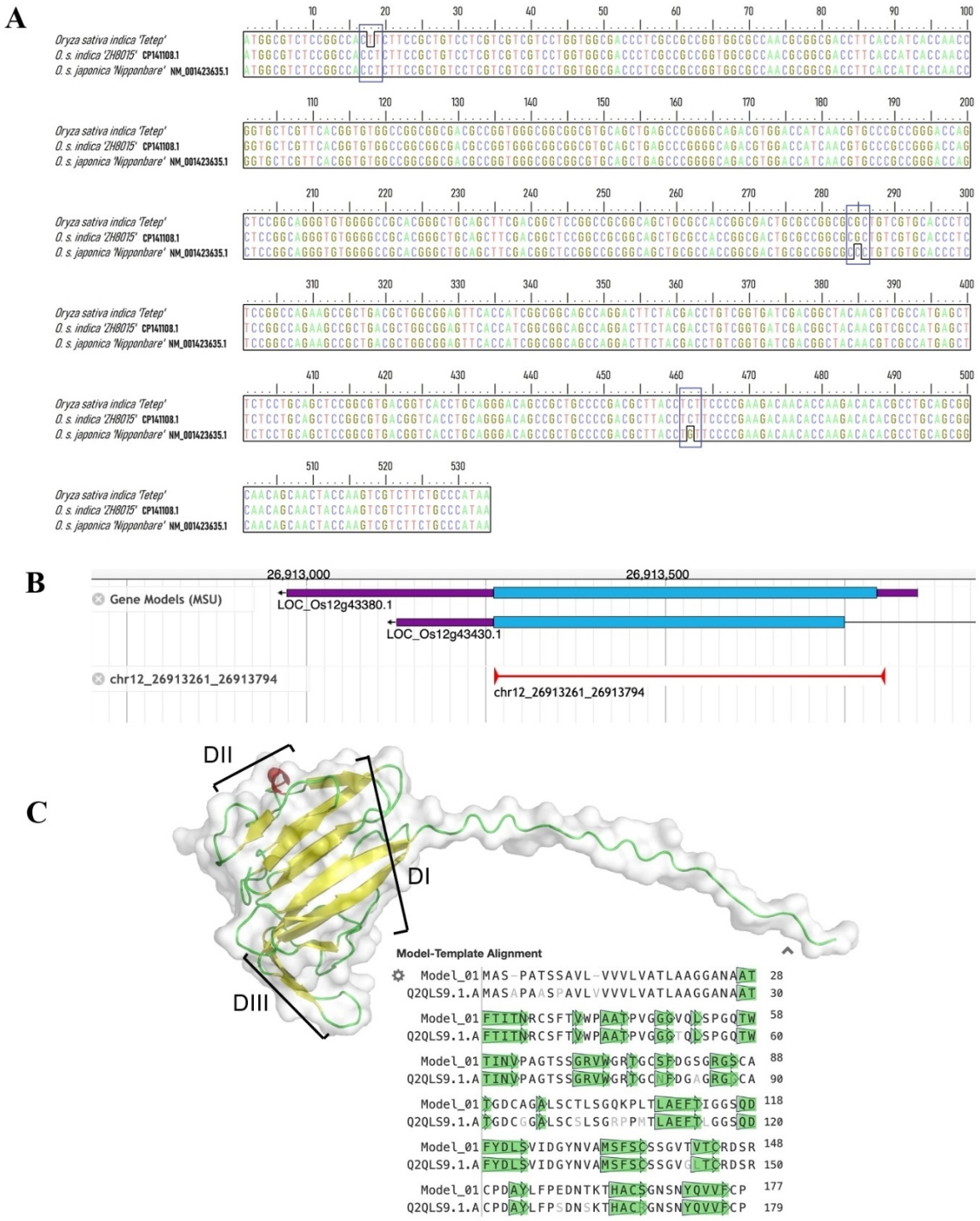

Figure 1. *In silico* analysis of the *OsTlp* sequence and three-dimensional (3D) structure prediction of the thaumatin-like protein isolated from Tetep. (A) The blue boxe indicates single nucleotide polymorphisms (SNPs) detected in Tetep's *OsTlp* (534 bp) through multiple alignments against orthologous *OsTlp* sequences from ZH8015 and Nipponbare rice. (B) The *OsTlp* gene is located on chromosome 12 and its CDS has a higher similarity to LOC_Os12g43380 than LOC_Os12g43430. (C) The predicted three-dimensional (3D) structure of OsTlp displays components of both α-helices and β-sheets, where the three conserved TLP domains (DI, DII, and DIII) are located.

