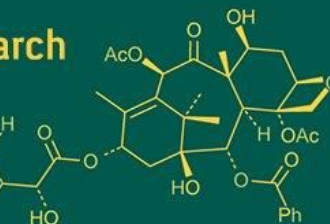


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Molecular characterization of sheath blight (*Rhizoctonia solani*) defense-responsive genes using QRT-PCR assay in rice (*Oryza sativa* L.)

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Abstract

Sheath blight, caused by *Rhizoctonia solani* AG1-IA, is a significant fungal disease affecting rice crops globally. Breeding for sheath blight resistance remains a key strategy for sustainable disease management. However, the quantitative inheritance and lack of complete resistance sources have limited progress through conventional breeding alone. Recent advances in molecular breeding have accelerated resistance improvement by identifying and mapping multiple quantitative trait loci (QTLs). But, understanding plant defense mechanisms at the molecular level is critical for developing disease-resistant cultivars. So, transcriptome analysis has emerged as a powerful tool to uncover gene expression changes in response to biotic stress. This study focuses on identifying and validating candidate genes associated with sheath blight resistance in rice from available transcriptomic data through quantitative real-time PCR (qRT-PCR). Transcriptome data analysis previously showed thousands of differentially expressed genes (DEGs) during *R. solani* infection in two rice TN1 (susceptible) and Tetep (resistant/tolerant) genotypes for sheath blight disease. Among thousands of DEGs identified, 11 DEGs were selected based on their fold change value and their functional annotation and conducted qRT-PCR expression analysis to know the expression of the genes during interaction of *R. solani* with rice in both tolerant (Tetep) and susceptible (TN1) genotypes. All the identified 11 candidate genes were shown high expression in Tetep than the TN1. The findings demonstrated that resistance involves rapid activation of calcium signaling, MAPK pathways, cell wall reinforcement, and ethylene-mediated responses, along with coordinated upregulation of multiple defense-related genes. These candidate genes serve as valuable molecular markers and potential targets for marker-assisted selection, genetic engineering, or genome editing to develop sheath blight-resistant rice varieties, contributing to more sustainable disease control and food security initiatives.

Keywords: *Rhizoctonia solani*, transcriptomic data, differentially expressed genes, qRT-PCR, Tetep and TN1

Introduction

Rice (*Oryza sativa* L.) is a staple food crop for more than half of the world's population, playing a critical role in global food security. However, its productivity is frequently threatened by various biotic stresses, among which sheath blight, caused by the necrotrophic fungal pathogen *Rhizoctonia solani* Kühn (AG1-IA), is one of the most destructive diseases. The disease manifests as lesions on leaf sheaths, which can coalesce and cause severe damage. Sheath blight can lead to yield losses ranging from 10% to 50% under favourable conditions, particularly in high-input, dense planting systems commonly used in intensive rice cultivation. Despite its economic importance, the development of sheath blight-resistant rice varieties remains a significant challenge due to the quantitative and complex nature of resistance (Wei *et al.*, 2021) [21].

Unlike other rice diseases such as bacterial blight or blast, no major gene conferring complete resistance to sheath blight has been identified to date. However, the quantitative inheritance and lack of complete resistance sources have limited progress through conventional breeding alone. Traditional approaches have focused on utilizing partially resistant donor cultivars such as Tetep and Jasmine 85, employing pedigree selection and recurrent backcrossing to transfer resistance into elite backgrounds. Despite some success, phenotypic selection for sheath blight resistance is complicated by strong environmental

interactions and the lack of consistent screening methods. Resistance is typically governed by multiple minor-effect genes or quantitative trait loci (QTLs), making conventional breeding approaches less effective and time-consuming. These limitations necessitate the integration of advanced molecular techniques for precise and efficient identification of resistance-associated genes (Wang *et al.*, 2022) [20]. Recent advances in molecular breeding have accelerated resistance improvement by identifying and mapping multiple quantitative trait loci (QTLs), notably qSBR11-1 and qSBR9-1, linked to partial resistance. Marker-assisted selection (MAS) has enabled precise introgression of these QTLs, improving efficiency in resistance breeding. Transcriptomic studies, genome-wide association studies (GWAS), and functional genomics have further elucidated key genes and defense pathways, including those related to chitinase activity, pathogenesis-related proteins, and hormonal signaling (e.g., jasmonic acid and salicylic acid pathways).

