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Research on the Mechanism of OsRLCK109 in Regulating Rice Blast Resistance

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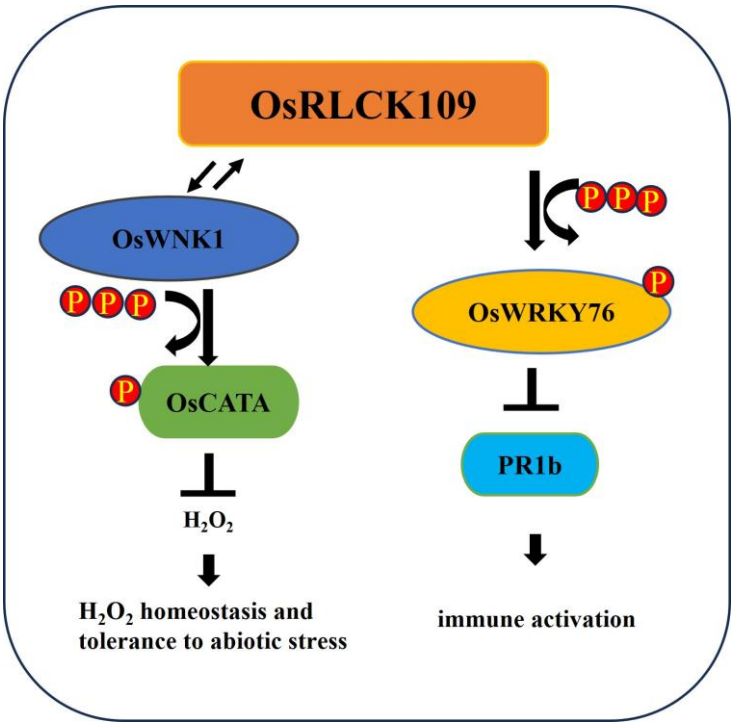

Promoteurs: Prof. Ludivine Lassois & Liyong Cao

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Yue Zhang

Dissertation originale présentée (ou essai présenté) en vue de l'obtention du grade de doctorat en sciences agronomiques et ingénierie biologique

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Abstract

Rice blast disease, caused by *Magnaporthe oryzae*, represents one of the most destructive diseases affecting rice production worldwide and poses a major threat to global food security. Improving our understanding of the molecular mechanisms underlying rice immunity is therefore essential for the development of durable disease-resistant varieties.

Receptor-like cytoplasmic kinases (RLCKs) have emerged as key regulators of plant immune signaling. Among them, OsRLCK109 was previously identified as a negative regulator of rice blast resistance; however, the molecular mechanisms by which it modulates immune responses remained largely unknown. The objective of this thesis was to elucidate the regulatory network controlled by OsRLCK109 through the identification and functional characterization of its interacting partners.

Using a combination of protein–protein interaction assays, biochemical analyses, gene expression studies, and functional characterization of mutant lines, we identified two major regulatory pathways mediated by OsRLCK109.

First, we demonstrate that OsWNK1 interacts with OsRLCK109 and plays a dual role in regulating both biotic and abiotic stress responses. OsWNK1 directly phosphorylates the catalase OsCATA, thereby modulating its enzymatic activity and regulating reactive oxygen species (ROS) homeostasis. This mechanism promotes ROS accumulation during pathogen infection, enhancing immune signaling and resistance to rice blast. Conversely, under abiotic stress conditions, reduced catalase activity impairs ROS scavenging, leading to increased oxidative damage and decreased tolerance to drought and cold stress. These results reveal a key role for the OsRLCK109–OsWNK1–OsCATA module in balancing immunity and stress adaptation.

Second, we identify the transcription factor OsWRKY76 as another interacting partner of OsRLCK109. We show that OsRLCK109 phosphorylates OsWRKY76 and enhances its ability to repress the transcription of the defense-related gene PR1b. Functional analyses indicate that OsWRKY76 acts as a negative regulator of rice blast resistance, and that OsRLCK109 reinforces this inhibitory effect. These findings uncover a novel regulatory pathway in which OsRLCK109 modulates immune responses through transcriptional control.

Taken together, this work demonstrates that OsRLCK109 acts as a central regulatory node integrating ROS-mediated signaling and transcriptional repression to fine-tune rice immune responses. By linking disease resistance with abiotic stress tolerance, this study provides new insights into the complex trade-offs governing plant adaptation. These findings also offer valuable molecular targets and genetic resources for the development of rice varieties with improved resistance to biotic and abiotic stresses.

Keywords: Rice, Rice blast, Receptor-like cytoplasmic kinases (RLCKs), With No-lysine Kinase, WRKY transcription factor

Résumé

La pyriculariose du riz, causée par Magnaporthe oryzae, constitue l'une des maladies les plus dévastatrices affectant la production rizicole à l'échelle mondiale et représente une menace majeure pour la sécurité alimentaire. Une meilleure compréhension des mécanismes moléculaires sous-jacents à l'immunité du riz est donc essentielle pour le développement de variétés durablement résistantes.

Les kinases cytoplasmiques de type récepteur (RLCK) sont reconnues comme des régulateurs clés des voies de signalisation immunitaire chez les plantes. Parmi celles-ci, OsRLCK109 a été précédemment identifié comme un régulateur négatif de la résistance à la pyriculariose ; toutefois, les mécanismes moléculaires impliqués restaient largement méconnus. L'objectif de cette thèse est d'élucider le réseau de régulation contrôlé par OsRLCK109, à travers l'identification et la caractérisation fonctionnelle de ses partenaires d'interaction.

Grâce à une combinaison d'approches incluant des analyses d'interactions protéine–protéine, des analyses biochimiques, des études d'expression génique et la caractérisation fonctionnelle de lignées mutantes, deux voies majeures de régulation médiées par OsRLCK109 ont été mises en évidence.

Dans un premier temps, il est montré que OsWNK1 interagit avec OsRLCK109 et participe à la régulation des réponses aux stress biotiques et abiotiques. OsWNK1 phosphoryle directement la catalase OsCATA, modulant ainsi son activité enzymatique et le contrôle de l'homéostasie des espèces réactives de l'oxygène (ROS). Ce mécanisme favorise l'accumulation de ROS lors de l'infection par le pathogène, renforçant les réponses immunitaires et la résistance à la pyriculariose. En revanche, en conditions de stress abiotique, la réduction de l'activité catalase altère l'élimination des ROS, entraînant une accumulation toxique et une diminution de la tolérance à la sécheresse et au froid. Ces résultats mettent en évidence le rôle du module OsRLCK109–OsWNK1–OsCATA dans l'équilibre entre immunité et adaptation aux stress abiotiques.

Dans un second temps, le facteur de transcription OsWRKY76 est identifié comme un autre partenaire d'interaction d'OsRLCK109. Il est montré qu'OsRLCK109 phosphoryle OsWRKY76 et renforce sa capacité à réprimer la transcription du gène de défense PR1b. Les analyses fonctionnelles indiquent qu'OsWRKY76 agit comme un régulateur négatif de la résistance à la pyriculariose, et que OsRLCK109 amplifie cet effet inhibiteur. Ces résultats mettent en évidence une nouvelle voie de régulation par laquelle OsRLCK109 module les réponses immunitaires via un contrôle transcriptionnel.

Dans l'ensemble, ce travail montre qu'OsRLCK109 agit comme un nœud central de régulation intégrant à la fois des mécanismes de signalisation dépendants des ROS et des mécanismes de régulation transcriptionnelle, afin d'ajuster finement les réponses immunitaires du riz. En mettant en évidence les compromis entre résistance aux pathogènes et tolérance aux stress abiotiques, cette étude apporte de nouveaux

éléments de compréhension des mécanismes d'adaptation des plantes. Elle fournit également des cibles moléculaires et des ressources génétiques prometteuses pour l'amélioration variétale du riz.

Mots clés: Riz, Pyriculariose du riz, Kinases cytoplasmiques de type récepteur (RLCK), Kinase sans lysine, Facteur de transcription WRKY

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Table of content

List of figures

List of tables

List of acronyms

PTI	Pattern-triggered immunity
ETI	Effector-triggered immunity
PRR	Pattern recognition receptors
PAMP	Pathogen-associated molecular patterns
DAMP	Damage-associated molecular patterns
RLK	Receptor-like kinases
RLP	Receptor-like proteins
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
EF-Tu	Elongation factor Tu
Pep	Plant elicitor peptides
PIP	PAMP-induced secreted peptides
OG	Oligogalacturonide
ROS	Reactive oxygen species
RLCK	Receptor-like cytoplasmic kinases
MAPK	Mitogen-activated protein kinase
NLR	Nucleotide-binding domain leucine-rich repeat containing receptors
Xoo	*Xanthomonas oryzae pv. Oryzae*
M.oryzae	*Magnaporthe oryzae*
R	Resistance
PR	Pathogenesis-related
SAR	Systemic acquired resistance
CDS	Complete coding sequence
CPK	Calcium-dependent kinase
WNK	With No-lysine kinase
CAT	Catalase
Co-IP	Co-immunoprecipitation
IP-MS	Immunoprecipitation coupled with mass spectrometry
LCI	Luciferase complementation imaging
PTM	Post-translational modification
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

Chapter 1

General introduction

1. Rice as a key crop for global food security

The Food and Agriculture Organization (FAO) reported in "The State of Food Security and Nutrition in the World 2025" that, despite some progress in recent years, global food security has not yet returned to pre-COVID-19 levels and remains far from achieving the goal of eliminating hunger by 2030.

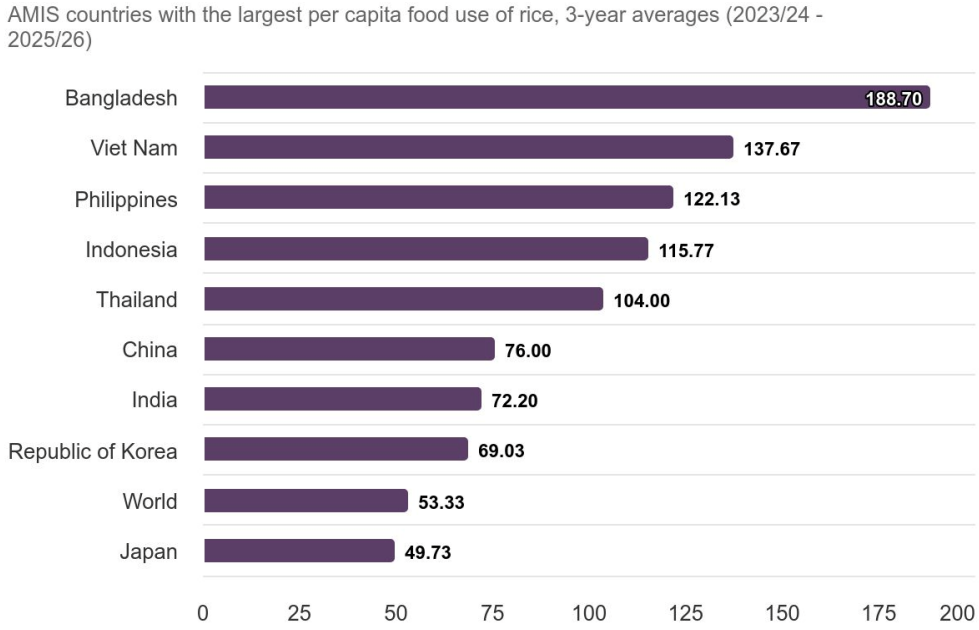


Figure 1: Countries with high consumption of rice. The Agricultural Market Information System (AMIS) has released a list of the countries with the highest per capita rice consumption, based on a three-year average. The horizontal axis represents per capita food use (kg/Yr). (https://www.amis-outlook.org/home).

Rice (Oryza sativa, 2n=2x=24) serves as a model organism for the study of monocotyledonous plants (Jain et al. 2019). It exists primarily in two varieties: indica and japonica (Wang et al. 2018). In 2005, the International Rice Genome Sequencing Project established that the genome size of japonica rice is approximately 389 megabases, comprising 37,544 protein-coding genes unrelated to transposable elements (International Rice Genome Sequencing Project and Sasaki 2005). Subsequently, in 2017, Chinese researchers determined that the genome size of indica rice is approximately 390.3 megabases, with an estimated 38,714 protein-coding genes identified with high confidence (Du et al. 2017).

Rice, a globally significant food crop, holds a prominent position in Asia. It is primarily categorized into two subspecies: indica and japonica. The cultivated area and total production of rice in Asia account for 87.02% and 89.99% of the global figures, respectively (Zhi et al. 2023). Asia, which hosts over 50% of the world's population, predominantly relies on rice as a staple food (Figure 1). The primary consumers of global rice encompass nations such as India and China, which also rank among the leading producers. In 2024, these countries together represented approximately 52% of the world's rice consumption. The most substantial trade flows of rice are primarily concentrated within the Asian continent. According to data from the United States Department of Agriculture, in the same year, the volume of trade among Asian nations amounted to 135.5 million tons, accounting for 23% of global trade (https://www.spglobal.com/en). Therefore, ensuring adequate rice production is essential for maintaining global food security.

While hybrid rice breeding has contributed to increased production, the unpredictable impacts of climate change continue to threaten rice cultivation. In recent years, environmental stresses—including elevated temperatures, cold spells, droughts, floods, and salinity—have intensified in severity and frequency. Additionally, climate change influences and amplifies biological stressors, such as bacterial, fungal, and viral infections, as well as infestations by insects and pests (Verma et al. 2021). Although it has been extensively studied in recent decades, rice remains an area with substantial research potential. Enhancing the understanding of gene functions in rice will additionally contribute to the formulation of innovative strategies for crop variety development.

2. Introduction to the plant immune system

Plants cannot move freely, have limited access to resources, and allocate resources and energy mainly for growth and development when there is no threat. However, they rapidly mobilize resources and energy for defense when attacked by pathogens. As scientific research advances, scientists have gradually come to understand the immune mechanisms in plants. The zigzag model (Jones and Dangl 2006) systematically delineates the bipartite plant immune system and its dynamic co-evolution with pathogens. Surface-localized pattern recognition receptors (PRRs) initially detect conserved microbial pathogen-associated molecular patterns (PAMPs), thereby initiating a mild, universal pattern-triggered immunity (PTI). Subsequently, effective pathogens deploy effectors to suppress PTI and establish effector-triggered susceptibility (ETS). Conversely, intracellular nucleotide-binding leucine-rich repeat (NLR) proteins recognize these virulence effectors and activate a robust, race-specific effector-triggered immunity (ETI), frequently characterized by hypersensitive response (HR) cell death. The pathogens continuously mutate their effectors to evade the recognition of NLRs, and plants also evolve new NLR proteins to reactivate ETI, thereby causing the periodic alternation of resistance and susceptibility in plants. The oscillation of immune status through successive host–pathogen interactions produces the characteristic zigzag trajectory (Figure 2).

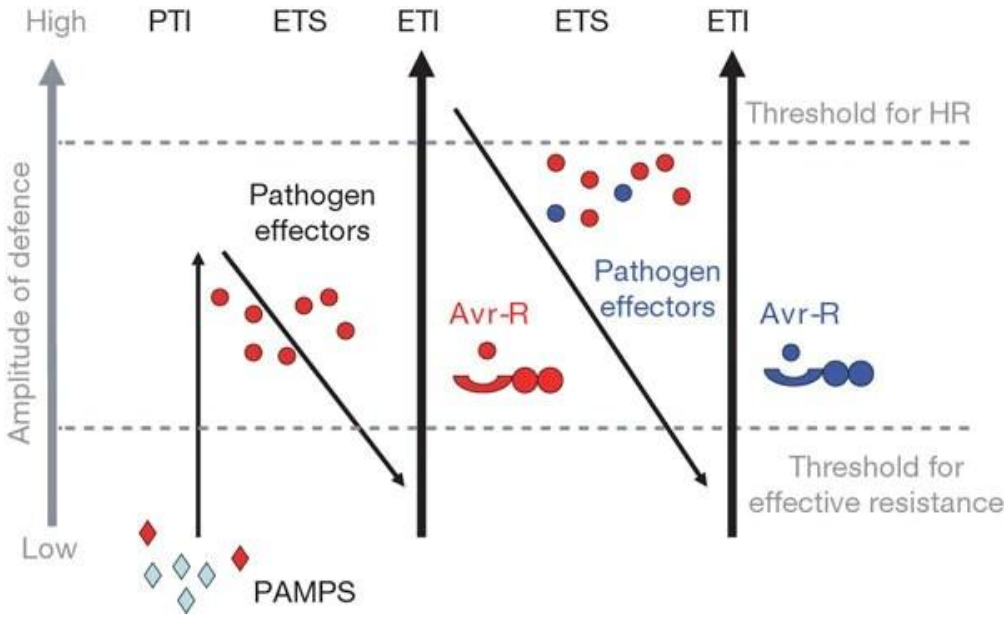


Figure 2: A zigzag model illustrates the plant immune system (Jones and Dangl 2006).

2.1 PTI

The fundamental principle of PTI involves the recognition of PRRs for PAMPs and DAMPs. A variety of PAMPs have been identified from pathogens capable of eliciting plant immune responses (Table 1), such as flagellin, cold-shock proteins, lipopolysaccharide (LPS), and elongation factor Tu (EF-Tu) from Gram-negative bacteria; and chitin, β-glucans, carbohydrate, and ergosterol from fungi (Nurnberger et al. 2004). In contrast to PAMPs, DAMPs are endogenous molecules released from cells that are infected with plant pathogens or subjected to injury. These include systemin, plant elicitor peptides (Peps), PAMP-induced secreted peptides (PIPs), extracellular ATP, and oligogalacturonide (OGs) (Song et al. 2021).PRRs encompass receptor kinases (RLKs) and receptor-like proteins (RLPs). RLK comprises an extracellular domain, a transmembrane domain, and a cytoplasmic kinase domain. RLP lacks a cytoplasmic kinase domain compared to RLK. Based on the extracellular domain of PRRs, PRRs are classified into leucine-rich repeat (LRR) PRRs, lysine motif (LysM) PRRs, lectin domain PRRs, and epidermal growth factor (EGF)-like domain PRRs (Tang, Wang, and Zhou 2017).

To realize the recognition of PAMPs and DAMPs, PRRs appear not to function independently but to form a complex enriched with immune receptors and associated proteins. For instance, flagellin-sensing 2 (FLS2) and the EF-Tu receptor (EFR), which detect flg22 and the bacterial elongation factor Tu epitope elf18, respectively, require BRI1-associated kinase 1 (BAK1) as a co-receptor. Likewise, receptor kinase

5 (LYK5), which contains a lysine motif (LysM), serves as a chitin receptor that requires another LysM-RK, chitin elicitor receptor kinase 1 (CERK1), as a co-receptor (Zhou and Zhang 2020). When PRR effectively detects PAMPs and DAMPs, thereby initiating a range of downstream physiological responses, plant PTI is formed. These responses include an influx of extracellular Ca^{2+} into the cytoplasm, extracellular alkalinization, rapid phosphorylation of receptor-like cytoplasmic kinase (RLCK), activation of calcium-dependent kinase (CPK), the mitogen-activated protein kinase (MAPK) cascade, activation of heterotrimeric G proteins, and the production of reactive oxygen species (ROS), nitric oxide (NO), phosphatidic acid (PA), phytoalexins, and phytohormones. Collectively, these responses enhance the plant's resistance to a broad spectrum of pathogens (Yu et al. 2017).