Analysis of the protein-protein interaction (PPI) network showed that OsTlp (or TLP_ORYSJ) interacts with 10 other proteins or interactors (Figure 2). The associated proteins are listed in Table 3, though not discussed in detail within this article. However, A0A0P0VAM2 (Os01g0857300 protein) is of particular interest, as additional STRING analysis revealed its interaction with LYP4 (LysM domain-containing GPI-anchored protein LYP4). LYP4, as reported by Liu et al. (2013), plays a role in plant innate immunity by recognising pathogens through its binding to bacterial peptidoglycans and fungal chitin elicitors. The predicted localisation of OsTlp within the apoplast appears to correlate with its function in binding to fungal chitin.

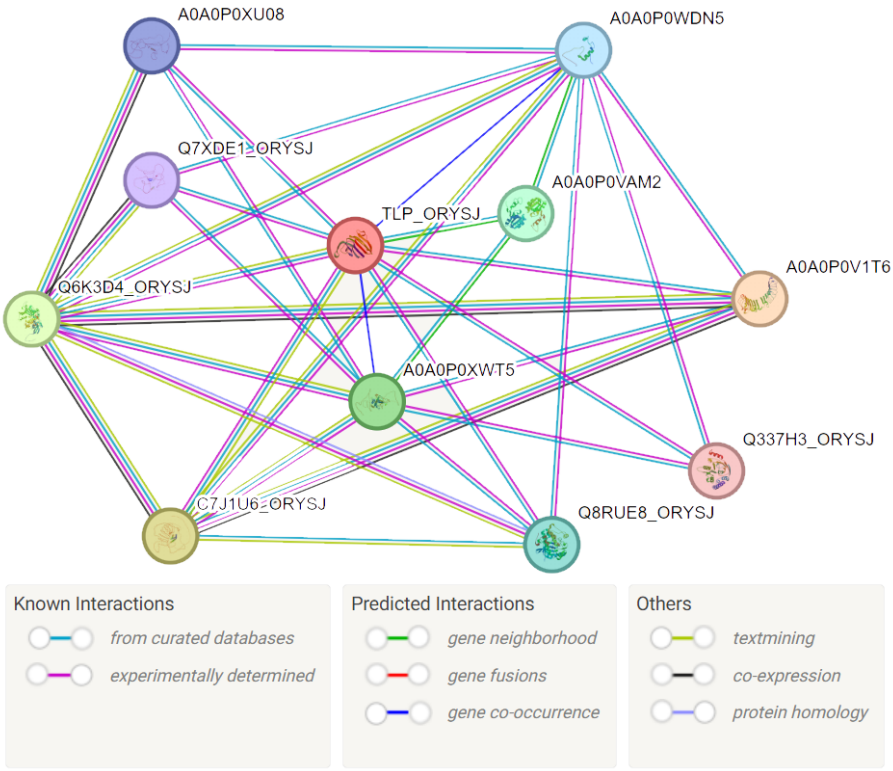

Figure 2. The protein-protein interaction (PPI) network of the query thaumatin-like protein (TLP_ORYSJ) with other ten interactors. The legend shows the coloured edges that represent protein-protein associations and interactions. The interaction network was analysed and generated using STRING (https://string-db.org/).

The Tlp/PR5 proteins are a highly complex protein family associated with host defence and developmental processes in plants, animals, and fungi. In plants, Tlp/PR5 proteins have various properties that are mostly associated with responses to biotic stresses and some predicted activities under drought and osmotic stresses (Zhang et al., 2018; de Jesús-Pires et al., 2020). Moreover, they have shown promise in enhancing disease resistance in various plant species and found to exhibit antifungal and antibacterial properties, making them effective in deterring the growth and development of pathogens (Xing et al., 2008; Liu et al., 2013; de Jesús-Pires et al., 2020). Acting as an antifungal protein, it would probably increase the permeability of the fungal membrane (Kalpana et al., 2006; Jesús-Pires et al., 2020). This notion was further supported by Zhang et al. (2018), as the authors reported restricted mycelial growth of *Puccinia triticina* (leaf rust fungal pathogen) when TaLr35PR5 protein was present. Dong (2001) also reported that accumulations of PR proteins in plants have been shown to induce systemic-acquired resistance that enables plants to survive against a broad range of pathogens. According to Jiao et al. (2018), the mechanism by which Tlp/PR5 disrupts fungal membranes and increases permeability involves several stages that ultimately lead to cell leakage and fungal cell death. Tlp/PR5 proteins destabilise the fungal