Transcriptome analysis using RNA sequencing (RNA-Seq) has become an invaluable tool for deciphering global gene expression profiles under pathogen challenge. It enables the identification of differentially expressed genes (DEGs) that play key roles in plant immune responses, such as those encoding nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins, receptor-like kinases (RLKs), transcription factors, and pathogenesis-related (PR) proteins. Such high-throughput data provide a genome-wide perspective on host-pathogen interactions and can be used to pinpoint candidate genes for functional validation (Shi *et al.*, 2020) [15]. Following transcriptome analysis, quantitative real-time PCR (qRT-PCR) remains the gold standard for validating the expression of selected DEGs due to its sensitivity, specificity, and quantitative accuracy. By comparing expression patterns between resistant and susceptible rice genotypes post-infection, researchers can confirm the involvement of candidate genes in resistance mechanisms. These genes may serve as targets for marker-assisted selection (MAS), genetic engineering, or genome editing, thus contributing to the development of rice cultivars with improved sheath blight resistance (Wang *et al.*, 2022) [20]. In this context, we aim to identify key candidate genes associated with sheath blight resistance from available transcriptomic data and validate their expression patterns using qRT-PCR. This integrative approach will deepen our understanding of the molecular basis of sheath blight resistance and offer novel genetic resources for rice improvement.

Materials and Methods

Plant Material

Two rice cultivars with contrasting responses to *Rhizoctonia solani* the resistant variety Tetep and the susceptible variety TN1 were procured from the ICAR-Indian Institute of Rice Research (ICAR-IIRR), Hyderabad, India. The plants were raised under controlled environmental conditions. The *R. solani* AG1-IA strain, known for its high virulence, was also obtained from ICAR-IIRR and maintained on suitable growth media for subsequent inoculations.

Pathogen Inoculation

The pathogenic *R. solani* AG1-IA strain was cultured on standard media and used for inoculation. The experiment was conducted in a glasshouse under controlled

environmental conditions, temperature maintained at 28-30 °C and relative humidity at 85-90% (Dutta and Deb, 2020). Inoculation was performed at the tillering stage by attaching mycelial plugs onto the leaf sheaths using cello tape. A minimum of five plants per genotype were inoculated to rice cultivars Tetep (Resistant) and TN1 (susceptible) (Fig. 2.). Specifically, inoculation at tillering stage by attaching mycelial plugs with cello tape, collecting symptomatic leaf and sheath tissues as per 0-9 scale SES of IRRI 2013 (Table 1), at 5 days post-inoculation, and pooling for RNA extraction, along with uninoculated controls, matches well with controlled glasshouse inoculation methods for transcriptomic or molecular pathology studies of *R. solani* AG1-IA infection (Abdelsalam *et al.*, 2020) [1].

RNA Isolation and Quality Assessment

Total RNA is isolated from plant tissues (e.g. fresh leaves) using a commercially available Nucleospin RNA plant kit (Macherey-Nagel) was employed to extract total RNA (20mg) from control and *R. solani* (AG-IA) VP-15 strain infected leaf tissue of Tetep (resistant) and TN1 (susceptible) plants. RNA quality and purity are assessed spectrophotometrically by measuring absorbance ratios OD260/OD280, with acceptable purity ratios ranging from 1.8-2.0 to indicate low protein contamination. Purity is also assessed via A260/A230 and A260/A280 ratios, which indicate contamination levels by proteins and organic compounds.

cDNA Synthesis

First-strand complementary DNA (cDNA) was synthesized from normalized RNA using oligo (dT) primers and Superscript II Reverse transcriptase (In vitrogen). The protocol follows manufacturer's instructions carefully for optimal yields.

Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR is used for expression profiling validation of 11 candidate genes. Primers were designed for all these 11 genes (Table 3). Careful selection and validation of reference genes (housekeeping genes) for normalization is critical for reliable data, ensuring reference gene (*β-Actin*) are stably expressed across experimental conditions. Data is analyzed using appropriate software for determination of relative expression levels. This technique has been extensively applied for analyzing plant-pathogen interactions and gene expression studies for anthocyanin synthesis and stress response genes.