Table 1: The PAMPs and DAMPs that have been reported in plants

PRR	PAMP/DAMP	Category	Plant Source	References
FLS2	Flagellin (flg22 epitope)	RLK	*Arabidopsis thaliana*	(Gómez-Gómez and Boller 2000)
EFR	EF-Tu (elf18 epitope)	RLK	*Arabidopsis thaliana*	(Zipfel et al. 2006)
XPS1	Xanthine/uracil permease (xup25 epitope)	RLK	*Arabidopsis thaliana*	(Mott et al. 2016)
PEPR1	Pep1-6	RLK	*Arabidopsis thaliana*	(Yamaguchi, Pearce, and Ryan 2006)
PEPR2	Pep1-2	RLK	*Arabidopsis thaliana*	(Yamaguchi et al. 2010)
RLK7	PIP1	RLK	*Arabidopsis thaliana*	(Pitorre et al. 2010)
WAK	Oligogalacturonides	RLK	*Arabidopsis thaliana*	(He et al. 1999)
AtLYK5	Chitin	RLK	*Arabidopsis thaliana*	(Cao et al. 2014)
AtCERK1	Chitin	RLK	*Arabidopsis thaliana*	(Miya et al. 2007)

XA21	RaxX	RLK	*Oryza longistaminata*	(Song et al. 1995)
OsCEBiP	Chitin	RLP	*Oryza sativa*	(Shimizu et al. 2010)
OsCERK1	Chitin	RLK	*Oryza sativa*	(Shimizu et al. 2010)
OsLYP4	Peptidoglycans/chitin	RLP	*Oryza sativa*	(Liu et al. 2012)
OsLYP6	Peptidoglycans/chitin	RLP	*Oryza sativa*	(Liu et al. 2012)
RLP1/ReMAX	eMaxc	RLP	*Arabidopsis thaliana*	(Jehle et al. 2013)
RLP23	nlp20	RLP	*Arabidopsis thaliana*	(Ono, Mise, and Takano 2020)
RLP30	SCFE1c	RLP	*Arabidopsis thaliana*	(Zhang et al. 2013)
AtLYM2	Chitin	RLP	*Arabidopsis thaliana*	(Narusaka et al. 2013)
AtLYM1	Peptidoglycans	RLP	*Arabidopsis thaliana*	(Jinrong et al. 2008)
AtLYM3	Peptidoglycans	RLP	*Arabidopsis thaliana*	(Jinrong et al. 2008)
RLP42/RBPG1	EndoPG	RLP	*Arabidopsis thaliana*	(Zhang et al. 2014)

2.2 ETI

PTI is mediated by receptors situated on the cell membrane. Similar to PTI, ETI can be simply understood as the immune response triggered by intracellular nucleotide-binding domain leucine-rich repeat containing receptors (NLRs). Due to their variable N-terminal domains, NLRs are classified into three major categories: helical coiled-coil NLRs (CNLs), RPW8-L-like coiled-coil domain NLRs (RNLs), and Toll/interleukin-1 receptor/resistance protein NLRs (TNLs) (Nabi et al. 2024). The LRR domain is responsible for the direct or indirect recognition of effectors. The NB-ARC region possesses ATP-binding activity, which regulates NLR activation. Additionally, the N-terminal domain plays a role in downstream signal transduction following NLR activation.

The earliest understanding of ETI originates from the gene-for-gene concept: an avirulence (Avr) gene present in the pathogen and a corresponding resistance (R) gene in the host. The *Avr* gene encodes an effector, while the *R* gene encodes an NLR. ETI is an immune response triggered by the R protein recognizing the Avr protein. When the host lacks an R gene corresponding to the Avr gene, the effector exhibits virulence and suppresses the host's defense mechanisms (Van der Biezen and Jones 1998). Through a comprehensive investigation of ETI, an increasing number of studies have demonstrated that the relationship between effectors and NLR is variable. Certain NLRs are capable of sensing specific effectors, while others can detect multiple effectors; likewise, some effectors can be recognized by multiple NLRs (Ngou, Jones, and Ding 2022). The rice *Piz-t* gene encodes a canonical NLR capable of inducing blast resistance, which is caused by the *Magnaporthe oryzae* avirulence gene *AvrPiz-t* (Li et al. 2009). ZAR1 recognizes multiple effectors, including AvrAC from *Xanthomonas* campestris and HopZ1a from *Pseudomonas syringae* (Wang et al. 2015). AvrRpm1 is recognized by two *Arabidopsis* CNLs, RPM1 and RPS2 (Redditt et al. 2019).

In numerous scenarios, NLRs do not operate independently but require the presence of ‘helper’ NLRs. NRG1 functions as a 'helper’ NLR for multiple NLRs in *Arabidopsis*. The ETI responses mediated by WRR4A, WRR4B, RPP1, RPP2, RPP4, RRS1/RPS4, RRS1B/RPS4B, CHS1/SOC3, and CHS3/CSA1 were all compromised in NRG1 loss-of-function mutants (Castel et al. 2019). In addition to helper NLRs, proteins belonging to the EDS1 family are also essential for responses mediated by sensor NLRs. EDS1-PAD4 forms interactions with ADR1, an accessory NLR family member, to facilitate plant resistance against disease. Conversely, EDS1-SAG101 interacts with NRG1, another accessory NLR family member, to induce rapid tissue necrosis at infected sites, known as a hypersensitivity reaction, thereby restricting pathogen proliferation (Huang et al. 2022).

The mechanism of ETI is presently not entirely comprehended; however, it may involve the activation of MAP kinase signaling, reprogramming of certain transcriptional events, alterations in Ca^{2+} levels both intracellularly and extracellularly, production of ROS, and programmed cell death as common mechanisms.

2.3 PTI and ETI influence each other

PTI and ETI induce immunity through different receptors; however, they are not two parallel pathways. Instead, they interact with each other to construct the plant immune network. The evidence that PTI and ETI interact is not simply that they share many immune signaling pathways; scientists have found some real evidence. On the one hand, ETI requires PRR receptors. The ETI responses triggered by avrRpt2, AvrPphB, and AvrRps4 were not effectively activated in two plant strains lacking PRR receptors (mutants *fec* and *bbc*). It indicates that PRR has a potentially widespread role in ETI (Yuan et al. 2021).On the other hand, activation of the NLR receptor can upregulate the expression of various PTI signaling components, including PRRs and their auxiliary receptors. The AvrRps4 protein alone does not induce the production of ROS. However, pre-activation of AvrRps4 enhances the ROS production elicited by flg22 and markedly increases the protein levels of BIK1, RBOHD, and MPK3 induced by these stimuli (Ngou et al. 2021). These studies offer evidence supporting the interaction between PTI and ETI, rather than the strict separation outlined in earlier models, thereby facilitating a robust and optimal immune response.

2.4 ROS in plant defence

ROS pertains to any oxygen derivative that exhibits greater reactivity than a solitary oxygen molecule. The primary ROS encompass singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH •) (Mittler 2017). ROS can be generated within diverse subcellular compartments. The extracellular matrix primarily experiences ROS production through membrane-bound NADPH oxidase, whereas intracellular ROS predominantly originate from chloroplasts, peroxisomes, and mitochondria.

The role of ROS in plant defense mechanisms is intricate and multifaceted. ROS can directly eliminate pathogens by oxidizing essential biomolecules such as proteins and nucleic acids, or by inflicting damage on pathogen cell membranes through lipid peroxidation, thereby hindering their proliferation and dissemination (Haghpanah et al. 2025). The deposition of lignin within the cell wall represents a crucial strategy employed by plants to resist pathogenic invasion. ROS may function as signaling molecules for lignin biosynthesis and also serve as substrates in lignin polymerization processes (Wang, Li, and Liang 2024). Furthermore, ROS can induce the production of antibacterial compounds, including phytoalexins, which directly suppress the growth and development of pathogenic bacteria (Ahuja, Kissen, and Bones 2012). ROS also activate signaling pathways involving Ca^{2+}, the MAPK cascade, and WRKY transcription factors, among others, to initiate PTI and ETI (Weralupitiya, Eccersall, and Meisrimler 2024). Localized high-intensity ROS signals can provoke programmed cell death, thereby limiting pathogen invasion and conferring disease resistance. Additionally, ROS facilitates the integration of plant hormone signals, such as salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) (Xu et al. 2024).

Whether during the PTI stage or the ETI stage, when pathogens invade plants, the production of ROS constitutes a vital cellular process. During the PTI process, NADPH oxidase located on the plasma membrane is responsible for initiating the ROS burst during PTI. After PRR detects PAMPs or DAMPs of the pathogen, it can activate NADPH oxidase through phosphorylation, producing ROS. In *Arabidopsis*, BIK1 forms a complex with FLS2 and the co-receptor BAK1. Following the detection of flg22, BIK1 undergoes phosphorylation and subsequently phosphorylates the essential enzyme NADPH oxidase RBOHD, thereby inducing ROS generation (Li et al. 2014) . The fungal chitin receptor CERK1 can also phosphorylate BIK1, which in turn phosphorylates RBOHD to regulate the ROS burst triggered by chitin (Yuan et al. 2021). Although the signaling pathways governing the initiation of ROS bursts in PTI have been elucidated, the regulation of ROS in ETI, particularly how it is modulated by NLRs, remains insufficiently understood. Recent investigations indicate that in cells infected by tobacco mosaic virus (TMV), NLR proteins enhance the phosphorylation of the transcription factor Alfin-like 7 (AL7) through activation of the MAPK cascade. This process inhibits the expression of downstream genes responsible for ROS detoxification, resulting in elevated ROS accumulation (Zhang et al. 2023). The surge of ROS is a shared process between PTI and ETI; however, disparities exist in the magnitude and duration of ROS generation. Current evidence demonstrates that ROS levels during PTI attain their zenith within 10 to 15 minutes and subsequently revert to baseline within one hour. Conversely, ROS production during ETI peaks between 1.5 to 3 hours, establishing a high-intensity plateau that persists for approximately 24 hours. These findings indicate that ROS generated via ETI generally exhibits a more pronounced and prolonged burst relative to that produced during PTI (Mittler et al. 2022).

Plants inherently possess the ability to coordinate defense mechanisms against diverse pathogens via a dynamic ROS signaling network. Nevertheless, this signaling network has become a target for pathogenic agents, which have evolved various secretion effectors aimed at disrupting ROS homeostasis. Facilitating the degradation of RBOH constitutes one of the molecular mechanisms through which these effectors induce susceptibility. This phenomenon is corroborated by evidence from *Fusarium graminearum* effector FgEC1, which interacts with the *Triticum aestivum* TaGF14b protein to induce degradation of TaRBOHD and inhibit the ROS production mediated by TaRBOHD, thereby facilitating infection (Shang et al. 2024).

3. Advances in rice disease resistance research

Rice is a vital crop facing numerous threats from various diseases. These include fungal blast caused by *Magnaporthe oryzae*, sheath blight from *Rhizoctonia solani*, false smut due to *Ustilaginoidea virens*, bakanae disease caused by *Fusarium fujikuroi*, bacterial blight from *Xanthomonas oryzae pv. oryzae* (*Xoo*), bacterial leaf streak caused by *Xanthomonas oryzae pv. oryzicola* (*Xoc*), and various virus infections (Liu et al. 2021). Significant advancements in rice molecular biology, functional genomics, and gene-editing technologies have accelerated the elucidation of the rice disease-

resistance mechanism. These advances include the discovery of some PTI and ETI pathways, the identification of PRRs and NLRs, and the functional analysis of other immune regulatory factors (Figure 3).

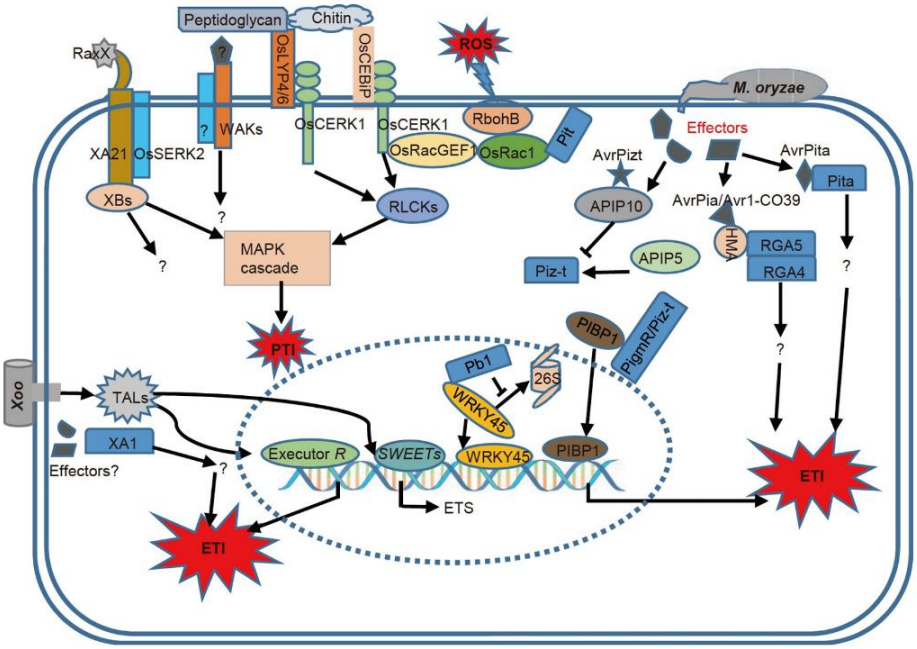


Figure 3: The PTI and ETI pathways have been reported in rice, including some identified PRRs and NLRs, as well as some immune regulatory factors (Chen et al. 2022).

3.1 PTI in rice

Several PRRs have been characterized in rice, including XA21, XA3/XA26, XA4, OsCEBiP, OsCERK1, LYP4, and LYP6. Among these, XA21, XA3/XA26, and XA4 are associated with *Xoo*. As the first PRR identified, it is extensively studied. The product encoded by *Xa21* is a receptor-like protein kinase comprising 1025 amino acids that recognizes the RaxX protein from *Xoo RaxX-RaxSTAB*. The helper PRR at the cell membrane for XA21 is OsSERK2, with which it forms a complex, phosphorylating each other to activate downstream PTI signaling. XA21 is cleaved by an unidentified peptidase to release an intracellular kinase domain, which translocates to the nucleus and interacts with the transcription factor OsWRKY62, a critical component for XA21-mediated immune responses (Park and Ronald 2012; Chen et al. 2014). The XA3/XA26 locus is believed to have originated approximately 7.5 million years ago, and its specific resistance has been maintained over time. Both OsSERK2 and OsTPI1.1 are proteins that interact with XA3/XA26, and their transcriptional repression or loss of function significantly impairs XA3/XA26-

mediated resistance to *Xoo* (Chen et al. 2014; Liu et al. 2018). *Xa4* encodes a cell wall-associated kinase that reinforces the cell wall by promoting cellulose synthesis and inhibiting cell wall loosening. This function can enhance resistance to bacterial infection and increase the mechanical strength of the culm, thereby improving lodging resistance in rice plants (Hu et al. 2017).

OsCEBiP and OsCERK1 constitute a group of PRR complexes located on the cell membrane, which recognize the fungal molecular pattern molecule, chitin. In the absence of chitin, OsCEBiP exists as a homooligomer. Upon the presence of chitin, OsCEBiP and OsCERK1 swiftly form a complex, resulting in the activation of OsCERK1. Subsequently, OsRacGEF1 undergoes phosphorylation and activation by OsCERK1, leading to the further activation of OsRac1. The OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 signaling pathway constitutes a vital mechanism for chitin-induced immunity in rice (Akamatsu et al. 2013). Additionally, OsLYP4 and OsLYP6 form both homodimers and heterodimers and function as dual PRRs for chitin and bacterial peptidoglycan, thereby initiating the basal defense response of rice against *M. oryzae* and *Xoo*. Furthermore, OsCERK1 is essential for the activation of immune pathways mediated by OsLYP4 and OsLYP6 (Liu et al. 2012).

3.2 ETI in rice

The rice genome encompasses over 400 genes encoding NLRs, of which only a limited number have been characterized as participating in ETI. The identified NLR genes are implicated in conferring resistance to rice blast disease, with the majority associated with rice blast resistance. Only Xa1 (Allele: Xa2, Xa14, Xa31, and Xa45) has been identified as an NLR related to *Xoo*. This observation may indicate that resistance to *M. oryzae* is predominantly governed by ETI, whereas resistance to *Xoo* could be regulated by alternative pathways.

The recognition of effectors by NLRs in rice follows varied patterns. The first pattern involves direct interaction, exemplified by the two spliced transcripts of RGA5, namely RGA5-A and RGA5-B. Genetic analysis reveals that only RGA5-A confers resistance, while RGA5-B remains inactive. The direct binding of AVR-Pia and AVR1-CO39 to RGA5-A is essential for the initiation of ETI (Cesari et al. 2013). The second pattern is the guard mode, where the pathogenic bacterial effector protein AvrPiz-t targets the host protease inhibitor APIP4, thereby attenuating the host's ETI response. Concurrently, the host NLR protein Piz-t stimulates the accumulation and activity of APIP4 (Zhang et al. 2020). The third pattern, decoy mode, involves two interacting NLRs. When the effector is absent, the NLR capable of activating ETI is inhibited, maintaining ETI in an inactive state. Upon effector presence, the decoy NLR binds to the effector, releasing the ETI-activating NLR to initiate immune responses. RGA4 and RGA5 constitute an NLR pair where RGA4 acts as a constitutively active disease resistance and cell death inducer, inhibited by RGA5 in the absence of a pathogen. RGA5 serves as the receptor for the effector proteins AVR1-CO39 and AVR-Pia from Mycobacterium. The direct interaction between RGA5 and these effectors alleviates RGA4 repression, thereby activating resistance

signaling (Cesari et al. 2014). The fourth pattern, the integrated decoy model, involves an NLR with an integrated domain that functions as a decoy NLR. This configuration facilitates interaction with the effector, enabling specific recognition and triggering ETI. In rice, the interaction between Pik-1 and the effector aligns with this model (Marchal et al. 2022).

3.3 Lesion mimic mutants

The term "lesion mimic mutant" was initially designated as "disease lesion mimics" and was first defined by Neuffer and Calvert (Neuffer and Calvert 1975). It pertains to mutants that spontaneously develop necrotic lesions resembling pathogen-induced hypersensitive responses in the absence of pathogen infection. In rice, at least 80 autoimmune mutants have been identified, which are designated as "lesion mimic mutants" (lmm) or "spot lesion mutants" (spl). These mutants typically display characteristics of activated immune responses, including bursts of ROS, activation of PR genes, and elevated levels of defense hormones such as salicylic acid (SA) and jasmonic acid (JA), all present under normal growth conditions. Furthermore, the majority of these mutants exhibit broad-spectrum resistance to rice blast fungus *M. oryzae* and *Xoo*. The characterization of these autoimmune mutants and the analysis of disease-resistant molecular mechanisms will facilitate a more comprehensive understanding of the rice immune system, identify key regulatory factors of immunity, and offer novel strategies for the selection of disease-resistant rice varieties.