membrane by binding to specific lipids, inserting into the membrane, and promoting ion and molecular leakage. This disruption leads to loss of cellular integrity, ion imbalance, and eventual fungal cell death, which supports the plant's immune response against pathogens.

Table 3. Information and functions of the proteins interacting with the pathogenesis-related thaumatin-like protein PR5 (P31110). The data was obtained from AlphaFold, UniProt, GenBank, Gene Ontology (GO), and InterPro databases.

	Protein identifier	Protein	Information
1.	TLP_ORYSJ, P31110	Pathogenesis-related thaumatin-like protein gene (PR5)	An extracellular, antifungal protein involved in plant defence response
2.	A0A0P0XU08	DNA polymerase III subunits τ and γ, Os10g0372500 protein	Involved in DNA replications
3.	A0A0P0WDN5	Glycoside hydrolase family 64 (beta-1,3-glucanases), Os04g0576600 protein	Produces specific pentasaccharide oligomers
4.	Q7XDE1_ORYSJ, XP_015613845.1	Formin-like protein 7	May be involved in the organisation and polarity of actin cytoskeleton
5.	A0A0P0VAM2	Lipid IV(A) 3-deoxy-D-manno-octulosonic acid transferase, Os01g0857300 protein	Participates in various metabolic processes of carbohydrates and sucrose
6.	A0A0P0V1T6	Os01g0314700 protein	Involved in defence response to other organisms
7.	Q6K3D4_ORYSJ	Protein-serine/threonine phosphatase, Os02g0281000 protein	Involved in ATP and metal ion binding; myosin phosphatase and protein kinase activities
8.	A0A0P0XWT5	Thaumatin family pfam00314, Os10g0524900 protein	Involved in plant defence response
9.	Q337H3_ORYSJ	1-acyl-sn-glycerol-3-phosphate acyltransferase,	Involved in embryo development ending in seed dormancy; lipid metabolism - CDP-diacylglycerol, phosphatidic acid, and phosphatidylglycerol syntheses
10.	Q8RUE8_ORYSJ	Putative 3-phosphoinositide-dependent protein kinase-1, non-specific serine/threonine protein kinase	Involved in protein serine/threonine kinase activity and phosphorylation
11.	C7J1U6_ORYSJ	Conserved hypothetical protein, contains the region of large tegument protein UL36, Os04g0589400 protein	Unknown

Construct Development

The plant binary vector pCAMBIA 1305.2 has the *hpt*II gene in the T-DNA region, which is driven by the CaMV35S promoter and NOS terminator and confers resistance to the antibiotic hygromycin as a plant

selection marker (Figure 3A). The orientation, position, and CDS of *OsTlp* inside the vector were verified using both PCR and sequencing analyses. Based on PCR analysis, the expected amplicon was obtained (Figure 3B), and the sequencing analysis validated that the *OsTlp* gene (534 bp) was properly ligated to the *Nco*I (CCATGG**)** and *Pml*I (CACGTG) junctions in sense orientation. The positive clones were then mobilised into *Agrobacterium tumefaciens* strain EHA105 and subsequently used in the *Agrobacterium*-mediated transformation experiment.