Quantitative PCR Conditions

Quantitative real-time PCR was carried out on a Bio-Rad CFX96 Real-Time PCR System using SYBR® Green Supermix (Bio-Rad, USA). Each 10 µL reaction mixture contained: 1 µL of cDNA, 5 µL SYBR Green Master Mix, 0.5 µL each of forward and reverse primers (10 µM), and 3 µL nuclease-free water (Figure 2). The thermal cycling conditions were as follows: initial denaturation at 98 °C for 1.5 min, followed by 44 cycles of denaturation at 95 °C for 5 s, annealing at 56.3-61.2 °C for 5 s, and extension at 72 °C for 10 s (Taylor *et al.*, 2019). A melting curve analysis was performed at the end of the amplification cycles to confirm the specificity of the amplicons. All reactions were conducted with two biological and two technical replicates.

qRT-PCR Analysis

Gene-specific primers were designed for 11 candidate defense-related genes associated with ethylene biosynthesis, jasmonic acid pathways, and pathogenesis-related proteins. Gene sequences were aligned using MEGA10 software to identify conserved regions. Primers were designed using Primer-BLAST and validated *in silico* to ensure target specificity. All primers were diluted to a working concentration of 10 pmol/ μ L. Amplification efficiency and expected amplicon sizes were confirmed prior to expression analysis. The β -Actin gene was used as the internal reference control (Udvardi *et al.*, 2008) [19]. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Standard errors were computed as described previously (Bhaskar Rao *et al.*, 2020) [2]. Expression data were obtained from two independent biological replicates.

Calculation of Real-Time PCR Data

Relative transcript abundance was calculated using the comparative C_t ($\Delta\Delta C_t$) method. The mean C_t value of each target gene was normalized against the C_t value of the internal reference (β -Actin) to obtain the ΔC_t value. The $\Delta\Delta C_t$ was determined using the formula:

$\Delta\Delta C_t = \Delta C_t$ (infected sample) - ΔC_t (control sample). Fold change in gene expression was calculated as $2^{-\Delta\Delta C_t}$. The standard error was computed using the standard deviation of ΔC_t values in Microsoft Excel (Sailaja *et al.*, 2014; Mangrauthia *et al.*, 2017; Prathi *et al.*, 2018) [14, 9, 12].

Results and Discussions

Differential Gene Expression

The differentially expressed genes (DEGs) from two genotypes *ie.*, Tetep (Resistant) and TN1 (susceptible) were studied from the available transcriptome data (ICAR-IIRR, Biotech lab) in both infected and control. Highest number of exclusive DEGs was in TN1 (1566), followed by Tetep (769) was observed previously in RNA seq data (Prati *et al.*, 2018). Among several thousands of identified DEGs, 11 candidate genes were commonly selected (Table 2) based on fold change value and also based on function related to sheath blight. Primers for all these 11 candidate genes were designed and sequenced (Table 3)

Validation of Transcriptomic Data

To confirm the accuracy of transcriptomic data associated with sheath blight resistance in rice, eleven differentially expressed genes (DEGs) were selected for validation through quantitative real-time PCR (qRT-PCR) (Fig. 1). These genes were chosen based on their putative roles in defense mechanisms, including ethylene biosynthesis, jasmonic acid signaling, and pathogenesis-related responses (Fig. 2.) (Chen *et al.*, 2013) [4]. Before going to expression studies, the genotypes were inoculated with the fungal culture. Infected leaf samples were collected at 5th day after infection and processed RNA isolations and cDNA synthesis, accurate concentrations were observed. Validated 11 candidate genes through qRT-PCR and the results revealed that the expression patterns of the selected genes were consistent with the transcriptomic data, confirming the reliability of the RNA-Seq analysis. All eleven genes exhibited significant upregulation in the resistant genotype Tetep upon *Rhizoctonia solani* infection compared to the susceptible genotype TN1 and their respective un-inoculated controls (Fig. 1.).