These self-immune genes involve multiple regulatory pathways. For example, transcription, ubiquitination, the MAPK pathway, ROS, and cell death. SPL7 encodes a heat shock transcription factor. The knockout of SPL7 leads to the development of lesions and an increased resistance to rice blast and bacterial blight, although it also results in inhibited growth. Moderate expression levels may enhance resistance without inducing lesions or causing significant growth inhibition defects (Hoang et al. 2019). OsCUL3a serves as a significant component of E3 ubiquitin ligase connectases. It engages in interaction with OsNPR1 and facilitates its degradation. OsNPR1 acts as a positive regulator of rice cell death. The loss of OsCUL3a function results in the excessive accumulation of OsNPR1, thereby causing abnormal activation of the immune response (Liu et al. 2017). The interaction between rice and *Xoo* involves OsMPKK6 phosphorylating and activating OsMPK4. Subsequently, OsMPKK6 phosphorylates OsVQ14 and OsVQ32 at T61 and S141, respectively, thereby augmenting salicylic acid-mediated resistance to bacterial blight(Li et al. 2021). OsCATC is a crucial peroxidase in rice and plays a negative regulatory role in resistance to rice blast disease. The effector AvrPiz-t enhances the activity of OsCATC, leading to the decomposition of hydrogen peroxide, which facilitates the infection process of the rice blast fungus (You et al. 2022). An increase in OsBAG4 levels results in the initiation of programmed cell death and the activation of immune self-response. OsOsBAG4 may undergo ubiquitination and subsequent degradation by EBR1. The E3-BAG module modulates the equilibrium of the plant's innate immune

response, whilst also balancing the plant's defense mechanisms and growth (You et al. 2016).

4. Trade-offs Between Growth and Defense in Plants

Achieving high yield and immunity against pathogens and pests are critical objectives in plant breeding. Nonetheless, immunity frequently results in yield reductions. This inverse correlation between growth and defense mechanisms is commonly termed the "growth-defense trade-off". The initial hypothesis suggested that damaged plants might lose substantial portions of photosynthetic tissues due to predation by insects and pathogens, consequently decreasing their capacity for light capture and photosynthesis, which impairs growth (Coley, Bryant, and Chapin 1985). However, the damage to photosynthesis may not constitute the primary explanation for the "growth-defense trade-off" phenomenon, as instances exist where insect or pathogen attacks do not lead to loss of photosynthetic tissues yet still impede plant growth. Plants are required to grow and defend concurrently to ensure survival and reproductive success in natural environments. Consequently, the equilibrium between growth and defense has profound ecological implications. During crop breeding, yield serves as the principal criterion for evaluating a superior variety. This selection process may favor the retention of genetic loci that positively regulate growth and development. Unfortunately, these loci often exhibit antagonistic relationships with those involved in defense, resulting in the loss of advantageous traits for biological resistance. Understanding the molecular mechanisms underlying the growth-defense trade-off is essential, as it may inform future crop breeding strategies aimed at developing high-yielding and robustly defended cultivars.

4.1 The hypothesis of trade-offs between growth and defense

A prevalent hypothesis concerning the trade-off between growth and defense pertains to the rational allocation of resources within set limits. In essence, the resources available to a plant are finite. The activation of defense mechanisms imposes a substantial demand on these resources. During an attack, plants can only reallocate resources that would otherwise be designated for growth towards defense. Research employing isotope-labeled nitrogen has demonstrated that nitrogen produced via photosynthesis is more heavily utilized for the synthesis of defense compounds when plants are subjected to simulated pest attacks. This empirical evidence corroborates the aforementioned hypothesis.

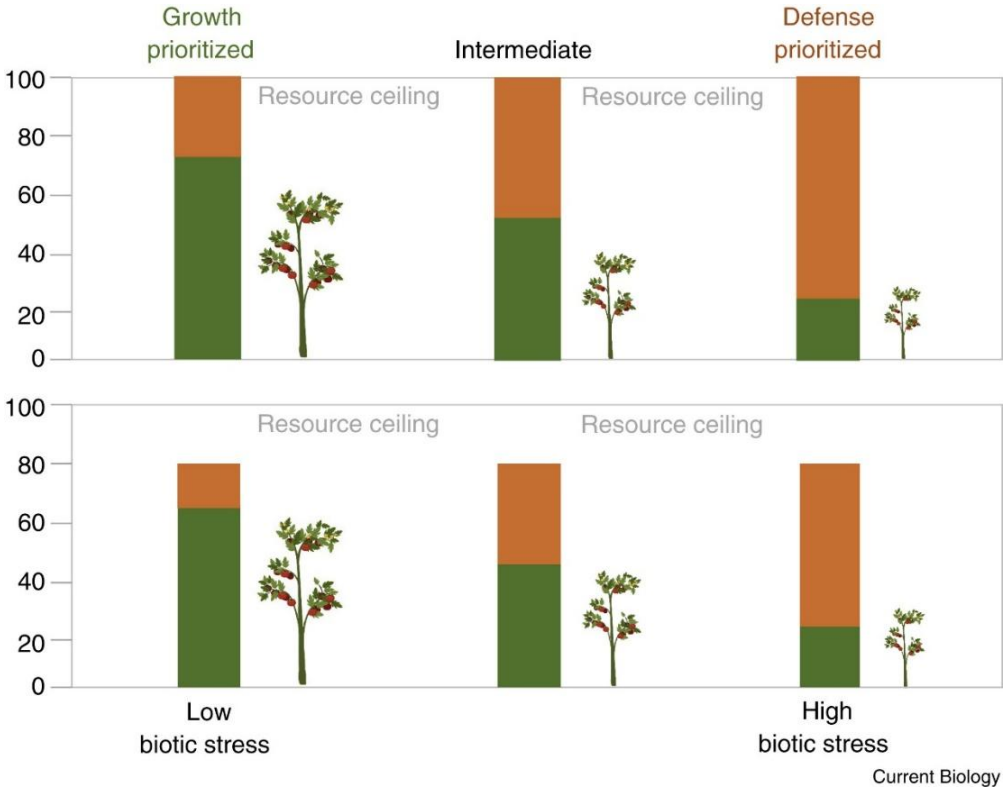


Figure 4: This illustration delineates two hypotheses concerning the trade-off between growth and defense. The upper section elucidates that the trade-off is attributable to resource limitations, whereas the lower section posits that it is a strategic adaptation by the plants themselves, independent of resource constraints (He, Webster, and He 2022).

Given that limited resources are the primary cause of the trade-off between growth and defense, this raises the question of whether providing ample resources would enable plants to simultaneously achieve strong growth and robust defense capabilities (Figure 4). Nonetheless, in a nutrient-rich laboratory environment or in agricultural settings with well-fertilized soil, the trade-off between growth and defense may still manifest even when resources are abundant. This suggests that the trade-off is not solely dictated by resource scarcity, and alternative biological explanations may exist.

4.2 The molecular mechanism of the trade-off between growth and defense

Plant hormones play a pivotal role in various physiological processes. The defense mechanisms mediated by plant hormones encompass jasmonic acid (JA), salicylic acid (SA), and ethylene (ET). Conversely, hormones such as auxin, cytokinin, abscisic acid (ABA), brassinosteroids (BR), and gibberellin (GA) are primarily involved in

plant growth and development. Jasmonic acid, recognized as a significant hormone in plant defense, has been extensively researched and is known to inhibit plant growth. Gibberellins, on the other hand, facilitate seed germination, leaf expansion, stem and root elongation, as well as the development of floral and fruit structures. A study published in the PNAS indicates that jasmonic acid induces the accumulation of SLR1, a key inhibitor in gibberellin signaling, thereby leading to plant dwarfism. Conversely, when the jasmonic acid pathway is attenuated, SLR1 undergoes rapid degradation, resulting in enhanced plant growth. This evidence demonstrates that the interaction mechanism between jasmonic acid and gibberellin maintains a balance between plant defense responses and growth (Yang et al. 2012). Furthermore, there exists a reciprocal interaction between SA and auxin, which influences growth and defense. SA induces the expression of the OsNPR1 gene in rice. The auxin content in the OsNPR1-OX strain is decreased, and consequently, the root system, seed number and weight, internode elongation, and tiller number are all diminished (Li et al. 2016). The transcription factor OsBIHD1 directly interacts with the promoter sequences of the ethylene synthase *OsACO3* and the BR degradation metabolic gene *CYP734A2*, thereby regulating the equilibrium between ethylene biosynthesis and BR degradation concerning growth and defense mechanisms (Liu et al. 2017).

The Pigm locus in the local variety of indica rice, Guimei No. 4, constitutes a gene cluster encompassing multiple NLRs and functions as an antitumor gene. Among these, only PigmR and PigmS encode functional proteins. PigmR demonstrates broad-spectrum disease resistance; however, it results in a decrease in grain weight per plant and a consequent reduction in rice yield. Epigenetic regulation of PigmS can enhance the grain setting rate of rice and mitigate the effects of PigmR yield (Deng et al. 2017).

The research conducted over the past three decades has advanced our molecular understanding of how plants balance growth and defense. These insights have started to elucidate the mechanistic basis of the trade-off between plant growth and defense. In the forthcoming decade, it is anticipated that this fundamental understanding will be translated into innovative breeding strategies, enabling the cultivation of optimal crops that are simultaneously high-yielding and highly resistant.

5. Research Progress on Rice Blast Disease

Rice, as a staple crop nourishing more than half of the global population, is susceptible to the most destructive fungal disease, rice blast. This disease is caused by *M. oryzae* and leads to a reduction in rice yield ranging from 10% to 35%. While chemical pesticides are effective in controlling rice blast disease, they clearly pose risks to environmental integrity and food safety. Consequently, understanding the pathogenesis of rice blast disease and cultivating varieties endowed with resistance genes constitute the most fundamental and efficacious strategies.

5.1 The infection process of *M. oryzae*

M. oryzae has the potential to impact the entire developmental cycle of rice. The infection process initiated by the rice blast fungus commences with the attachment of conidia to the rice plant. The ascospores germinate within a few hours, generating germ tubes. These germ tubes subsequently elongate to form melanized appressoria. It requires approximately 24 hours for the appressorium to mature and amass sufficient glycerol, thereby generating an expansion pressure of 8.0 MPa. The penetration peg then breaches the epidermis to access the underlying epidermal cells. Internally, the penetration peg differentiates into filamentous primary hyphae, which subsequently develop into bulbous invasive hyphae (IH). The IH absorbs nutrients from the host and proceeds to expand within the host tissues until it causes the death of host cells and induces necrotic lesions (Figure 5) (Wei et al. 2023).

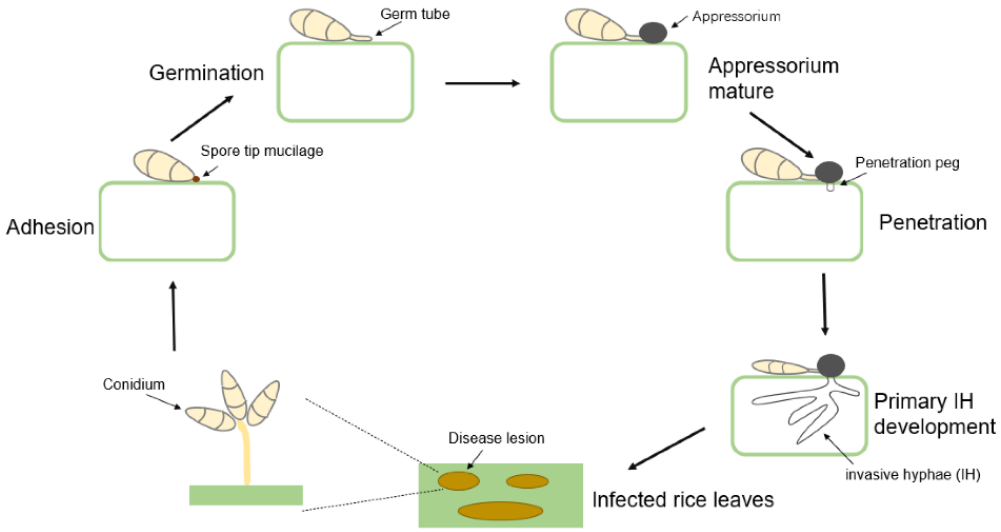


Figure 5: A schematic diagram illustrating the process by which *M. oryzae* infects rice (Wei et al. 2023).

5.2 Genomics of *M. oryzae*

To effectively prevent and manage rice blast disease, it is essential to acquire a comprehensive understanding of the rice blast fungus. Currently, the genome assemblies of more than 50 isolates of *M. oryzae* have been successfully completed, significantly advancing our knowledge of the mechanisms by which rice blast infects the host and disrupts the host's immune response (Sahu et al. 2022). These genomes are characterized by numerous repetitive sequences, contributing to notable genomic instability. Each subspecies possesses distinct genes and genomic regions that are unique to it, which determine its evolutionary classifications, environmental adaptability, and host specificity. The genome size of *M. oryzae* is approximately

40.12 Mb, and it contains 12,684 genes (Devanna et al. 2022). Li et al. reanalyzed 109 complete genome sequences of rice blast fungus from rice, wheat, and other hosts, and found that strains with rice as the host contained fewer conserved genes and exhibited stronger selection pressure. This suggests that the *M. oryzae* adapts to rice through gene loss and rapid evolution at specific loci. A total of 228 genes related to host adaptation in *M. oryzae* involve transposons, transcription factors, thiol metabolism, and nucleotide metabolism. The adaptation of *M. oryzae* to rice occurs through a process of fine-tuning, characterized by both the increase and loss of these genes (Wu et al. 2021).

5.3 Research on positive regulators of rice blast resistance

Over 140 rice blast resistance (R) genes have been identified. Additionally, at least 126 molecular maps of these R genes have been constructed (Figure 6), of which 38 have been cloned (Younas et al. 2023; Huang et al. 2025). The majority of the cloned R genes encode NLRs. Among the NLR-type R genes, those conferring broad-spectrum resistance to rice blast disease include *Pi1, Pi2, Pi5, Pi56(t), Pi64, Pi9, Piz-t, Pizh, Pi50, Pigm,* and *Pijx*. NLR-class R genes are typically organized in tandem repeat formations within the rice genome. For example, clusters of 21, 27, and 27 R genes are located on chromosomes 6, 11, and 12, respectively (Kou et al. 2024).

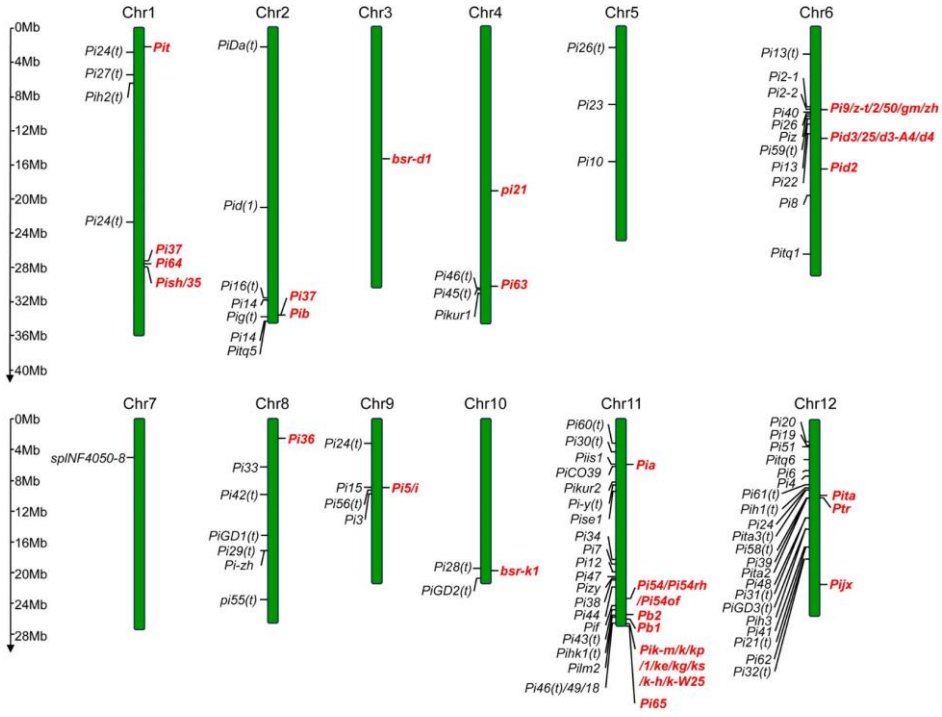


Figure 6: The distribution of the R gene on rice chromosomes. Cloned genes are shown in red (Huang et al. 2025).

In addition to the enhancement of rice's blast resistance via NLR-mediated ETI, many other factors have been identified that influence rice blast resistance, encompassing various physiological mechanisms. Transcription factors are instrumental in regulating rice's response to blast disease. A study conducted by Tang et al. investigated the transcriptional interaction between rice and the rice blast fungus and identified a NAC-type transcription factor gene named *OsNAC29*, which is markedly upregulated upon fungal infection (Lu et al. 2024). The knockout of *OsNAC29* in rice resulted in diminished resistance to rice blast disease, whereas the augmentation of *OsNAC29* expression augmented resistance. Further analysis demonstrated that *OsNAC29* binds to the CACGTG motif within the promoters of genes associated with 5,10-ricinoleic acid synthesis, such as *OsTPS28* and *OsCYP71Z2*, thereby promoting their transcription. Both *OsTPS28* and *OsCYP71Z2* positively influence rice's resistance to rice blast disease, and the resistance mediated by *OsNAC29* is contingent upon these genes.

Phenylalanine ammonia-lyase (PAL) is an enzyme catalyzing the deamination of phenylalanine, which releases ammonia and forms a carbon-carbon double bond to generate trans-cinnamic acid (t-CA). t-CA serves as a precursor for the biosynthesis of various phenolic compounds, including lignin. The expression of the *OsPAL* gene family members enhances lignin biosynthesis, and a higher lignin content fortifies rice's resistance to disease. Overexpression of *OsPAL1* and *OsPAL6* notably improved rice resistance to rice blast disease (Wang et al. 2022).

The PR gene (Pathogenesis-related gene) is a specific gene induced by pathogenic bacteria or exogenous hormones. Research indicates that it is also closely associated with systemic acquired resistance (SAR) and is frequently employed as a molecular marker for establishing SAR in plants. These genes are categorized into multiple subfamilies and exhibit a broad spectrum of functions, including acting as chitinases, peroxidases, antibacterial agents, hydrolases, and protease inhibitors, among others. Members of the PR-1 family are among the most prevalent proteins in plants responding to pathogen attack. The expression of the PR-1 gene has long served as a marker for salicylic acid-mediated disease resistance. *OsPR1a*, *OsPR1b*, and *OsPR1aL* have all been demonstrated to positively regulate resistance to rice blast (Liu et al. 2018; Qiu et al. 2024; Zhang et al. 2024).