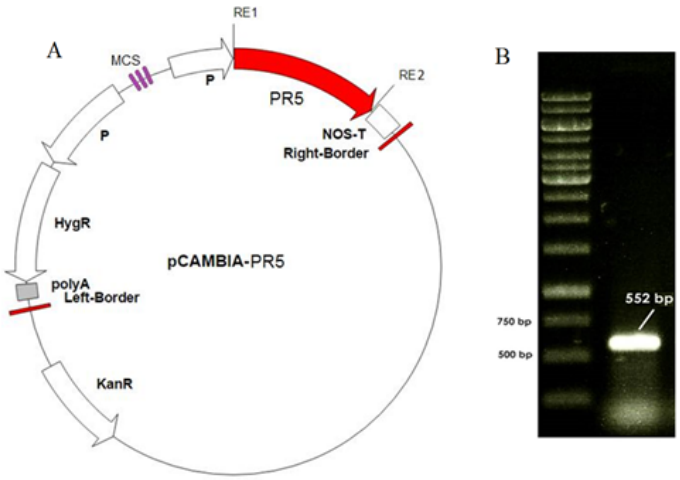

Figure 3. Development of the gene construct for MR 219 transformation. (A) The map of pCAMBIA-PR5 (10,336 bp) indicates the gene of interest *OsTlp* (in sense orientation) is controlled by CaMV35S promoter (P) and *nos* terminator (NOS-T). (B) The PCR amplification (Ta = 58 °C) of the gene insert (552 bp) from the vector validates the presence of the transgene.

Agrobacterium-Mediated Transformation, Regeneration of Transgenic Lines, and Molecular Analysis

In this study, over 98% of the cultured seeds produced embryogenic calli and the developed calli were friable, granular, and yellow in colour, which later transformed and regenerated into plantlets (Figure 4A-C). Based on the PCR analysis of the regenerated transformed T_0 plants, it was validated that a total of 11 T_0 lines contained the *hpt*II gene, whereas non-transgenic and control plants showed no amplification (Figure 4D). The transgenic lines were then transplanted into soil in pots and placed in a controlled and enclosed environment at the transgenic glasshouse for evaluation to determine the degree of resistance against *R. solani*. The first generation (T_1) seeds were harvested and replanted to carry out a resistance assessment study against the *R. solani* fungus.

Rice transformation with *A. tumefaciens* is the preferred method and was employed in this study because it enables stable and low-copy number integration of transfer-DNA (T-DNA) into the plant chromosome (Hiei and Komari, 2008). Recently, we have developed a simple method to deliver a plasmid containing gene cassettes into Malaysian rice, which bypassed callus induction and transformation (Tamizi et al., 2023). This advancement opens new prospects for robust and rapid genetic manipulation for rice, encompassing both genetic modification (transgenesis) and genome editing.

Screening and Evaluation of the Transgenic MR 219 T_3 Rice Lines for Resistant Against *R. solani*

An effective inoculation method is a critical component of a precise disease assay for quantifying ShB resistance levels during the screening process. The percentage of disease incidence that occurred in control plantlets was 89.1% after 30 days of infection, while the disease incidence in transgenic plants was 29.4%. The symptoms of infection started appearing in the wild type (WT) at 3 days post-infection (dpi), whereas

blighting was delayed by up to 14 dpi in transgenic T_3 lines expressing the *OsTlp* gene. Fungal infection or lesion spread was severe on the control WT plants compared with the transgenic plants (Figures 4F and 4G). According to the Standard Evaluation System (SES) for rice, non-transgenic plants (WT and VC) had an average RLH score ≥ 30% at 30 dpi, whereas three transgenic lines had an RLH score ≤ 15%. The most potential line, PL3-4, had an average scored relative lesion height (RLH) of 11% at 30 dpi (Figure 5).

From our study, it has been shown that the engineered MR 219 displayed an enhanced resistance against the pathogenic fungus compared to the control and showed no phenotypic or physiological abnormalities that could hamper their productivity. Our study provides additional evidence for the role of rice thaumatin-like protein in conferring tolerance to *R. solani* in non-resistant rice varieties, somehow consistent with the findings of Shah et al. (2013) and Naseri et al. (2012).

Gene Expression Profile of Transformed Transgene in Transgenic Rice Lines

For quantitative gene expression analysis, primers specific for the transgene and the housekeeping genes were designed as listed in Table 2. The results obtained indicated that the designed primer sets were sensitive and accurate for the qRT-PCR. Moreover, a single peak melting profile for each primer set obtained from melting curve analysis of the PCR product revealed the specificity of the designed primers. Quantitative real-time PCR (qPCR) was carried out on the cDNA of the T_3 generation of highly potential transgenic rice lines by using the dye-based detection SYBR Green approach.