Expression analysis of candidate defense-related genes in rice genotypes

Quantitative real-time PCR (qRT-PCR) was conducted to analyze the expression of selected candidate genes in resistant (Tetep) and susceptible (TN1) rice genotypes following infection with *Rhizoctonia solani*. Gene expression levels were normalized to the internal reference gene β -Actin to ensure reliable comparison across treatments and genotypes (Zhang *et al.*, 2017) [23]. The results revealed significant variation in transcriptional responses between the two cultivars. In general, Tetep showed markedly higher induction of defense-related genes upon infection, whereas TN1 exhibited minimal or basal expression changes (Richa *et al.*, 2017; Yang *et al.*, 2023) [13, 22].

Among the analyzed genes, *ERG1* (LOC_Os01g62430.1) displayed the highest upregulation (~8-fold) in Tetep, implying a potential role in sterol or secondary metabolite biosynthesis linked to defense activation. A similar trend was reported by Richa *et al.* (2017) [13], who observed that elevated *ERG1* expression in Tetep contributes to rapid defense signalling against *R. solani*. *ACO1* (LOC_Os09g27820.1), a critical component of the ethylene biosynthetic pathway, showed approximately 4-fold higher expression in Tetep compared to less than 2-fold in TN1, suggesting that ethylene signalling participates in early resistance responses. This observation is consistent with findings by Molla *et al.* (2020) [10, 11], indicating that ethylene enhances resistance by activating early defense signalling.

Calcium-mediated signalling components such as *CML31* (LOC_Os01g72530.1) and *CBL4* (LOC_Os03g44820.1) were induced 3-5-fold in Tetep but only about 1-fold in TN1, indicating that Ca^{2+} -dependent signalling plays a crucial role in Tetep's rapid defense activation. Similarly, the signalling kinase *MPK7* (LOC_Os05g49140.1) exhibited strong induction (~7-fold) in Tetep, supporting its involvement in pathogen-triggered immunity (Koornneef and Pieterse, 2008; Li *et al.*, 2021) [6, 7]. *HOS66* also showed moderate (~3-fold) upregulation in Tetep relative to ~1.3-fold in TN1, implying a role in general stress tolerance. Genes associated with structural defense exhibited pronounced induction in Tetep. *LAC19* (LOC_Os11g42200.1), involved in lignin biosynthesis, was upregulated about 6-fold in Tetep but only 1.5-fold in TN1, suggesting enhanced cell wall reinforcement in the resistant genotype. Likewise, *BGLU28* (LOC_Os08g39870.1) increased nearly 5-fold in Tetep, implicating its participation in the hydrolysis of defense-related glycosides. *SAP7* (LOC_Os03g57900.1), a stress-associated protein, showed 4-fold induction in Tetep but negligible change in TN1. In addition, *PCF8* (LOC_Os12g42190.1) was significantly induced (~5-fold) in Tetep infected plants compared to ~1.5-fold in TN1, suggesting its role as a transcriptional regulator mediating defense gene activation. Collectively, Tetep exhibited 3-8-fold higher expression levels across most defense-associated genes upon *R. solani* infection, whereas TN1 showed only slight (1-2-fold) induction (Fig. 1.).

Overall, these findings demonstrate that Tetep mounts a rapid, coordinated, and robust defense response upon *R. solani* infection. Enhanced activation of Ca^{2+} - and MAPK-mediated signaling, together with increased expression of genes involved in cell wall reinforcement, sterol biosynthesis, and ethylene signaling, underpins its resistant phenotype. In contrast, the limited transcriptional response

in TN1 reflects delayed or weak activation of these pathways, consistent with its susceptibility. These results corroborate previous reports suggesting that early and strong transcriptional activation of multiple small-effect genes

contributes to the quantitative resistance observed in Tetep (Channamallikarjuna *et al.*, 2010; Molla *et al.*, 2020; Liu *et al.*, 2022; Kamboj *et al.*, 2017) ^[3, 10, 11, 8, 5].