Exposure to light influences the resistance of rice to blast disease. Certain rice varieties experience severe symptoms of rice blast disease when exposed to low light conditions, but exhibit reduced symptoms under high light intensity. What is the underlying relationship between sunlight and rice resistance? In the rice plant, a family of rice light-harvesting complexes (LHC), responsible for absorbing and transmitting light energy, exists. One member of this family, *LHCB5*, typically functions in conjunction with *PsbS*, a protein involved in electron transfer within the chloroplast. Zhang et al. discovered that under light conditions, when rice blast fungus infects the rice plant, the 24th threonine of *LHCB5* undergoes phosphorylation and accumulates rapidly within the chloroplast, dissociating from the *PsbS* complex and migrating swiftly within the chloroplast. This disrupts the normal electron transfer

process, reduces the transfer rate, and leads to the accumulation of electrons in the chloroplast. Consequently, reactive oxygen species are released in the chloroplast, activating genes related to disease resistance within the chloroplast and thereby enhancing the rice plant's resistance to rice blast fungus (Liu et al. 2019)

MicroRNA-mediated Resistance to Rice Blast Disease. MicroRNAs (miRNAs) constitute a class of small, non-coding RNA molecules ranging from 20 to 24 nucleotides in length. They are encoded by miRNA genes (MIRs) and undergo a maturation process that includes splicing and methylation. The mature miRNAs are incorporated into ARGONAUTEs (AGO), forming miRNA-induced gene silencing complexes (miRISCs). These complexes specifically target miRNA-guided messenger RNAs (mRNAs) through sequence complementarity, thereby inhibiting gene expression via transcriptional cleavage or translational repression. Notably, *miR398b* targets four genes within the superoxide dismutase family: *OsCCSD*, *OsCSD1*, *OsCSD2*, and *OsSODX*. Among these, *OsCCSD* negatively influences rice blast resistance, whereas *OsCSD1*, *OsCSD*2, and *OsSODX* play positive roles in conferring resistance (Li et al. 2019).

5.4 Research on negative regulators of rice blast resistance

Compatible pathogens are capable of penetrating multiple layers of plant defenses and successfully infecting the host. Once the compatibility between the host and the pathogen is disrupted, their interaction becomes incompatible, thereby rendering the plant immune to the pathogen. Any plant gene that facilitates a compatible interaction with the pathogen is referred to as a susceptibility (S) gene. To date, more than one hundred S genes have been identified in rice. The S gene includes those that promote the fundamental compatibility of pathogenic bacteria, as well as those that support the sustained compatibility of pathogenic bacteria (Huang et al. 2024). The small G protein (Rho-GTPase and RAC/ROP) is crucial for cytoskeletal dynamics and vesicle transport, and it also plays a role in basic compatibility. Three RAC/ROP proteins in rice, *OsRacB*, *OsRac4*, and *OsRac5*, have been identified as susceptibility factors for *M. oryzae* infection (Chen et al. 2010). The genes *OsDjA2* and *OsERF104* encode chaperone proteins and APETELA2/ethylene response factor, respectively. They are markedly induced during compatible interactions with rice blast disease. The knockout of *OsDjA2* and *OsERF104* via CRISPR/Cas9 significantly improves the disease resistance of the plants (Távora et al. 2022).

In nature, due to genetic mutations, certain plant materials may spontaneously develop phenotypes resembling disease spots on various parts, such as leaves, leaf sheaths, and stems, even in the absence of pathogenic bacteria. These materials have been designated as LMM by researchers. The majority of these pseudo-disease spot mutants are closely associated with plant disease resistance, markedly increasing the expression of defense-related genes, activating defense mechanisms, inhibiting the proliferation of pathogenic bacteria, and thereby enhancing disease resistance. To date, approximately 100 mutant varieties of rice disease spots have been documented, and over 40 associated genes have been cloned. The majority of these genes encode

negative regulatory factors for rice blast disease. The proteins encoded by these genes are diverse, and the mechanisms they involve in conferring disease resistance are also quite extensive, including the ROS signaling pathway, plant hormones, and protein ubiquitination, among others. The *oscatc* mutant of rice accumulates a substantial amount of hydrogen peroxide, which induces cell death and enhances resistance to rice blast fungus (You et al. 2022). The *sl-MH-1* gene modulates the concentrations of salicylic acid, jasmonic acid, ethylene, and abscisic acid in rice, thereby effectuating the regulation of cell death and the augmentation of resistance (Tian et al. 2022). OsUbc13 encodes a ubiquitin conjugating enzyme. The *OsUbc13-RNAi* plants exhibit a disease-like spot phenotype. After induction by the flg22 polypeptide and chitin, the accumulation of reactive oxygen species in these plants rapidly increases, the expression levels of defense-related genes rise, and the resistance to pathogenic bacteria also significantly enhances (Liu et al. 2023).

5.5 Strategies for developing rice varieties with resistance to rice blast

The R gene is employed in the breeding of rice cultivars with resistance to rice blast. Over the past decade, the most frequently utilized alleles have been the broad-spectrum resistance complementing alleles of the Piz gene cluster located on chromosome 6 and the Pik gene cluster situated on chromosome 11. A research team led by Professor Zuo Shimin from Yangzhou University introduced Pigm into two high-yielding, high-quality japonica rice varieties, successfully developing a new cultivar resistant to rice blast with enhanced yield and superior quality (Feng et al. 2022). Furthermore, Indian researchers integrated the Pi1 and Pi54 genes from the Pik gene cluster into the high-quality and high-yield variety "BPT5204". The resulting genetically modified variety exhibited a significant enhancement in its resistance to rice blast disease (Kumar et al. 2018). The consolidation of R genes with diverse disease resistance signaling pathways constitutes a means to attain durable broad-spectrum resistance. Li et al. discovered that Pijx and Pigm/Piz-t occupy distinct branches on the phylogenetic tree and are associated with separate signaling pathways. Following the combination of Pijx and Pigm/Piz-t, a more enduring and broad-spectrum resistance can be achieved (Xiao et al. 2021).

The CRISPR/Cas9 genome editing technology facilitates targeted mutagenesis of DNA. Due to its simplicity, feasibility, and versatility, it is increasingly employed across various organisms, including applications in improving crop traits. By editing the S gene with CRISPR/Cas9, resistance to rice blast disease was enhanced. Bsr-d1, Pi21, and OsERF922 are three genes associated with susceptibility to rice blast disease. Researchers generated mutant strains with each of these three genes individually knocked out using CRISPR/Cas9 technology. All three mutant strains exhibited increased resistance to rice blast disease. Furthermore, these mutant strains demonstrated enhanced resistance to rice blast disease and bacterial blight without compromising key agricultural traits (Zhou et al. 2022). The two genes, RNG1 and RNG3, exhibit polymorphisms within their 3'-untranslated regions (3'-UTRs), which influence their expression variability. Alterations to these 3'-UTRs via CRISPR/Cas9

technology affect their expression levels, demonstrating a positive correlation with susceptibility to rice blast disease. The knockout of RNG1 or RNG3 in rice results in increased resistance to rice blast disease without sacrificing essential agronomic traits (Xu et al. 2023).

Modify the NLR protein to attain broad-spectrum resistance. *RGA5* encodes an NLR protein and features an integrated heavy metal-associated (HMA) domain, which is essential for the recognition of the rice blast effector AVR-CO39. The researchers engineered the HMA domain, resulting in the RGA5-HMA2 protein, capable of recognizing AVR-Pib and initiating the disease resistance response induced by AVR-Pib. Through the alteration of specific domains or critical amino acids within disease-resistant proteins, it is feasible to effectively broaden their resistance spectrum by enhancing their effector recognition capabilities (Liu et al. 2021).

5.6 Research progress on QTL related to rice blast disease

Quantitative Trait Loci (QTL) primarily influence complex quantitative traits such as disease resistance, stress tolerance, and crop yield, which are affected by multiple genes and environmental factors. Conducting QTL research aids in elucidating the genetic principles underlying these traits, facilitates the analysis of gene effects and interactions, reduces environmental interference in traditional phenotypic assessment through linkage molecular markers, improves the efficiency of germplasm screening and breeding programs, and offers vital theoretical and technical support for crop genetic mechanism analysis and molecular precision breeding.

In different environments, using different distribution populations, more than 500 QTLs related with rice blast resistance across different environments and diverse populations (Huang et al. 2025). Evidently, due to the origin of these QTLs from distinct genetic materials, rice blast strains, and cultivation settings, the same disease resistance intervals are frequently identified. Research dedicated to refining the reported QTLs and determining stable meta-QTLs (MQTLs) pertinent to rice blast disease demonstrated that 435 original QTLs were consolidated across 12 rice chromosomes, ultimately revealing 71 stable MQTLs (Devanna et al. 2024). This process significantly reduced the initial number of QTLs. Within the 53 MQTL segments, 199 candidate genes linked to rice blast resistance were identified, including 48 recognized R gene analogs. Another study identified a new QTL for rice blast disease through Pangenome-Wide Association Study and Transcriptome Analysis. This QTL has no significant negative impact on yield and is expected to be utilized to breed new rice varieties with lasting resistance (Devanna et al. 2024).

The accumulation of multiple QTLs leads to broader resistance. Utilizing indica rice varieties IR64 and JHN, which possess inherent disease resistance traits, four QTLs associated with resistance to rice blast were identified and integrated through molecular marker-assisted selection. The resulting plants exhibited resistance to all 11 strains of rice blast (Sreewongchai et al. 2010). These rice varieties, endowed with resistance genes against rice blast disease, sustained their resistance over extended periods of cultivation, with the elevated resistance levels primarily attributable to the

combined effect of multiple QTLs. Four resistance-associated QTLs (pi21, Pi34, qBR4-2, qBR12-1) were incorporated into various polygenic lines. It was confirmed that the resistance conferred by these QTLs was more durable and non-specific to specific pathogen races (Fukuoka et al. 2015).

Conducting research on QTLs for rice blast resistance facilitates the precise identification of resistance genetic segments and the analysis of the underlying genetic mechanisms of disease resistance. By employing marker-assisted polymerization to integrate multiple resistance QTLs, it is possible to develop broad-spectrum and durable disease-resistant rice varieties, thereby decreasing pesticide use, enhancing yield stability, and reducing production costs. This approach holds substantial practical significance for the safe cultivation and environmentally sustainable breeding of rice.

Chapter 2

Objectives of thesis

1. Objectives of thesis

In previous studies, OsRLCK109 was identified as a negative regulator of rice blast resistance. However, the molecular mechanisms underlying its regulatory role remain largely unclear. In particular, the identity of its interacting partners and the signaling pathways through which it modulates rice immunity have not been fully elucidated.

The overall objective of this thesis is to decipher the molecular mechanisms by which OsRLCK109 regulates rice blast resistance, with a particular focus on its interaction network and downstream regulatory pathways.

To achieve this goal, the specific objectives of this work are:

1. To identify and characterize proteins interacting with OsRLCK109, in order to uncover key components of its regulatory network involved in rice immunity.

2. To investigate the role of OsRLCK109 in the regulation of reactive oxygen species (ROS) homeostasis by analyzing its interaction with protein kinases and downstream targets involved in oxidative stress responses.

3. To elucidate the transcriptional regulatory mechanisms mediated by OsRLCK109, particularly through its interaction with WRKY transcription factors and their downstream target genes.

4. To determine how these pathways contribute to the balance between biotic and abiotic stress responses, especially in the context of the trade-off between disease resistance and stress tolerance.

5. To explore the potential implications of these findings for rice improvement by identifying candidate genes and regulatory modules that could be targeted to enhance disease resistance.

Altogether, this study aims to provide a comprehensive understanding of OsRLCK109 as a central regulatory node integrating multiple signaling pathways involved in rice immunity.

2. Outline of thesis

This thesis is structured into five chapters, each addressing specific aspects of the objectives described above.

• Chapter 1 presents the general background of the study. It introduces the importance of rice for global food security, the fundamentals of the plant immune system (PTI and ETI), and current knowledge on rice blast disease. It also discusses the trade-off between growth and defense, thereby providing the conceptual framework supporting Objectives 2 and 4.

• Chapter 2 defines the research objectives and outlines the structure of the thesis.

• Chapter 3 addresses Objectives 1 and 2, focusing on the identification and functional characterization of OsRLCK109-interacting proteins involved in ROS regulation. This chapter demonstrates that OsWNK1 interacts with OsRLCK109 and

regulates ROS homeostasis through phosphorylation of OsCATA, thereby influencing both rice blast resistance and tolerance to abiotic stresses.

• Chapter 4 addresses Objectives 1 and 3, and partially Objective 4, by investigating the transcriptional regulatory pathway mediated by OsRLCK109. This chapter shows that OsRLCK109 interacts with and phosphorylates the transcription factor OsWRKY76, enhancing its repression of the PR1b gene and thereby negatively regulating rice blast resistance.

• Chapter 5 integrates the findings of the previous chapters and addresses Objectives 4 and 5. It provides a general discussion of the dual regulatory role of OsRLCK109 in coordinating ROS signaling and transcriptional control, highlights the trade-off between biotic and abiotic stress responses, and proposes perspectives for future research and potential applications in rice breeding.

Chapter 3

With No-lysine Kinase 1 regulates biotic and abiotic stresses tolerance in rice by targeting OsCATA to alter H_2O_2 homeostasis

To address the first objective of this thesis, Chapter 3 focuses on the identification and functional characterization of proteins interacting with OsRLCK109. In particular, this chapter aims to elucidate how OsRLCK109 contributes to the regulation of reactive oxygen species (ROS) homeostasis, a key component of plant immune responses. By investigating the interaction between OsRLCK109 and the kinase OsWNK1, as well as its downstream target OsCATA, this work explores a signaling module linking phosphorylation events to ROS dynamics. Through a combination of molecular, biochemical, and genetic approaches, Chapter 3 provides mechanistic insights into how this regulatory module influences rice blast resistance while also impacting tolerance to abiotic stresses. These results contribute to a better understanding of the role of OsRLCK109 in coordinating multiple stress response pathways.

This chapter is based on the following publication:

Zhang, Y., Liu, Q., Kang, Y., Wang, G., Duan, W., Zhang, Y., Wu, W., Chen, D., Hong, Y., Sun, L., Shen, X., Zhan, X., Cheng, S., Ludivine, L., & Cao, L. (2026). **With No-Lysine Kinase 1 Regulates Biotic and Abiotic Stress Tolerance in Rice by Targeting OsCATA to Alter H_2O_2 Homeostasis.** *Molecular plant pathology*, *27*(4), e70256. https://doi.org/10.1111/mpp.70256

1. Abstract

Receptor-like cytoplasmic kinases (RLCKs) play pivotal roles in regulating plant responses to both biotic and abiotic stresses. Although numerous RLCKs have been identified, their underlying regulatory mechanisms remain incompletely understood. In our previous work, we identified the rice OsRLCK109 as a negative regulator of blast resistance. In this study, we aimed to identify interaction partners of OsRLCK109 and to investigate their role in rice responses to both biotic and abiotic stresses. We identified OsWNK1 as an interacting partner of OsRLCK109 and characterized its inhibitory role in regulating rice blast disease, as well as drought and cold stress responses. OsWNK1 directly phosphorylates OsCATA, modulating its enzymatic activity and leading to elevated Reactive Oxygen Species (ROS) levels during pathogen infection. This enhances immune signaling and improves disease resistance. However, under abiotic stress conditions, reduced catalase activity in oswnk1 mutants impairs ROS scavenging, resulting in ROS toxicity and decreased stress tolerance. These findings reveal the dual regulatory role of the OsRLCK109–OsWNK1–OsCATA module in both biotic and abiotic stress responses, thereby offering new insights into a more comprehensive understanding of the stress response mechanism in rice through ROS signaling.

2. Introduction

Protein kinases constitute a large family of enzymes that primarily catalyze protein phosphorylation, a post-translational modification essential for regulating a wide range of biological processes (Lehti-Shiu and Shiu 2012; Zhang et al. 2023). A specific subset of receptor-like kinases (RLKs) that lack an extracellular portion is classified as receptor-like cytoplasmic kinases (RLCKs). Given that RLCKs lack extracellular structures, they are unable to directly sense extracellular signals to initiate intracellular reactions. Research has demonstrated that when RLKs detect extracellular signals, they phosphorylate RLCKs to modulate various cellular activities (Liang and Zhang 2022). Recently, there has been an increased number of reports detailing the role of RLCKs in mediating immunity in plants. For example, GhRLCK7 is significantly upregulated by Verticillium dahliae, and its silencing increases cotton susceptibility to infection (Cen et al. 2023). In Arabidopsis, BOTRYTIS-INDUCED KINASE 1, a member of the RLCK subfamily VII, phosphorylates SHOU4 and SHOU4L, thereby regulating cellulose synthesis and contributing to immunity (Wang et al. 2023). In maize, the ZmWAKL-ZmWIK complex phosphorylates ZmBLK1, which in turn activates NADPH oxidase ZmRBOH4, modulating ROS bursts and conferring resistance to gray leaf spot (Zhong et al. 2024). In rice, mutations in BSL2, which encodes an RLCK, cause programmed cell death and increased disease susceptibility (Wang et al. 2025). Collectively, these instances highlight the crucial role of RLCK in immune signaling across various plant species.

With No-lysine kinase (WNK), which belongs to a distinct subgroup of serine/threonine kinases, its name is derived from the absence of lysine at the N-terminus subdomain II of the protein. This lysine residue typically facilitates ATP binding and phosphorylation in other kinases. In plants, the conserved lysine is substituted by asparagine, serine, and glycine, whereas in animals, it is replaced by cysteine or asparagine (Verissimo and Jordan 2001). Since their first identification in mice, WNK-encoding genes have been found in both animals and plants. Furthermore, a greater number of WNK-encoding genes exist in plants compared to animals. Specifically, Arabidopsis possesses 11 WNK-encoding genes (Wang Y 2008), papaya contains 8, soybeans exhibit 26 (Su et al. 2024), and rice has 9 (Manuka, Saddhe, and Kumar 2015). Recently, WNK has also been reported in calamus and upland cotton (Zhang et al. 2023). Research on WNK in humans primarily focuses on its regulation of ion homeostasis, angiogenesis, and cancer metastasis (Arreola 2024). In the realm of botany, the cellular functions associated with the WNK family exhibit greater complexity. In Prunus persica, varying expression levels of WNK in the fruit have been documented at different stages of ripening, suggesting that WNK may be implicated in fruit development (Cao et al. 2019). In soybeans, GmWNK1 governs the morphological structure of the roots, and the overexpression of GmWNK1 in Arabidopsis enhances tolerance to NaCl and osmotic stress (Wang et al. 2011). AtWNK2, AtWNK5, and AtWNK8 regulate flowering in Arabidopsis (Li et al. 2021). Disruption of AtWNK8 and overexpression of AtWNK9 enhance tolerance to salt, osmotic, and drought stress (Zhang et al. 2013; Xie et al. 2014). OsWNK9 also confers salt and drought tolerance in rice (Negi and Kumar 2025), and OsWNK1 responds to environmental stress, displaying rhythmic expression patterns (Kumar et al. 2011). In bamboo, WNKs are also responsive to stress (Liu et al. 2022). However, little is currently known about the role of WNKs in plant disease resistance.