Figure 4. *Agrobacterium*-mediated transformation of MR 219 rice based on embryogenic rice calli. (A) Embryogenic calli; (B) *Agrobacterium* infection of rice calli; (C) Putative transformed tissues with regenerated plantlets; (D) Molecular analysis of transformed rice lines (T_0) showing the amplicons of *hpt*II (984 bp) gene; (E) Planting of transgenic rice lines at the transgenic glasshouse; (F) Performance of

resistant transgenic and (G) control plants against *R. solani*, with lesions on the leaf sheaths indicated by white arrows.

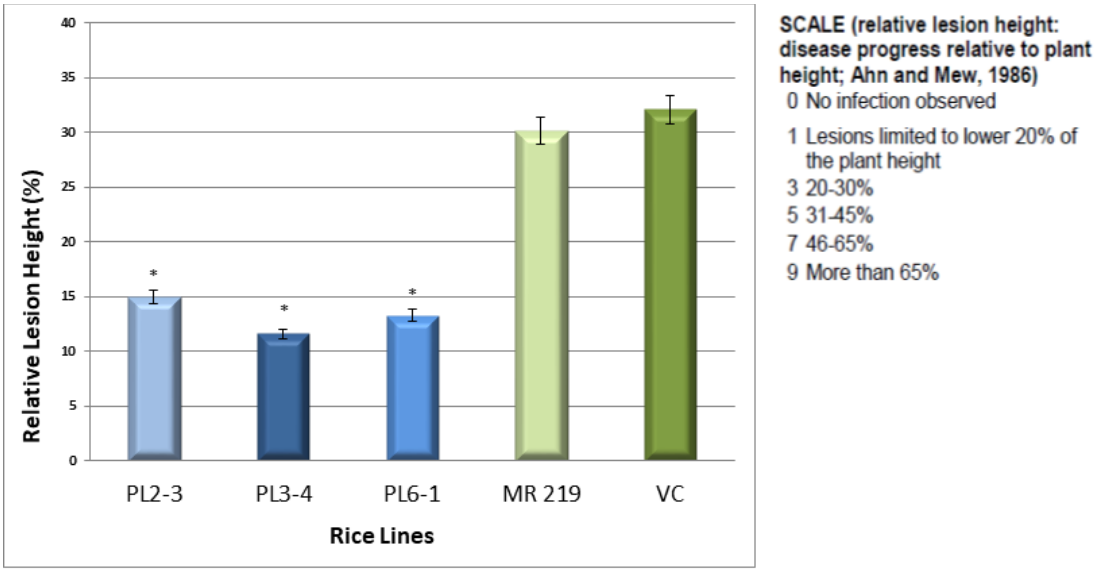

Figure 5. Disease progress relative to plant height in transgenic lines (PL2-3, PL3-4, and PL6-1) and controls (MR 219 wild type and vector control VC plants) at Day 30 post infection. The asterisks (*) indicate significant disease reduction in the transgenic lines.

A comparative gene expression method using ΔΔCp calculation with geometric mean from multiple reference genes was used for the gene expression analysis. In this study, three housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (*OsGADPH*), nucleic acid binding protein (*OsNABP*), and elongation factor 1-alpha (*OsEF1α*), were used as internal controls and for normalising the gene expression level. The reaction was carried out in tri-replicate to minimise technical error and variation. Constitutive, high-level expression of *OsTlp* was observed in transgenic plants, with the highest expression detected in line PL3-4; no amplification was observed from non-transgenic control samples (Figure 6). This analysis has concretely confirmed that *OsTlp* (transgene) was overexpressed only in transgenic lines. Hence, it showed that the level of resistance in transgenic plants was linked to the level of *OsTlp* overexpression.