Table 1: SES scale of IRRI 2013 for sheath blight disease

Score	Observations
0	Free from infection
1	lesion limited to lower 20% of the plant height
3	20–30%
5	31–45%
7	46–65%
9	more than 65%

Table 2: Genes selected for qRT-PCR and their Fold change in different genotypes

Gene	Gene ID	Genotype	Fold Change	Function
TUBB5	LOC_Os02g07060.1	TN1	-2.03	Tubulin beta-5 chain
		TETEP	6.76	
PCF8	LOC_Os12g42190.1	TN1	-2.53	Transcription factor PCF8
		TETEP	4.31	
ACO1	LOC_Os09g27820.1	TN1	-3.04	1-aminocyclopropane-1-carboxylate oxidase 1
		TETEP	10.69	
CML31	LOC_Os01g72530.1	TN1	-6.47	Probable calcium-binding protein CML31
		TETEP	5.66	
LAC19	LOC_Os11g42200.1	TN1	-5.09	Laccase-19
		TETEP	5.91	
ERG1	LOC_Os01g62430.1	TN1	-3.54	Elicitor-responsive protein 1
		TETEP	8.6	
HOS66	LOC_Os03g03164.1	TN1	-7.33	Homeobox protein knotted-1-like 3
		TETEP	7.68	
CBL4	LOC_Os03g44820.1	TN1	-5.91	Calcineurin B-like protein 7
		TETEP	3.24	
MPK7	LOC_Os05g49140.1	TN1	-2.46	Mitogen-activated protein kinase 7
		TETEP	2.54	
BGLU28	LOC_Os08g39870.1	TN1	-4.27	Beta-glucosidase 28
		TETEP	8.68	
SAP7	LOC_Os03g57900.1	TN1	-1.97	Zinc finger A20 and AN1 domain-containing stress-associated protein 7
		TETEP	2.61	

Table 3: Primers used for RT-qPCR analysis

S. No.	Gene ID Name	Forward F/Reverse R primer	Sequence
1	OS05G0399300 (HOS66)	F	5'GCAGGGCTTCAAGTCAAGGA 3'
		R	3'GAGTGTGCTGCCACCATTG 5'
2	OS08G0509400 (BGLU28)	F	5'GTTTCGACTTCATCGGCCTCA 3'
		R	3'GTCGCGTTGACCCAATTGTC 5'
3	OS05G0566400 (MPK7)	F	5'AGATCGTGTGCAGTACAGGC 3'
		R	3'TCTGGGAGCACTCCTCGTAA 5'
4	OS09G0451400 (ACO1)	F	5'CGATGGAGCAGCTGGATGAT 3'
		R	3'CACACGCTTGTAGTGGTCCT 5'
5	OS12G0616400 (PCF8)	F	5'TCCACTGCTCGAACACCAAC 3'
		R	3'GCCATCACCTCGAACGACAT 5'
6	OS02G0167300 (TUBB5)	F	5'ACCCGGACAGGATGATGTTG 3'
		R	3'CCATGCACTCATCGGCATTC 5'
7	OS02G0291000 (CBL7)	F	5'ATTTTTCACCCGGAACGCC 3'
		R	3'AATGGCGAGCACCATTTCGT 5'
8	OS03G0793000 (SAP7)	F	5'AACAACATGTGCTCCCGGTG 3'
		R	3'GCCTTCTTCGTCGTCGTCTC 5'
9	OS11G0641500 (LAC19)	F	5'TTCGTGGTGCACGAGATGAA 3'
		R	3'ATGACATGGACAACCACCGT 5'
10	OS01G0955100 (CML31)	F	5'TCGGCATGTACGAGATGGAAG 3'
		R	3'CGCCGTTGAGATCGAACCT 5'
11	OS01G0841700 (ERG1)	F	5'TTCTCACGGACGACTTCCT 3'
		R	3'CGCCGTGGTATGTTTTGTCC 5'