Reactive oxygen species (ROS) play a vital role in plant immunity, primarily encompassing hydrogen peroxide (H_2O_2), singlet oxygen, superoxide anions, and hydroxyl radicals. Among them, H_2O_2 is widely recognized as a key signaling molecule due to its electrical neutrality, relative stability, and reactivity (Wang et al. 2024). ROS are produced not only under stress conditions but also as part of normal physiological processes in plants (Kou, Qiu, and Tao 2019). However, excessive levels of ROS can be detrimental to plant health, making tight regulation of ROS homeostasis essential l (Mittler et al. 2022). This regulation relies on a balance between ROS-generating and ROS-scavenging enzymes (Yang et al. 2017). Catalase (Cat) constitutes a significant enzyme responsible for H_2O_2 scavenging (Riseh et al. 2024). In rice, three significant genes encode catalase: OsCATA, OsCATB, and OsCATC(Jiang et al. 2023). Ning Yuese et al. reported that during rice blast fungus infection, the pathogen effector protein AvrPiz-t enhances OsCATC activity, facilitating the removal of H_2O_2 and promoting pathogen colonization. Conversely, APIP6 degrades OsCATC, reducing its activity and thus limiting pathogen success. Rice Oscatc mutants accumulate high levels of H_2O_2, leading to cell death and enhanced resistance to the rice blast disease (You et al. 2022). In contrast, much less is known about the roles of OsCATA and OsCATB in rice-pathogen interactions, with

existing studies primarily linking them to abiotic stress responses (Asif et al. 2023; Wang et al. 2024; Shi et al. 2025).

According to current research, various plants respond to pathogenic bacterial invasion by using RLCK to activate downstream substrates, which leads to phosphorylation and activation of NADPH oxidase and the generation of ROS bursts both intracellularly and extracellularly (Li et al. 2014; Zhong et al. 2024). The rapid elimination of ROS is essential for plants to withstand biological and abiotic stresses and ensure survival. CAT is an important enzyme in plants for removing ROS. Zhang et al. reported that maintaining elevated levels of CAT is one of the reasons for the enhanced salt and osmotic stress tolerance observed in the *wnk8* mutant (Zhang et al. 2013). Nevertheless, it remains unclear whether the ROS burst triggered by RLCK to activate immune signaling depends on the transient inhibition of CAT activity and whether WNK regulates CAT enzyme activity via phosphorylation. These issues are currently unresolved, and investigating the interaction mechanisms among RLCK, WNK, and CAT holds significant importance.

In our previous work, we identified the rice receptor-like cytoplasmic kinase OsRLCK109 as a negative regulator of blast resistance (Zhang et al. 2019). However, the downstream regulatory mechanisms of OsRLCK109 remained unclear. In this study, we aimed to identify interaction partners of OsRLCK109 and to investigate their role in rice responses to both biotic and abiotic stresses. Specifically, we hypothesized that OsRLCK109 may modulate H_2O_2 homeostasis by phosphorylating antioxidant enzymes. To test this hypothesis, we focused on the potential role of OsWNK1, a kinase candidate identified through protein–protein interaction screening. The remainder of this work explores whether OsWNK1 regulates catalase activity, modulates ROS levels, and thereby contributes to both disease resistance and stress tolerance in rice.

3. Materials and Methods

3.1 Plant materials and growth conditions

oswnk1 and *oscata* rice plants were generated using the CRISPR/Cas9 method in the ZH11 context. The authors performed vector construction, and the genetic transformation was carried out by Biorun BioSciences. Plants used for rice blast inoculation and catalase activity assays were grown in sterilized soil within an incubator under a 14-hour light/10-hour dark cycle at 30°C. All other plant materials were cultivated under natural conditions in either Hangzhou or Lingshui experimental fields of the China National Rice Research Institute.

3.2 Pathogen inoculation and quantification

The plants that had been cultivated in an incubator (with 14 hours of light at 30°C and 10 hours of darkness at 25°C) for 40 days were utilized for inoculation. The *Magnaporthe oryzae* strain RB22 was grown on oat tomato medium in the dark for 5 days at 25°C, then exposed to light for 7 days. Spores were washed with sterile water

and adjusted to a final concentration of 5×10^5 spores mL^{-1}. A punch inoculation method was used: a 2 mm diameter hole was made in the leaf with a puncher, and 10 μL of spore solution was added. The wound was sealed with tape to maintain humidity. After inoculation, plants were kept in the dark for 24 hours and then transferred to a 12h light/12h dark photoperiod cycle at 25°C for 14 days. Lesion areas were quantified using ImageJ. Total DNA from the inoculated site was extracted using the CTAB method and was utilized to estimate fungal biomass through DNA-based qRT-PCR.

3.3 Yeast two-hybrid analysis

The vectors pGADT7 and pGBKT7 were used. Fusion plasmids carrying the coding sequences of the target genes were transformed into Y2HGold yeast cells. Yeast cultures were incubated for 4 days at 30°C. Monoclonal yeast colonies were diluted in sterile water in a serial gradient, and 5μL of each dilution was spotted onto selection medium to evaluate protein-protein interactions.

3.4 Quantitative RT-PCR analysis

Total RNA was extracted from rice leaves using the TIAGEN RNAprep Pure Plant Kit (Cat. no. 4992237). cDNA was obtained with the TOYOBO ReverTra Ace® qPCR RT Master Mix and gDNA Remover kit (Cat. no FSQ-301). PCR reagents were sourced from the TaKaRa TB Green® Premix Ex Taq™ II (Tli RNaseH Plus Cat. no RR820A). Each experiment included three biological replicates and three technical replicates, with Actin1 serving as the reference gene. Primer information is included in the Supplementary Material.

3.5 Co-immunoprecipitation (Co-IP) assay

HA-tagged constructs were driven by the Ubi promoter, as previously developed in vectors (He et al. 2018). The GFP-tag vector uses pYBA-1132, which is initiated by the CaMV 35S promoter. Full-length cDNA fragments were PCR-amplified and ligated into the corresponding vectors. Fusion constructs were transformed into rice protoplasts. Total protein was extracted after 16 hours of incubation at 25 °C.

The protein extraction method was previously described in works (Liu et al. 2017). Protein samples were incubated with Bimake Anti-HA magnetic beads (Cat: B26201), following the manufacturer's instructions. Eluted proteins were analyzed by Western blot using antibodies from TransGen Biotech (Cat. no. HT301-01 and HT801-01).

3.6 Immunoprecipitation coupled with mass spectrometry (IP-MS)

The immunoprecipitation experiment followed the same protocol as that for Co-IP. After immunoprecipitation, the protein was subjected to SDS-PAGE gel electrophoresis. Gel pieces were digested with trypsin overnight at 37 °C. Peptides were extracted with 50% acetonitrile/5% formic acid, followed by 100% acetonitrile. Peptides were dried to completion and resuspended in 2% acetonitrile/0.1% formic

acid. The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo) coupled online to the UPLC. The resulting MS/MS data were processed using Proteome Discoverer 2.1. The resulting MS/MS data were processed using Proteome Discoverer 2.1.

3.7 Luciferase complementation imaging (LCI) assay

The pCAMBIA1300-cLUC and pCAMBIA1300-nLUC vectors were used. The cDNAs of OsRLCK109 and OsCATA were fused into the nLUC vector, while OsWNK1 was fused into the cLUC vector. The recombinant plasmids were transformed into Agrobacterium GV3101. The cultures were grown at 28°C, and the OD600 was adjusted to 1.2 to 1.6 before injection. Equal volumes of the two bacterial solutions were mixed, and acetosyringone was added to a final concentration of 150μM. Equal volumes of Agrobacterium containing nLUC, cLUC, and Renilla luciferase (Ren) were combined and injected into Nicotiana benthamiana leaves. D-luciferin was injected 48 hours later for fluorescence imaging, analysis of luciferase activity, and detection of protein expression levels. The relative luciferase activity was calculated as the Luc/Ren ratio. Luciferase activity was then detected using a GloMax 96 microplate luminometer (Promega). Anti-luciferase antibody (Sigma, cat. no. L0159) and Anti-CLuc antibody (Sigma, cat. no. L2164) were used to detect proteins labeled with nLuc and cLuc, respectively.

3.8 Subcellular localization assay

OsWNK1 cDNA was fused to pYBA-1132 for GFP expression. RFP tag vectors were provided by the Yuese Ning research group (Institute of Plant Protection, CAAS). The fusion vectors were transformed into rice protoplasts and Nicotiana benthamiana leaves as described above. COX4 (Cytochrome c oxidase subunit 4) and PTS1 (Peroxisome targeting signal 1) were used as mitochondrial and Peroxisomal markers, respectively.

3.9 Chitin-induced H_2O_2 assay

Intact leaves from 40-day-old incubating plants were punched into 4 mm discs, avoiding veins. Any interference from mechanical damage was eliminated by immersing them in sterile water in the dark for 12 hours. Sterile absorbent paper was employed to remove excess water from the leaves, after which they were placed into a 1.5mL sterile transparent centrifuge tube. A reaction solution comprising 100μl of luminol was added, along with 1μl of horseradish peroxidase and either 1μl of distilled water (control) or 0.8μM chitin. Chemiluminescence was recorded every minute for 30 minutes using a GloMax 20/20 luminometer (Promega).

3.10 In vivo phosphorylation assays

OsCATA cDNA was cloned into the GFP vector (pYBA-1132) and expressed in rice protoplasts. Total protein was extracted, and OsCATA-GFP was immunoprecipitated using Anti-GFP magnetic beads. An Anti-OsCATA antibody was used to detect the

total amount of OsCATA protein, and an Anti-Phospho-(Ser/Thr) Phe antibody (ab17464) purchased from Abcam was used to detect the phosphorylated protein. The phosphorylation level was calculated as the ratio of phospho-OsCATA to total OsCATA using grayscale analysis with ImageJ. The gray value was calculated with reference to the previous (Stael et al. 2022).

3.11 Extracellular phosphorylation experiment

The expression vector OsRLCK109-GST employs pGEX-4T-1, whereas the vector His-OsCATA/OsCATC utilizes the pCold™ TF DNA vector. The purified protein was incubated in a reaction mixture comprising 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM ATP, and 10 mM MgCl2 at 30°C for a duration of 1 hour. Protein detection was carried out using the Anti-His Mouse Monoclonal Antibody and Anti-GST Mouse Monoclonal Antibody (TransGen HT601-01, HT501-01). Conversely, the Anti-Phospho-(Ser/Thr) Phe antibody (Abcam ab17464) was employed to identify phosphorylated proteins.

3.12 Pull-down assay

Following the introduction of the fusion vector into the BL21 strain, induction was conducted at 16°C with agitation at 120 rpm for a duration of 16 hours. The protein was subsequently purified and utilized in subsequent experimental procedures. The mixture of the protein and Glutathione High-Capacity Magnetic Agarose Beads (Sigma G0924) was incubated at room temperature for 30 minutes, after which Western blot analysis was performed.

3.13 Statistical analysis

Statistical significance was evaluated using Student's t-test or one-way ANOVA (GraphPad Prism). A p-value < 0.05 was considered significant. Details of statistical parameters are provided in the figure legends.

3.14 Gene ID

OsRLCK109 (LOC_Os03g24930), *OsWNK1* (LOC_Os07g38530), *OsWNK3* (LOC_Os07g08750), *OsCATA* (LOC_Os02g02400), *OsCATC* (LOC_Os03g03910), *PR1b* (LOC_Os07g03600), *PR1a* (LOC_Os07g03710), *PR10a* (LOC_Os12g36880), *PAL1* (LOC_Os02g41630)

4. Results

4.1 Screening of OsRLCK109 interacting proteins

The binding domain of the transcription factor GAL4 can bind to the upstream activating sequence independently without inducing transcription. However, specific proteins possess intrinsic activation or transcriptional functions, which enable the hybrid protein (BD-bait), formed through recombination of the DNA binding domain, to induce downstream reporter gene expression in the absence of a specific activation

domain. This phenomenon renders it challenging to ascertain whether the reporter gene expression results from the interaction between the bait and the prey. Consequently, the exclusion of self-activation is essential to accurately determine whether an interaction occurs between the bait and the prey. In library screening experiments, failure to detect self-activation prior to screening significantly increases the likelihood of obtaining numerous false-positive transformants.

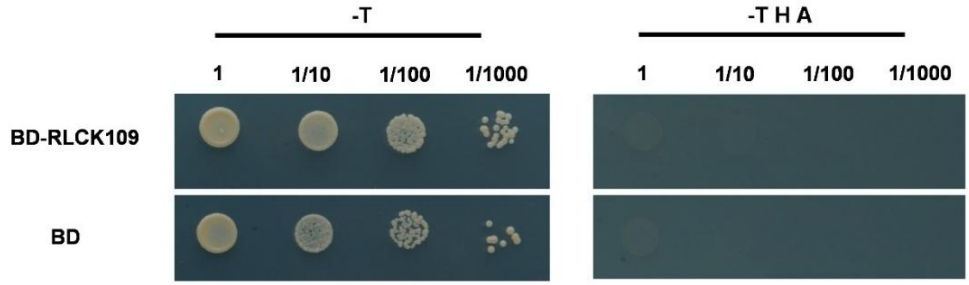


Figure 7: Detection of Self-Activation Activity of OsRLCK109 in the Yeast Two-Hybrid System. Yeast cells were cultured on SD/-Trp (-T) and SD/-Trp/-His/-Ade (-T H A) media at dilutions of 1, 1/10, 1/100, and 1/1000. The pGBKT7 (BD) empty vector served as the control.

To determine whether OsRLCK109 possesses self-activating activity, the complete coding sequence (CDS) of OsRLCK109 was subjected to PCR amplification and subsequently inserted into the pGBKT7 vector. The recombinant plasmid was then transformed into the Y2HGold strain. *HIS3* and *ADE2* were selected as reporter genes. The results demonstrated that BD-OsRLCK109 did not grow on the selective medium (SD/-Trp/-His/-Ade), indicating that OsRLCK109 lacks self-activation activity (Figure 7). The recombinant BD-OsRLCK109 protein can be utilized as bait for subsequent yeast library screening.

Utilizing BD-OsRLCK109 to screen a yeast library containing rice genomic cDNA, a total of 64 positive clones were obtained. Yeast plasmids were extracted from these clones and subsequently sequenced. The Blast function on the Gramene website (https://www.gramene.org/) was employed to identify a total of 29 candidate interacting proteins (Table 2).

Among the observations, OsWNK1 was more frequently identified in the positive clones during the screening procedure. It has been documented that RLCK receives phosphorylation signals from receptor protein kinases located on the cell membrane and subsequently activates the MAPK pathway to modulate immune responses (Lin et al. 2013). Consequently, our subsequent step will be to concentrate on OsWNK1 as the primary research focus and to investigate the role of the OsRLCK109-OsWNK1 module in the immune response of rice.

Table 2: List of Candidate Proteins Interacting with OsRLCK109

Number	ID	Protein type
1	LOC_Os06g02620	small heat shock protein
2	LOC_Os07g38530	With No Lysine Kinase 1
3	LOC_Os01g03390	Bowman-Birk type bran trypsin inhibitors
4	LOC_Os03g07880	CCAAT-box-binding transcription factor
5	LOC_Os04g46390	small heat shock protein
6	LOC_Os03g15890	RNA recognition motif-containing protein
7	LOC_Os09g24820	ZF-HD protein dimerization region containing protein
8	LOC_Os07g32880	ATP synthase gamma chain
9	LOC_Os11g33030	retrotransposon protein
10	LOC_Os08g01380	photosynthetic ferredoxin
11	LOC_Os05g49350	DUF1645 domain-containing protein
12	LOC_Os06g42810	UBA and UBX domain-containing protein
13	LOC_Os12g20150	phosphoglucan, water dikinase
14	LOC_Os08g36170	cytokinesis negative regulator RCP1

15	LOC_Os08g33050	MYB family transcription factor
16	LOC_Os01g2488	RING-finger E3 ligase
17	LOC_Os04g55159	alpha-amylase inhibitor 1
18	LOC_Os02g10480	ARF-GTPase-activating protein
19	LOC_Os01g72340	beta-galactosidase
20	LOC_Os12g08260	dehydrogenase E1 component domain-containing protein
21	LOC_Os08g45130	Histone H3K9 Methyltransferase Gene
22	LOC_Os01g03310	Bowman-Birk type bran trypsin inhibitor precursor
23	LOC_Os02g49190	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1
24	LOC_Os03g15890	RNA recognition motif-containing protein
25	LOC_Os09g33500	transketolase
26	LOC_Os03g53140	protein transport protein Sec23B
27	LOC_Os05g33360	transmembrane BAX inhibitor motif-containing protein
28	LOC_Os07g32880	ATP synthase gamma chain
29	LOC_Os11g47970	ribulose-1,5-bisphosphate carboxylase/oxygenase activase

4.2 OsWNK1 and OsRLCK109 directly interact

In order to confirm the interaction between OsRLCK109 and OsWNK1, first of all, full-length OsWNK1 was ligated to the bait vector (AD-OsWNK1) and transformed into yeast cells along with the prey vector (BD-OsRLCK109). As a specificity control, OsWNK3, the most similar WNK family member to OsWNK1 in rice, was tested in parallel. Only yeasts containing both BD-OsRLCK109 and AD-OsWNK1 were able to grow on SD/-Leu/-Trp/-His/-Ade medium, while those containing BD-OsRLCK109 and AD-OsWNK3 were not viable (Figure 8A). This implies that OsWNK1 interacts specifically with OsRLCK109.

We then used luciferase complementation imaging (LCI) assay to evaluate the interaction between OsWNK1 and OsRLCK109 in *Nicotiana benthamiana*. OsRLCK109 was fused to the N-terminal of Luc (OsRLCK109-nLuc), and OsWNK1 was fused to the C-terminal of Luc (cLuc-OsWNK1). The results showed that the samples co-expressing cLuc-OsWNK1 and OsRLCK109-nLuc displayed strong luminescence signals (Figure 8B, C, and S1), indicating that the interaction between OsWNK1 and OsRLCK109 could be detected in planta.

To further demonstrate the interaction in rice, fusion proteins OsWNK1-GFP and HA-OsRLCK109 were expressed in rice protoplasts for co-immunoprecipitation (Co-IP) experiments. When OsWNK1-GFP and HA-OsRLCK109 were co-expressed, OsWNK1-GFP could be co-immunoprecipitated by HA-OsRLCK109, but GFP protein without OsWNK1 could not be co-immunoprecipitated by HA-RLCK109 (Figure D), indicating that OsWNK1 and OsRLCK109 interact in rice. The yellow fluorescence signal of OsRLCK109 and OsWNK1 interaction was also observed in the BiFC experiment (Figure 9). Taken together, these results confirm a specific and direct physical interaction between OsRLCK109 and OsWNK1, suggesting that OsWNK1 may function as a downstream effector or modulator within OsRLCK109-mediated signaling pathways.