Several reports have indicated the potential of thaumatin-like protein genes (often grouped into the PR5 family) in enhancing resistance against ShB pathogens in various crops, including rice. In terms of application, rice thaumatin-like protein, *OsTlp*, has been evaluated in several genetic modification (transgenesis) studies for broad fungal resistance. For instance, Mahdavi et al. (2012) discovered that transgenic banana overexpressing *OsTlp* exhibited enhanced resistance to *Fusarium oxysporum* sp. *cubensec* (race 4), causing Fusarium wilt. Moreover, Ojola et al. (2018) found that *OsTlp* overexpression conferred enhanced tolerance against anthracnose disease caused by *Colletotrichum gloeosporioides* f. sp. *Manihotis* in cassava. The same application in *indica* rice was also reported by Shah et al. (2013), in which *tlp-D34* was overexpressed individually or in pair with the chitinase gene to enhance the resistance of 'White Ponni' rice against sheath blight disease. Interestingly, the deduced amino acid sequence of *OsTlp* isolated from Tetep exhibits a remarkable similarity to *tlp-D34* (Shah et al. 2013). This suggests its potential to overcome ShB through overexpression in a susceptible local rice cultivar, especially considering the findings from earlier studies on various pathogenesis-related (PR) genes (Ahmad Redzuan et al., 2015). Additionally, our analysis of the promoter sequence of the *OsTlp* gene revealed the presence of *cis*-acting regulatory elements and motifs associated with various plant hormones and environmental stresses (Nazaruddin et al., 2015). This information from our studies greatly contributed to developing strategies for enhancing resistance in susceptible rice cultivars through transgenesis, as what we have

achieved in our current study. Exploring the genetic basis of *PR5* expression and its regulation has provided valuable information for breeding programmes aimed at developing blight-resistant rice cultivars.

Overall, our findings were promising and suggested that the genetic modification has successfully enhanced the ability of rice to combat the fungal pathogen. Further studies and field trials will be conducted to validate these results and assess the long-term effectiveness of this resistance in different environmental conditions.

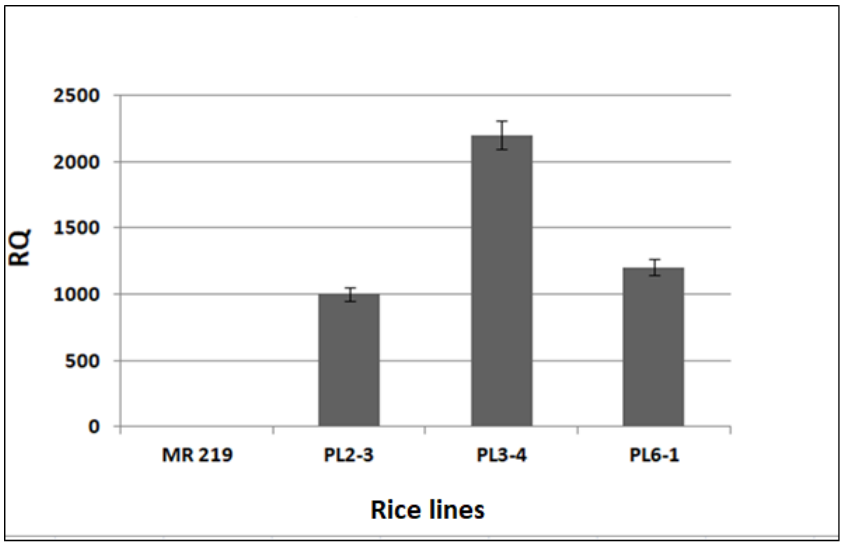

Figure 6. Relative quantification (RQ) of *OsTlp* gene expression against housekeeping genes in T_3 transgenic rice lines and non-transgenic MR 219 (wild type, WT). Overexpression of the transformed gene was observed in three transgenic rice lines, with the highest expression detected in PL3-4.

CONCLUSION

All three MR 219 transgenic lines (PL2-3, PL3-4, and PL6-1) expressing the *OsTlp* gene demonstrated increased tolerance to the fungal pathogen. Among these lines, PL3-4 exhibited the most promising performance, showing an RLH of 11% at 30 dpi. The seeds harvested from all transgenic lines have been preserved for further confined field evaluation. This additional evaluation in the confined field trial is crucial to confirm the observed resistance trait and to collect the necessary biosafety data for future research. The findings from this study may also contribute to the identification of marker genes associated with ShB resistance, which could be invaluable in future rice breeding programmes. Furthermore, the insights gained from studying the transgenic T_3 plants could contribute towards the development of sustainable farming techniques that reduce reliance on chemical inputs and minimise the environmental impact of agriculture. By harnessing the potential of genetic modifications and integrating them with sustainable practices, we can move towards a more secure and resilient food system for future generations.

ETHICAL APPROVAL

No ethical approval was required.

AUTHORS CONTRIBUTION

RS conceptualised and designed the work. All authors conducted experiments, planted rice, and analysed data. RS, AAT, and NHN conceived, drafted, edited, and reviewed the manuscript. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest regarding the publication of this paper.

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