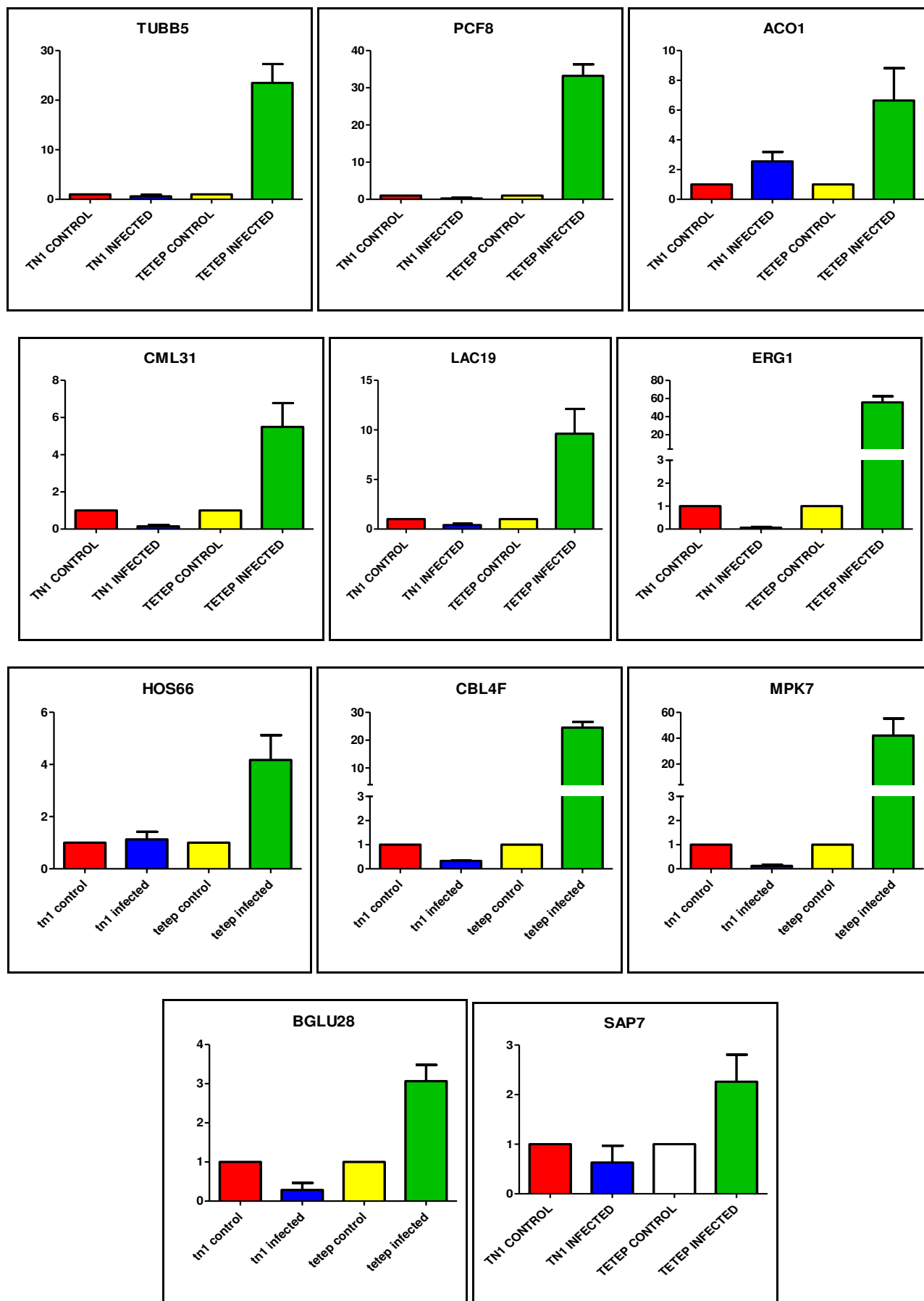


Fig 1: Validation of identified candidate genes through quantitative real-time PCR (qRT-PCR) assay