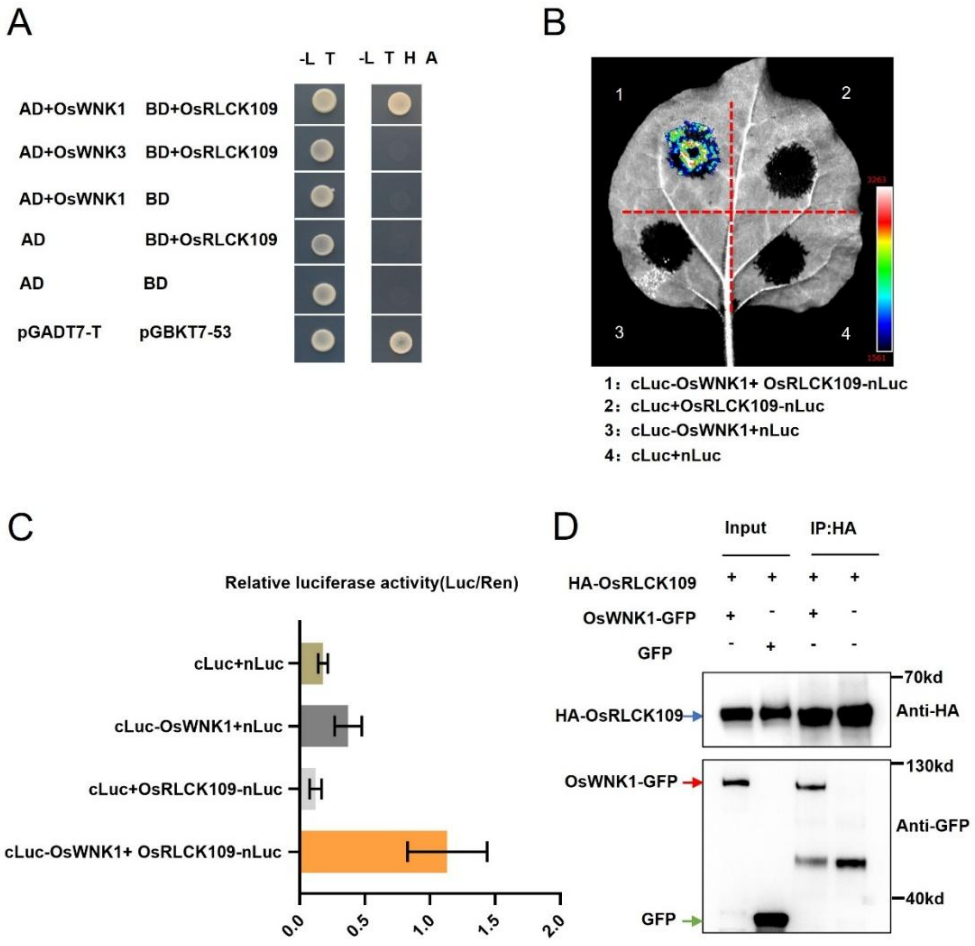


Figure 8: OsRLCK109 and OsWNK1 interact physically. (A) OsRLCK109 and OsWNK1 engage in the yeast two-hybrid system. Yeast cells were cultured on SD/-Leu/-Trp (-L T) and SD/-Leu/-Trp/-His/-Ade (-L T H A) media. OsWNK3 served as a specific control. (B) and (C) LCI assays were utilized to analyze the interaction between OsRLCK109 and OsWNK1. (B) A fluorescence image illustrates the interaction between OsRLCK109 and OsWNK1 in tobacco leaves. (C) Data from the luciferase activity assay in part B. The relative luciferase activity was calculated as the Luc/Ren ratio. Bars represent means ± SE, n = 3. (D) A Co-IP assay was conducted to detect the interaction between OsRLCK109 and OsWNK1 in rice protoplasts. Total protein was extracted 16 hours after plasmid transformation for immunoprecipitation. The immunoprecipitation was carried out using Anti-HA Magnetic Beads. The GFP protein acted as a negative control.

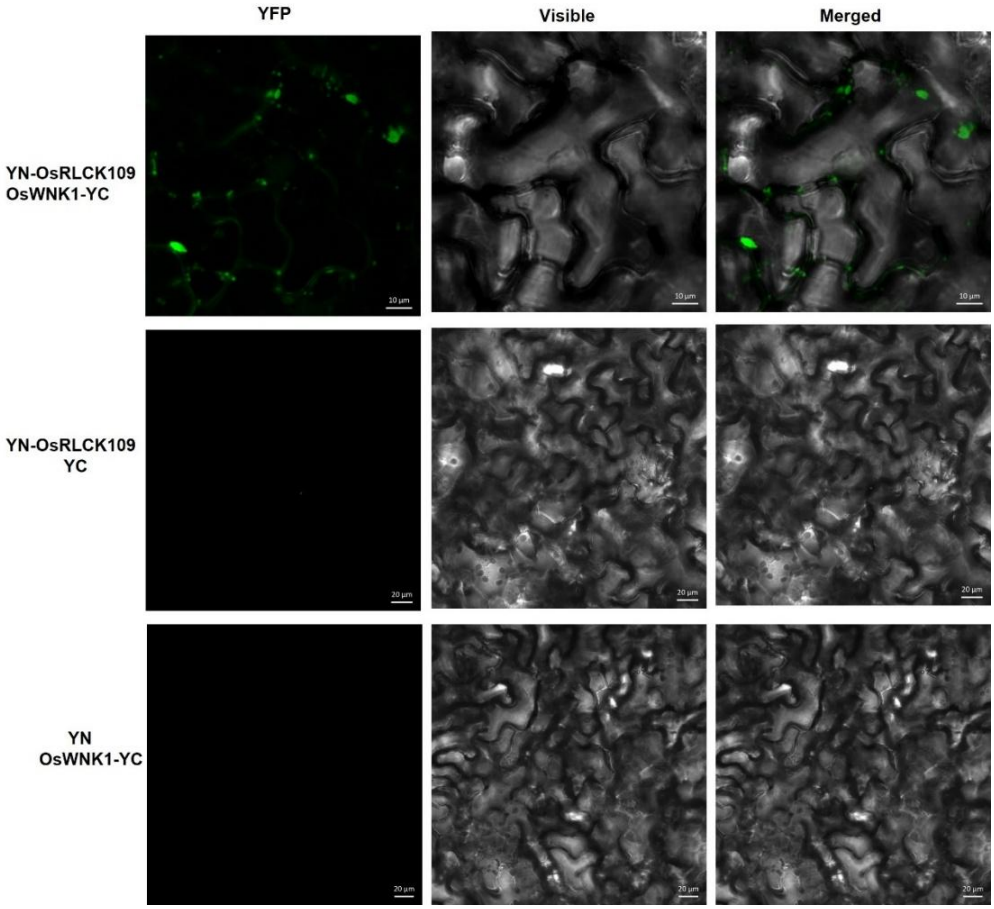


Figure 9: BiFC assay. To generate BiFC vectors, the full-length CDS of *OsRLCK109* was amplified via PCR and cloned into the pSPYNE-35S-pUC-SPYNE vector, and the full-length cDNA sequences of *OsWNK1* were cloned into the pSPYNE-35S-pUC-SPYCE. The constructs were transiently expressed in *N. benthamiana* by agroinfiltration. Three days after infiltration, fluorescence was observed using a laser scanning confocal microscope (ZEISS 750). Using an argon laser, GFP was excited at 488 nm and RFP at 552 nm. The interaction signal (yellow light point) is visible in the figure.

4.3 Analysis of subcellular localization and bioinformatics of OsWNK1

To investigate the biological function of OsWNK1, we analyzed its amino acid sequence using the CDD database from the NCBI database. OsWNK1 consists of 704 amino acids, with amino acids 25-284 predicted to encode the catalytic domain of the Serine/Threonine kinase, With No Lysine (WNK) kinase (STKc_WNK) (Figure 10). This finding provides an essential clue for its classification within the WNK family.

Additionally, amino acids 377-434 were predicted to encode the C-terminal domain of Oxidative-stress-responsive kinase 1 (OSR1_C) (Figure 10). These results suggest that OsWNK1 is a Serine/Threonine kinase potentially involved in oxidative stress.

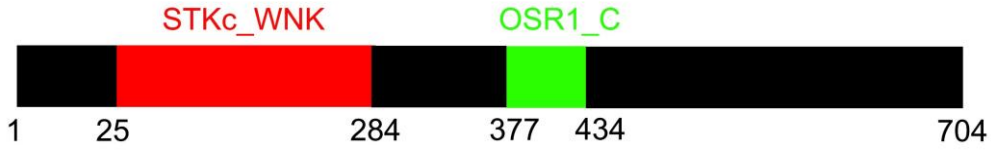


Figure 10： Schematic diagram of the structure of the OsWNK1 protein. Predicted OsWNK1 protein structure from the NCBI website. The WNK kinase domain is shown in red, and the oxidative stress kinase domain is represented in green. Other domains are indicated in black. The numbers indicate the positions of the amino acids.

We compared the amino acid sequence of OsWNK1 with other rice varieties using the UniProt database. OsWNK1 showed over 95% identity with wild rice accessions, particularly in the kinase domain (Figure 11). This conservation indicates that OsWNK1 may play a significant role in species evolution and is therefore subject to intense natural selection pressure. Mutations in the kinase domain may lead to abnormal protein function and, consequently, less accumulation of variation. Multiple sequence alignment with other WNK family members revealed that the WNKs in rice were primarily conserved in the kinase domain (Figure 12), with substantial differences in other domains. OsWNK3 was the least different from OsWNK1, showing 35% identity.

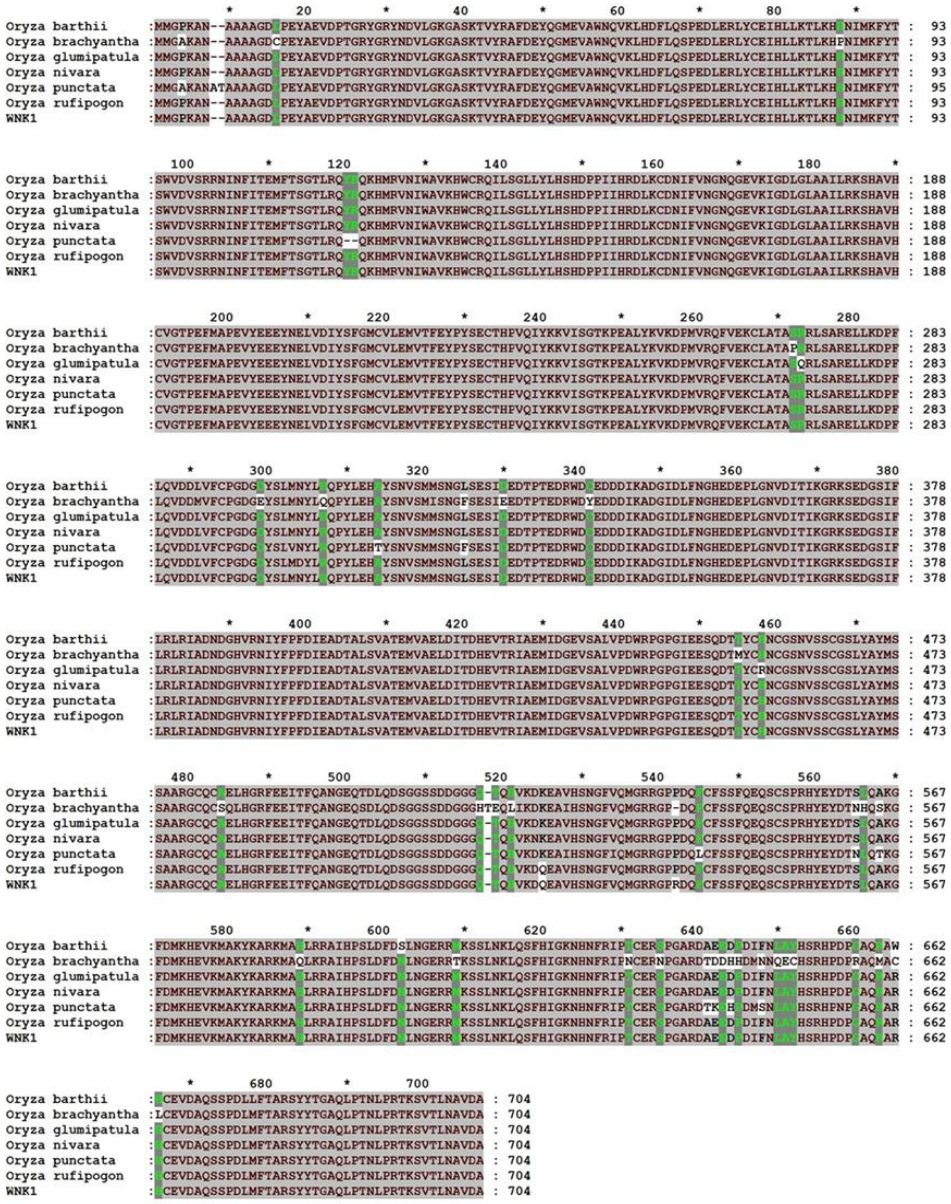


Figure 11: Amino acid sequences of *OsWNK1* were compared with those of wild rice. Differential amino acids are shown in green, and identical amino acids are in brown. Amino acid sequence data were obtained from UniProt, and sequence alignment was executed using MEGA software.

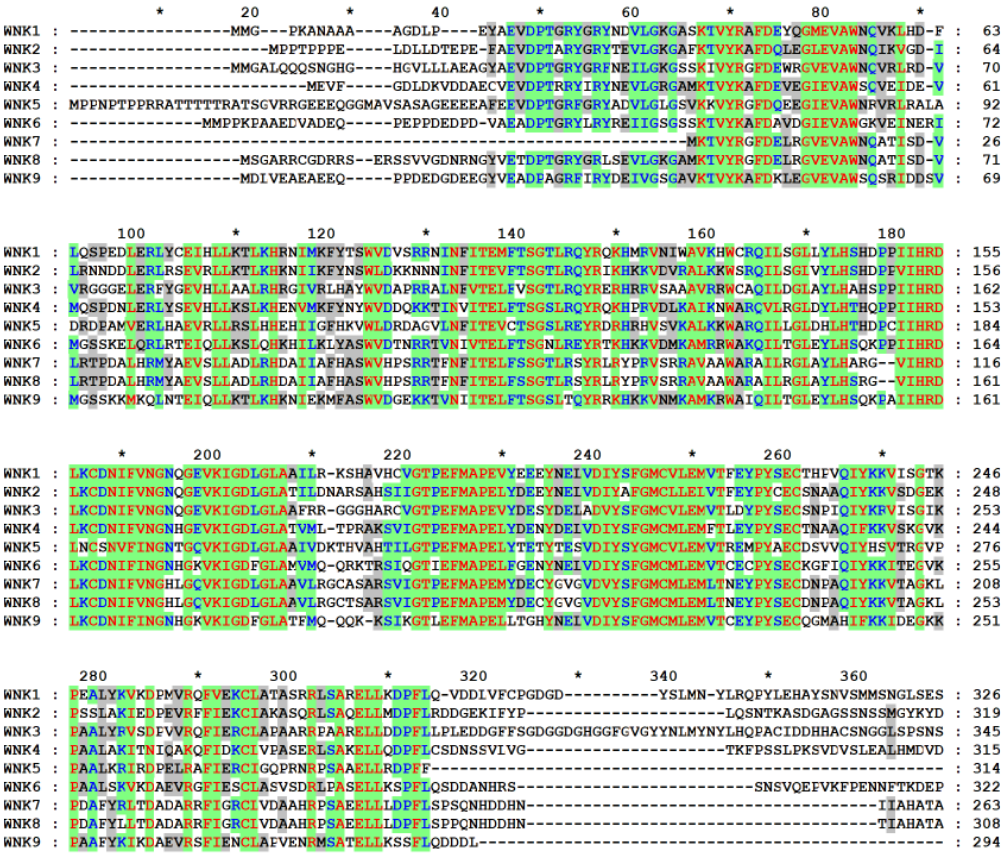


Figure 12: Sequence comparison of the amino acids encoding the kinase domain of OsWNK1 and other OsWNKs in rice.

4.4 Subcellular localization of OsWNK1

To clarify the biological function of OsWNK1, rice protoplasts and tobacco leaves expressing the OsWNK1-GFP fusion protein were used for subcellular localization experiments. In both systems, punctate green fluorescence signals were observed. Co-expression with organelle-specific markers showed clear co-localization of OsWNK1-GFP with RFP-PTS1 (peroxisome marker) and RFP-COX4 (mitochondrial marker). Similarly, OsRLCK109 exhibits both mitochondrial and peroxisomal localization (Figure 13). These findings indicate that OsWNK1 and OsRLCK109 primarily perform their functions within mitochondria and peroxisomes. Finally, we examined the expression pattern of OsWNK1 in various rice tissues. The results showed that OsWNK1 was constitutively expressed, with the highest transcript level detected in leaves (Figure S2).

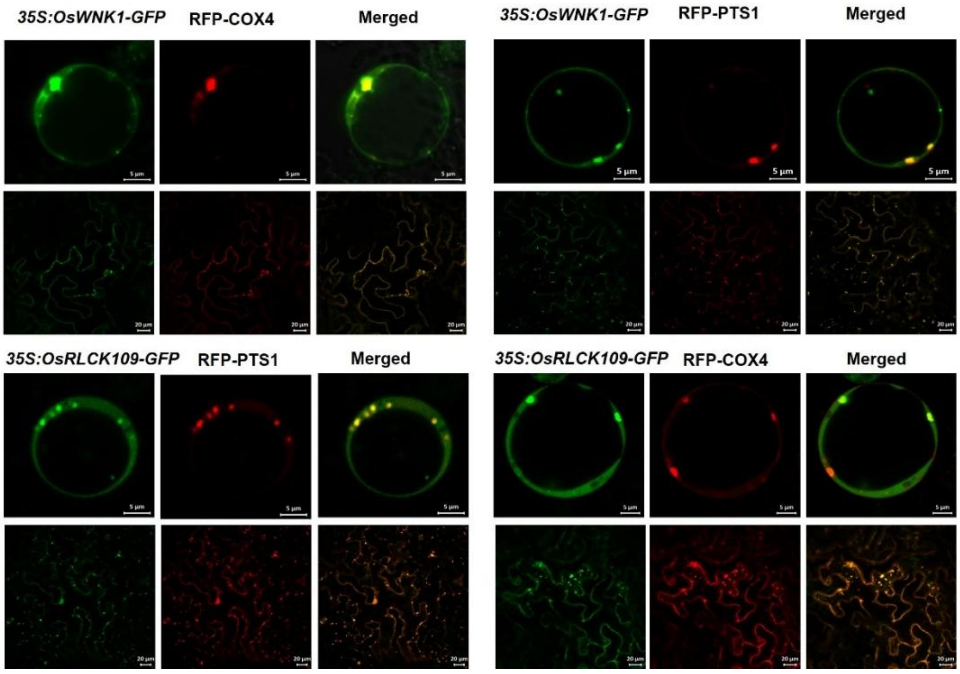


Figure 13: Subcellular localization of OsWNK1 and OsRLCK109. The green fluorescence signal indicates 35S: OsWNK1-GFP and 35S: OsRLCK109-GFP, while the red fluorescence signal denotes the specifically expressed protein. COX4 and PTS1 were used as standard markers for mitochondria and peroxisomes, respectively. The upper row of the image represents rice protoplasts, and the lower row illustrates the lower epidermal cells of tobacco leaves.