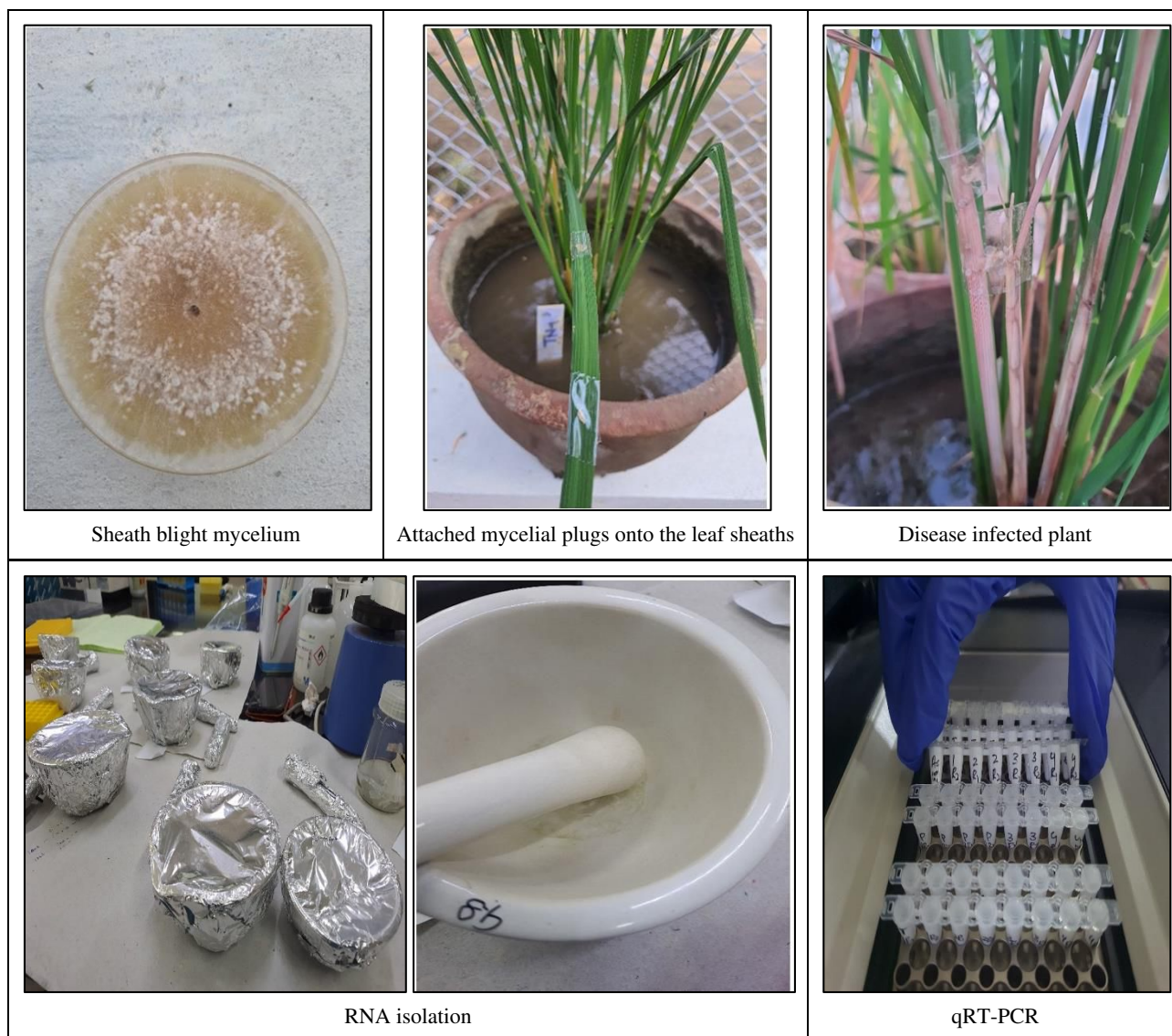


Fig 2: Screening of TN1 (susceptible) and Tetep (resistant) genotypes against Sheath blight disease

Conclusion

Integrating transcriptomic analysis with quantitative real-time PCR (qRT-PCR) has successfully identified and validated eleven candidate genes associated with sheath blight resistance in rice. These genes showed significantly higher expression in the resistant cultivar Tetep compared to the susceptible TN1 when challenged with the pathogen *Rhizoctonia solani*. The findings demonstrate that resistance involves rapid activation of calcium signaling, MAPK pathways, cell wall reinforcement, and ethylene-mediated responses, along with coordinated upregulation of multiple defense-related genes. These candidate genes serve as valuable molecular markers and potential targets for marker-assisted selection, genetic engineering, or genome editing to develop sheath blight-resistant rice varieties, contributing to more sustainable disease control and food security initiatives.

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