4.5 OsWNK1 negatively regulates blast resistance in rice

AtWNK1 regulates circadian rhythms in Arabidopsis, while the function of OsWNK1 in rice remains unknown. To investigate the role of OsWNK1 in rice disease resistance, we employed CRISPR-Cas9 technology to generate OsWNK1 functionally deficient mutants (*oswnk1*). Two types of T2 generation homozygous mutants were obtained, including one with a single-base deletion and another with a 15-base deletion, both of which led to premature termination of translation (Figure 14).

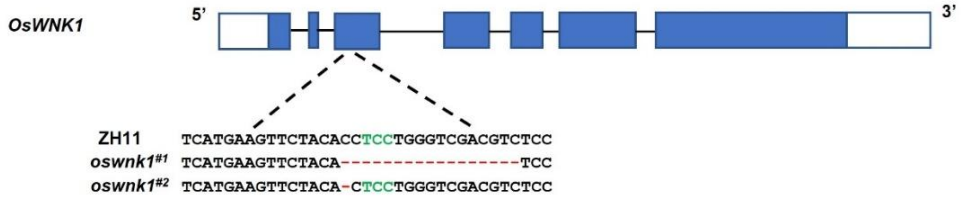


Figure 14: The *OsWNK1* knockout mutation type. The red horizontal line signifies the deletion of a gene.

Interestingly, at the seedling stage, the *oswnk1* plants displayed brown disease spots on the leaves, similar to those observed in *osrlck109* mutants (Figure 15A). However, at a later developmental stage, lesions in *osrlck109* were more severe and visibly affected growth (Figure 15B), whereas those in *oswnk1* remained less extensive and did not impact growth significantly. Diaminobenzidine (DAB) staining indicated that *oswnk1* leaves accumulated more H_2O_2 than the wild type ZH11 (Figure 15A), a phenotype commonly associated with lesion mimic mutants and enhanced disease resistance. Following inoculation with rice blast fungus, *oswnk1* mutants showed reduced lesion size and fungal biomass compared to wild-type (Figure 15C–E), indicating increased resistance. We also observed elevated expression of pathogenesis-related genes (*PR1a, PR1b, PR10a*) and a lignin biosynthesis gene (*PAL1*) in *oswnk1* (Figure 15F), further supporting an enhanced immune response. These results demonstrate that OsWNK1 functions as a negative regulator of rice blast resistance, possibly by modulating ROS levels and activating defense gene expression.

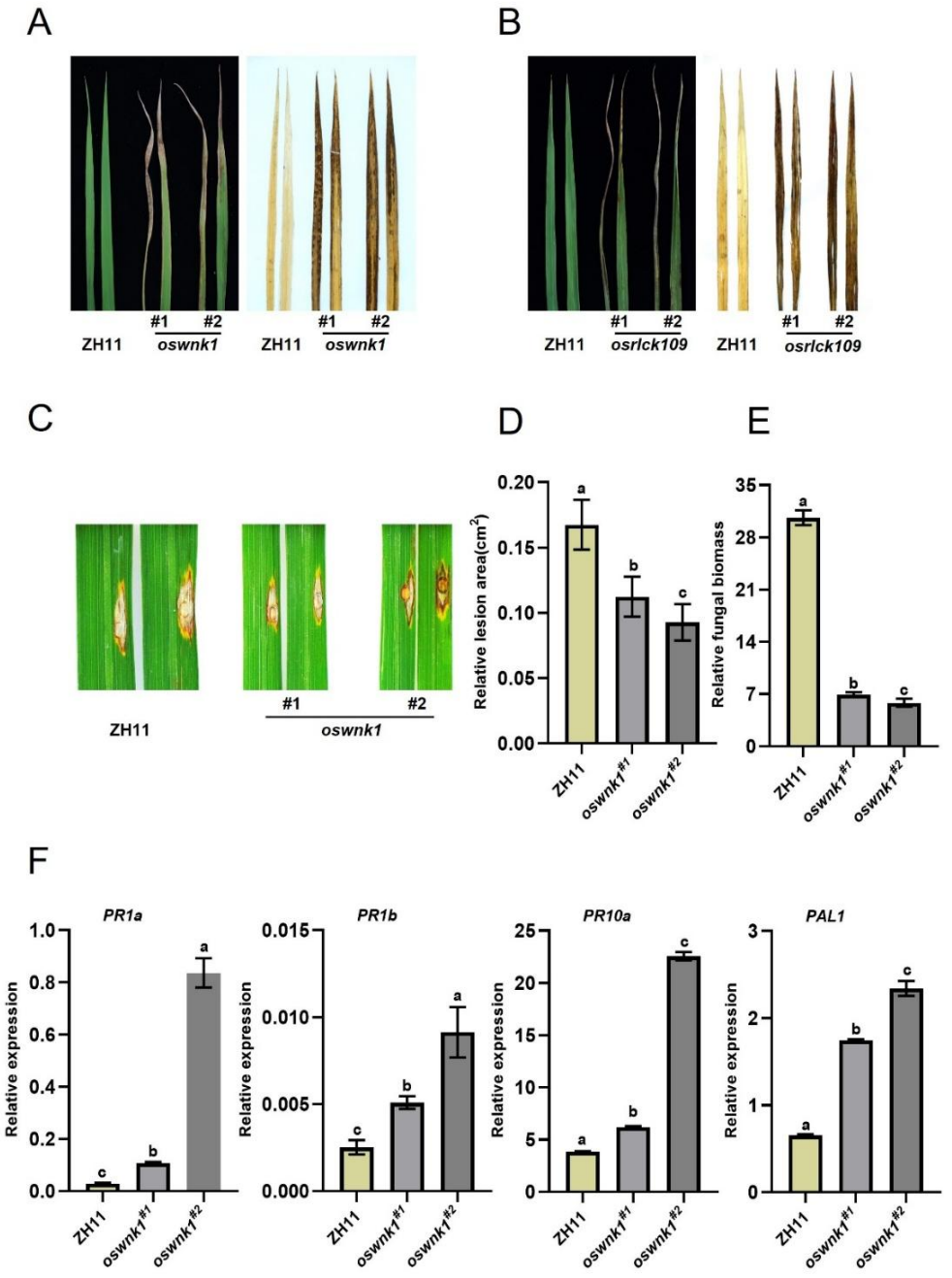


Figure 15: Resistance performance of rice blast inoculated with *oswnk1*. (A) and (B) *oswnk1* and *osrlck109* induce cell death phenotypes. Leaves from rice plants grown in the field for

40 days were utilized for DAB staining. (C) Punch inoculation of the ZH11 and the *oswnk1* mutant. Leaves were photographed 14 days after inoculation to document the phenotype. (D) and (E) Analysis of relative lesion area and relative fungal biomass post-inoculation. (F) qRT-PCR analysis of genes related to pathogenesis and lignin synthesis. Total RNA was extracted from leaves of 40-day-old non-inoculated plants grown in the growth chamber. Bar values (D, E, F) represent mean ± SD from three biological replicates.

AtWNK1 regulates the circadian rhythm in *Arabidopsis thaliana*; however, it remains unclear whether OsWNK1 performs a similar function in rice. Field-grown *oswnk1* plants showed no difference in heading date compared to wild-type ZH11, suggesting that OsWNK1 does not play a major role in rice flowering time (Figure 16).

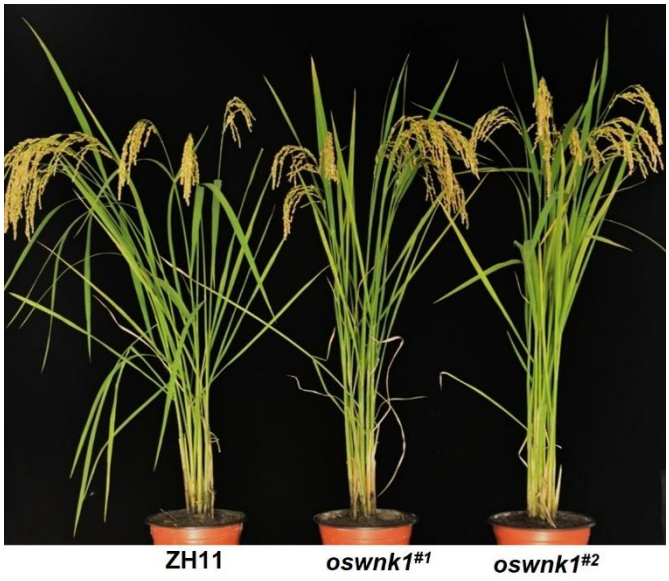


Figure 16: Phenotypes of ZH11 and *oswnk1* in the field.

4.6 OsWNK1 interacts with catalase OsCATA

To search for evidence that OsWNK1 regulates immunity, we aimed to identify OsWNK1-interacting proteins. Immunoprecipitation followed by mass spectrometry (IP-MS) identified 631 peptides corresponding to 226 proteins. Among these, rice catalases OsCATA and OsCATC emerged as candidate interactors. Given the previous observation of H_2O_2 accumulation in *oswnk1* leaves, we further investigated whether OsWNK1 could interact with OsCATA and OsCATC.

First, the interaction of OsWNK1 with OsCATA and OsCATC was assessed using a yeast two-hybrid system. Results indicated that co-transformed yeast cells with

AD+OsWNK1 and BD+OsCATA were able to grow on SD/-Leu/-Trp/-His medium, whereas co-transformed yeast cells with AD+OsWNK1 and BD+OsCATC were unable to grow (Figure 17A). This suggests that OsWNK1 and OsCATA interact. LCI analysis demonstrated that tobacco leaves co-transformed with nLuc-OsCATA and OsWNK1-cLuc displayed a clear interaction fluorescence signal. When nLuc-OsCATC and OsWNK1-cLuc were co-expressed, no signal was detected (Figure 17B and Supplementary Figure S2). A luciferase activity assay confirmed that the combination of nLuc-OsCATA and OsWNK1-CLuc exhibited the strongest enzyme activity (Figure 17C), indicating that OsWNK1 and OsCATA interacted in planta, but OsCATC does not. To confirm this interaction in rice, we performed Co-IP in protoplasts. OsWNK1-GFP was coimmunoprecipitated by HA-OsCATA, while GFP alone was not (Figure 17D), demonstrating that OsWNK1 interacts with OsCATA in *vivo*. Taken together, these results show that OsWNK1 specifically interacts with the catalase isoform OsCATA, supporting its role as a redox regulatory kinase. This interaction supports the hypothesis that OsWNK1 could directly influence ROS detoxification by targeting catalase activity, potentially linking kinase signaling to redox regulation.

Similarly, we examined whether OsRLCK109 interacts with OsCATA and OsCATC. The results showed that OsRLCK109 interacted with OsCATA and OsCATC in both the yeast system and in *vitro* (Supplementary Figure S3). We also conducted an in *vitro* phosphorylation experiment to determine whether RLCK109 phosphorylates OsCATA and OsCATC. Regrettably, no phosphorylation of OsRLCK109 on OsCATA and OsCATC was detected in *vitro*.

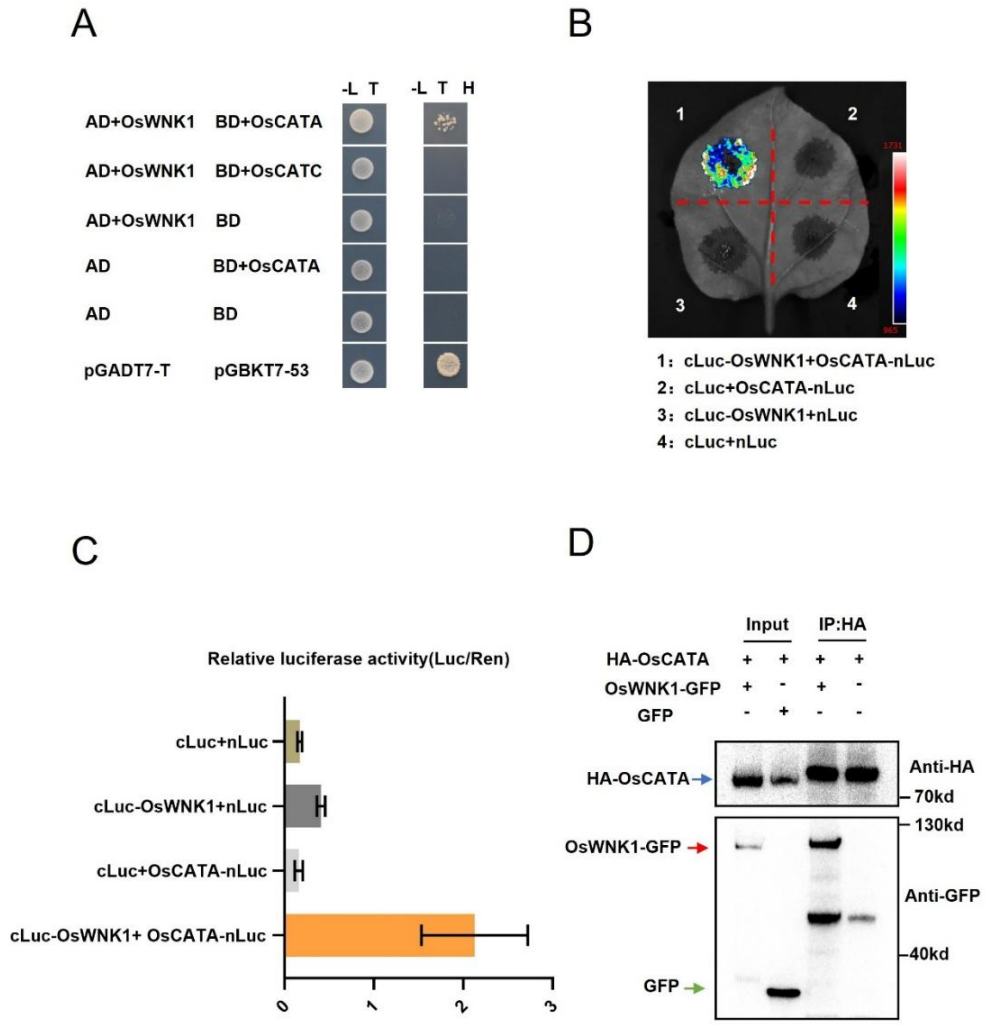


Figure 17: OsCATA and OsWNK1 physically interact. (A) OsCATA and OsWNK1 interact in the yeast two-hybrid system. Yeast cells were grown on SD/-Leu/-Trp (-L T) and SD/-Leu/-Trp/-His (-L T H) medium. (B) and (C) LCI assays were used to analyze the interaction between OsCATA and OsWNK1. (B) Fluorescence image of the interaction between OsCATA and OsWNK1 in tobacco leaves. (C) Data from the luciferase activity assay in B. Bar values represent mean ± SD from three biological replicates. (D) Co-IP assay was used to detect the interaction between OsCATA and OsWNK1 in rice protoplasts. Total protein was extracted 16 h after plasmid transformation for immunoprecipitation. Immunoprecipitation was performed using Anti-HA Magnetic Beads. GFP protein was used as a negative control.

4.7 Knockout OsCATA enhances rice blast resistance

To elucidate the function of *OsCATA* in rice disease resistance, we developed *OsCATA* knockout lines (*oscata*) utilizing CRISPR/Cas9 technology. We acquired two variants of genetic modifications featuring deletions of 8 and 2 bases, respectively, within the second exon of *OsCATA* (Figure 18), resulting in premature termination of protein synthesis. These two variants were employed in subsequent experimental investigations.

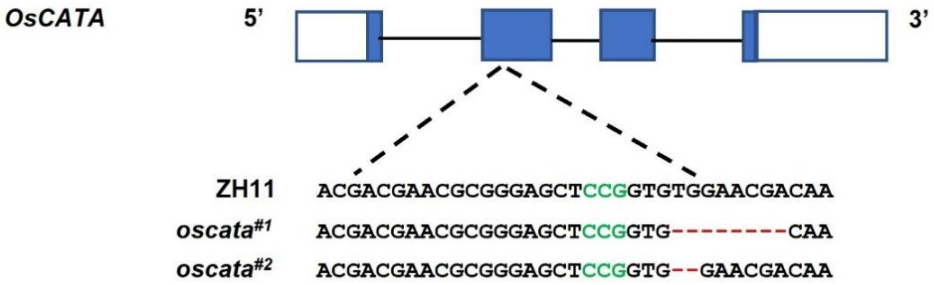


Figure 18: The *OsCATA* knockout mutation type. The red horizontal line signifies the deletion of a gene.

oscata exhibited the same self-inflicted lesions in the leaves due to excessive accumulation of H_2O_2, as observed in *oswnk1* and *osrlck109* (Supplementary Figure S4). Rice blast inoculation experiments were conducted on the T1 generation plants obtained. Two independent *oscata* lines showed visibly smaller lesions compared to the wild type, and fungal biomass quantification confirmed significantly reduced pathogen growth (Figure 19A–C). In a manner analogous to the previously mentioned experiments, we additionally assessed the expression levels of defense-related genes. As expected, *oscata* plants exhibited elevated expression of *PR1a, PR1b, PR10a*, and *PAL1* (Figure 19D), consistent with enhanced resistance. These results indicate that OsCATA acts as a negative regulator of blast resistance and that its disruption leads to constitutive activation of immune responses.

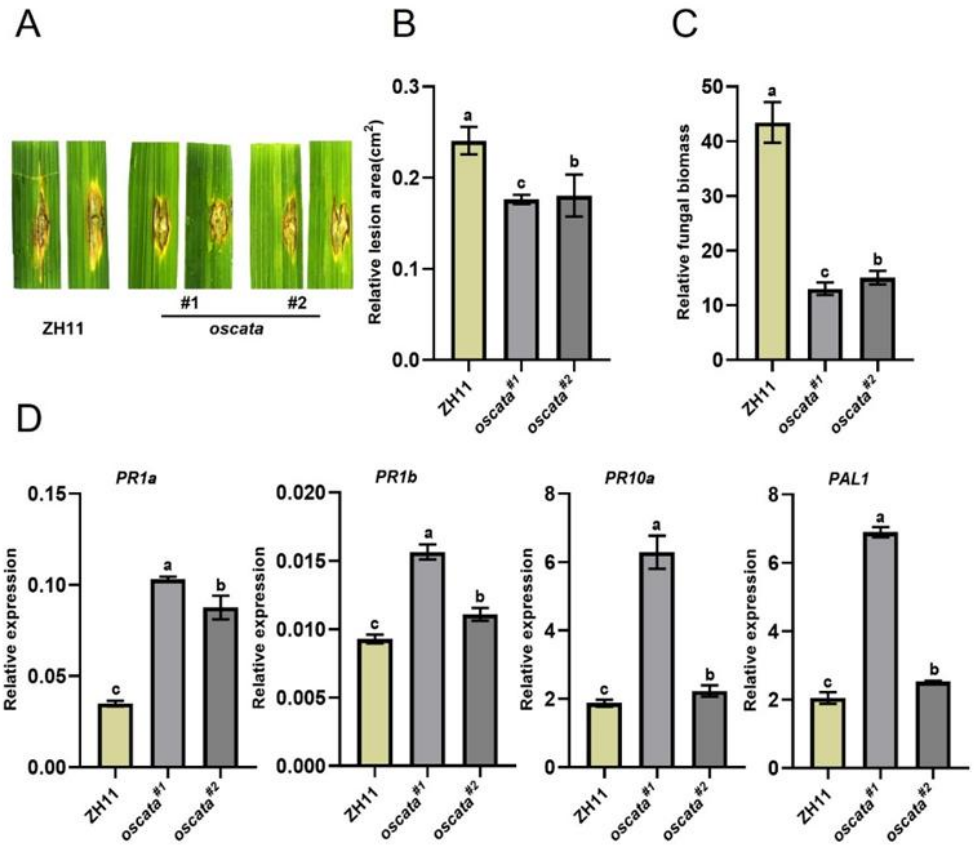


Figure 19: The *oscata* plants were resistant to rice blast. (A) Punch inoculation of the ZH11 and the *oscata* mutant. Leaves were photographed 14 days after inoculation to document the phenotype. (B) and (C) Analysis of relative lesion area and relative fungal biomass post-inoculation. (D) qRT-PCR analysis of genes related to immune responses in ZH11 and *oscata*. Total RNA was extracted from leaves of 40-day-old non-inoculated plants grown in the growth chamber. Bar values (B, C, D) represent mean ± SD from three biological replicates.

4.8 OsWNK1 phosphorylates OsCATA in vivo and regulates catalase activity

WNKs are serine-threonine kinases that regulate downstream targets through phosphorylation. Since we could not express OsWNK1 in *vitro*, phosphorylation experiments could not be performed in *vitro*. To determine whether OsWNK1 phosphorylates OsCATA and modulates its activity, we examined the phosphorylation state of OsCATA in *oswnk1* and wild-type ZH11 plants. The results showed that the

phosphorylation rate of OsCATA in *oswnk1* was lower than that in ZH11 when OsCATA-GFP was expressed alone (Figure 20A and B). However, it significantly increased in *oswnk1* when Myc-OsWNK1 was co-expressed, while it remained almost unchanged in ZH11. These results indicate that OsWNK1 directly phosphorylates OsCATA in *vivo*.

To understand whether the loss of function of OsWNK1 directly impacted OsCATA catalase activity, we next examined the catalase activity of *oswnk1*. The results indicated that the catalase activity in *oswnk1* was significantly lower than that in ZH11 and was similar to that observed in *oscata* (Figure 20C and D), suggesting that OsWNK1 positively regulates OsCATA enzymatic function. Based on these observations, we hypothesized that decreased catalase activity in *oswnk1* and *oscata* mutants leads to greater H_2O_2 accumulation during pathogen challenge, which in turn enhances the immune response. To test our hypothesis, H_2O_2 accumulation experiments were conducted. Rice leaves were treated with chitin to simulate pathogen infection and detect H_2O_2 accumulation. The results demonstrated that *oswnk1* and *oscata* accumulated higher levels of H_2O_2 than ZH11 when leaves were treated with chitin (Figure 20E and F), supporting our hypothesis. Taken together, these findings provide functional evidence that OsWNK1 not only binds to but also post-translationally modifies OsCATA, thereby actively shaping ROS homeostasis in rice.

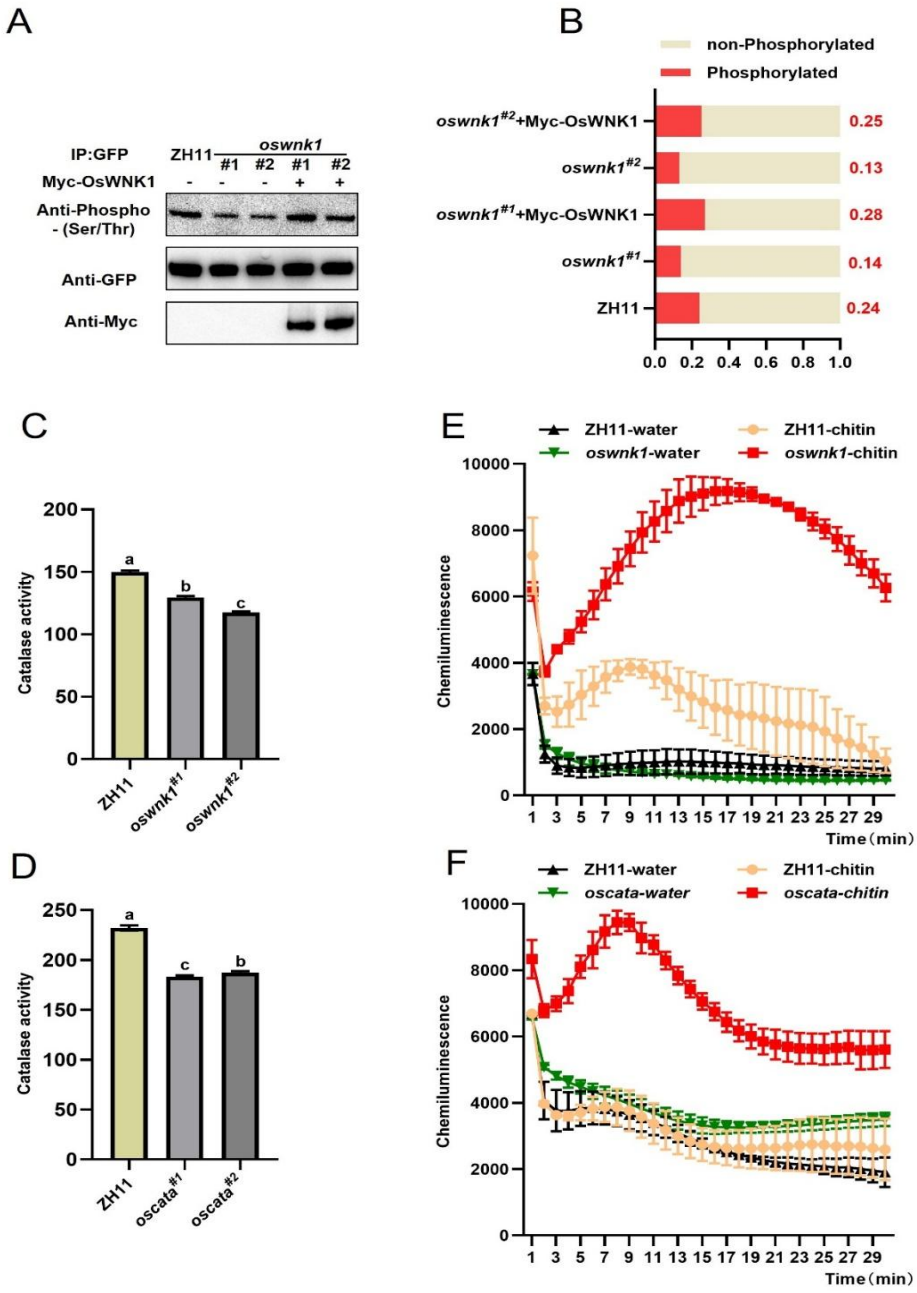


Figure 20: OsWNK1 phosphorylates OsCATA in *vivo* and affects catalase activity. (A) The in *vivo* phosphorylation of OsCATA was measured. OsCATA-GFP protein was expressed in

rice protoplasts for 16 hours following the extraction of total protein and immunoprecipitation with Anti-GFP magnetic beads. Total OsCATA was detected using the Anti-GFP antibody, while phosphorylated OsCATA was detected with the Anti-Phospho-Ser/Thr Phe antibody. Protein quantification was carried out using ImageJ for gray value calculation. (B) Data in A were visualized with GraphPad Prism. The total OsCATA protein was treated as 1. Numbers represent the ratio of the OsCATA phosphorylated band to the total protein band. (C) and (D) Catalase activity assays were performed in *oswnk1* and *oscata* mutants. Plants grown in an incubator for 20 days were used for the experiments. ZH11 served as a control. Bar values represent mean ± SD from three biological replicates. (E) and (F) Chitin-induced H_2O_2 burst in oswnk1 and *oscata* leaves. Water treatment was used as a negative control. Bars are shown as means ± SE, n = 3.

4.9 OsWNK1 negatively regulates drought stress and cold stress

Previous studies have reported that OsWNK1 is induced to express in response to various abiotic stresses (Kumar et al. 2011). To better understand its functional role, we evaluated the performance of *oswnk1* plants under drought and cold stress treatments. The results demonstrated that under conditions of drought and cold stress, *oswnk1* mutants showed increased sensitivity compared to the wild type control ZH11, with visible stress symptoms and significantly reduced survival rates (Figure 21A–D). Additionally, the H_2O_2 content was measured subsequent to stress exposure. The findings revealed that, when subjected to cold and drought stress, *oswnk1* accumulated a higher concentration of H_2O_2 (Figure 21E and F), which may contribute to the observed stress sensitivity. These findings highlight the role of OsWNK1 in abiotic stress responses: its loss enhances ROS accumulation, but simultaneously impairs tolerance to abiotic stress due to disrupted ROS homeostasis and insufficient detoxification capacity.

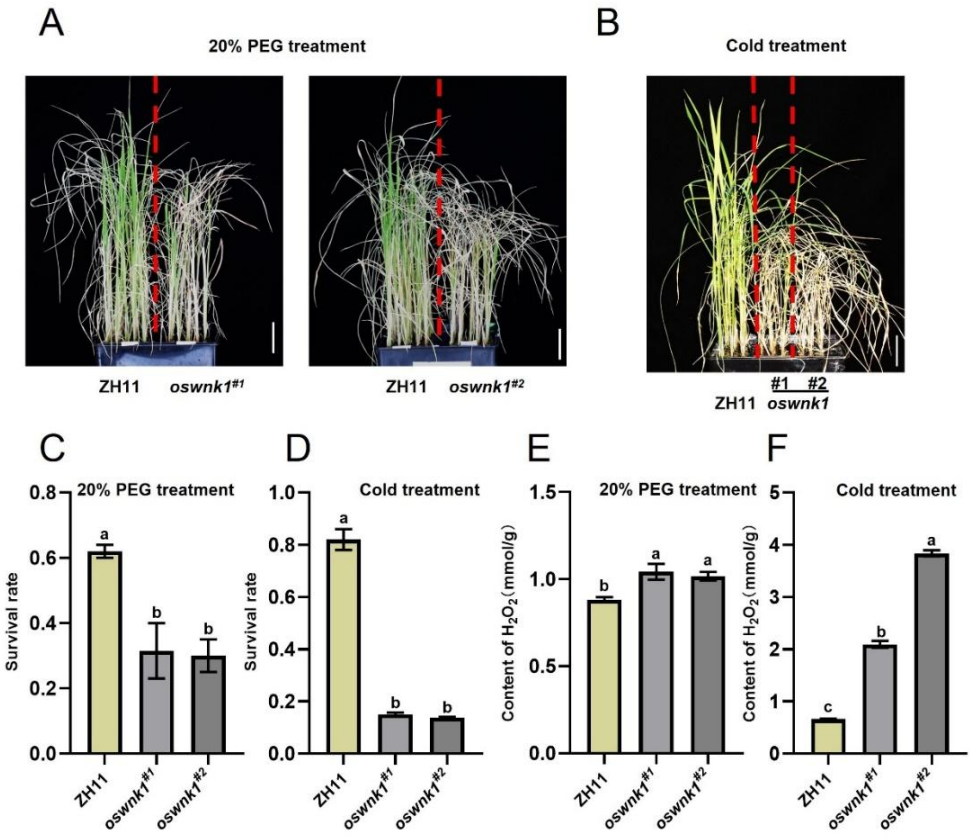


Figure 21: OsWNK1 is a negative regulator of drought and cold stress. (A) Under drought stress for 7 days, the phenotypes of *oswnk1* and ZH11 were observed after 7 days of recovery. The plants that had been grown in the incubator for 15 days were used for the experiment. 20% PEG6000 solution simulates drought. (B) The phenotypes of oswnk1 and ZH11 under 4°C treatment for 4 days after 10 days of recovery. (C) The survival rate statistics are shown in A. (D) The survival rate statistics are shown in B. (E) The content of H_2O_2 in leaves was determined 7 days after drought stress. (F) The content of H_2O_2 in the leaves was determined 4 days after cold stress. Bar values (C, D, E, F) represent mean ± SD from three biological replicates. Scale bar: 5 cm in (A) and (B).

5. Discussion

In contrast to animals, plants do not possess specialized immune cells (Ngou, Jones, and Ding 2022). Nevertheless, they exhibit distinct response mechanisms to counteract pathogen attacks, which encompass PTI and ETI (Delplace et al. 2022). During PTI, PRRs located on the cell surface detect PAMPs and subsequently transmit signals to the cell interior to initiate an immune response (Zhou and Zhang 2020). In

this process, RLCKs generally function as intermediaries in immune signaling, receiving signals from PRRs (Vij et al. 2008; Sun and Zhang 2020). Numerous studies have confirmed that RLCKs can phosphorylate downstream proteins to facilitate signal transduction. The activation of the MAPK cascade is a crucial step in the immune pathway (Liang and Zhou 2018). Functional variations of RLCKs are frequently associated with alterations in intracellular ROS homeostasis during pathogen infection. However, direct evidence linking RLCKs to ROS regulation remains limited. In this study, it was observed that the catalase activity showed a decrease in the knockout mutant of *osrlck109*. Furthermore, chitin significantly induced the accumulation of ROS in *osrlck109* (Supplementary Figure S5), indicating that the ROS homeostasis of the *osrlck109* was compromised. Then we determined that the OsRLCK109-interacting factor OsWNK1 directly phosphorylates OsCATA and enhances its ability to remove H_2O_2. Although subsequent studies revealed that OsRLCK109 also interacts with OsCATs, it does not mediate the phosphorylation of OsCATs. We speculate that OsWNK1 serves as an intermediary through which OsRLCK109 influences ROS homeostasis during the immune response.

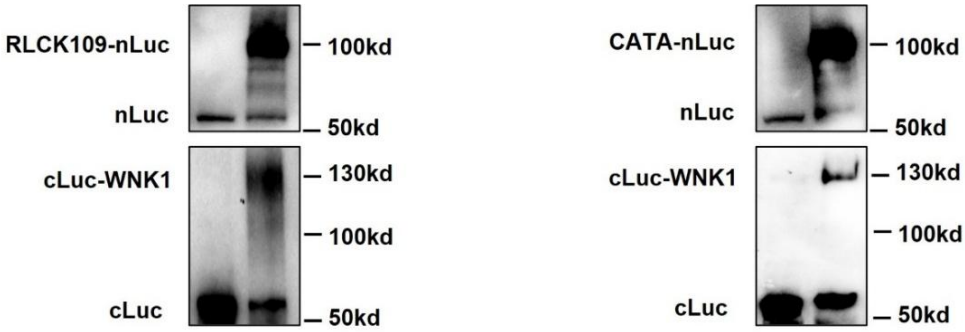
The role of WNK kinases in animal physiology is well documented, particularly in the regulation of cellular ion homeostasis (Furusho, Uchida, and Sohara 2020). Pseudohypoaldosteronism type II (PHAII), a rare hereditary hypertensive disorder in humans, is characterized by hyperkalemic and hyperchloremic hypertension, which is mediated by the WNK-OSR1/SPAK-NCC signaling cascade (Brown et al. 2021). Consequently, WNKs are frequently considered as target genes for the therapeutic management of these conditions (AlAmri et al. 2017). In plants, the WNK gene family is larger, and their functional diversity is more complex. Previous studies have linked WNKs to the regulation of circadian rhythms and abiotic stress responses (Xie et al. 2014; Negi and Kumar 2025). However, there are few reports on the involvement of WNKs in biotic stress, with only *AtWNK2* currently known to participate in plant immunity (Dunker et al. 2020). Using CRISPR/Cas9, we generated *oswnk1* mutants in rice. Upon infection with rice blast fungus, these mutants exhibited enhanced resistance. These findings support a role for OsWNK1 in regulating biotic stress responses in rice. Furthermore, we investigated whether OsWNK1 also regulates abiotic stress, as shown for other WNKs. As expected, OsWNK1 was found to regulate both drought and cold stress tolerance positively. These findings address a notable gap in the research concerning WNKs and their influence on biotic and abiotic stresses in rice.

The production and scavenging of ROS are critical processes during the interaction between *Magnaporthe oryzae* and rice. On one hand, the generation of ROS within *Magnaporthe oryzae* facilitates the formation and successful invasion of its appressorium. Following successful penetration into host cells, *Magnaporthe oryzae* must secrete ROS-scavenging enzymes to eliminate host-generated ROS, thereby contributing to its pathogenesis and subsequent progression (Zhao et al. 2023). On the other hand, rice produces a burst of ROS upon infection, which can directly eliminate pathogens and act as a signaling molecule to trigger programmed cell death and

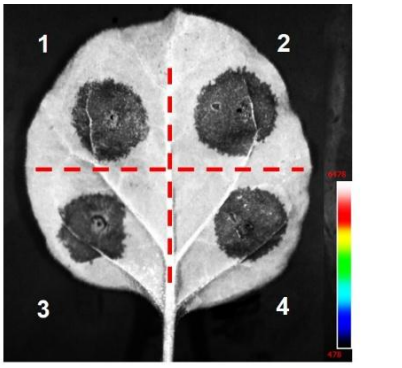
activate the synthesis of antimicrobial compounds. Although the ROS burst plays a significant role in improving plant immunity, extended exposure to elevated concentrations of ROS within cells may result in oxidative damage (Baker et al. 2023). Catalase, which breaks down hydrogen peroxide into water and oxygen, plays a key role in limiting ROS toxicity (Sepasi Tehrani and Moosavi-Movahedi 2018). In this study, we identified rice catalase OsCATA as a negative regulator of blast resistance. OsCATA physically interacts with OsWNK1 and is phosphorylated by it. The activity of OsCATA was significantly reduced in *oscata* mutant plants, which showed an H_2O_2 burst upon pathogen infection. These observations suggest that OsCATA contributes to the rice immune response by modulating ROS levels.

Through analysis of OsRLCK109-interacting partners, we identified OsWNK1 as a new regulatory kinase that, like OsRLCK109, negatively regulates rice blast resistance. It was subsequently found that OsWNK1 altered the H_2O_2 homeostasis by regulating the H_2O_2 scavenging ability of OsCATA to endow rice with the ability to cope with biotic and abiotic stresses. Certainly, there are still unresolved issues, such as whether OsRLCK109 will phosphorylate WNK1 and the consequent effects on its kinase activity. We also endeavored to find a definitive answer; however, our inability to successfully express OsWNK1 in the laboratory heightened the difficulty of conducting the experiment. Our research has only established that OsWNK1, as an interacting factor of OsRLCK109, is involved in both biological and abiotic stress conditions.

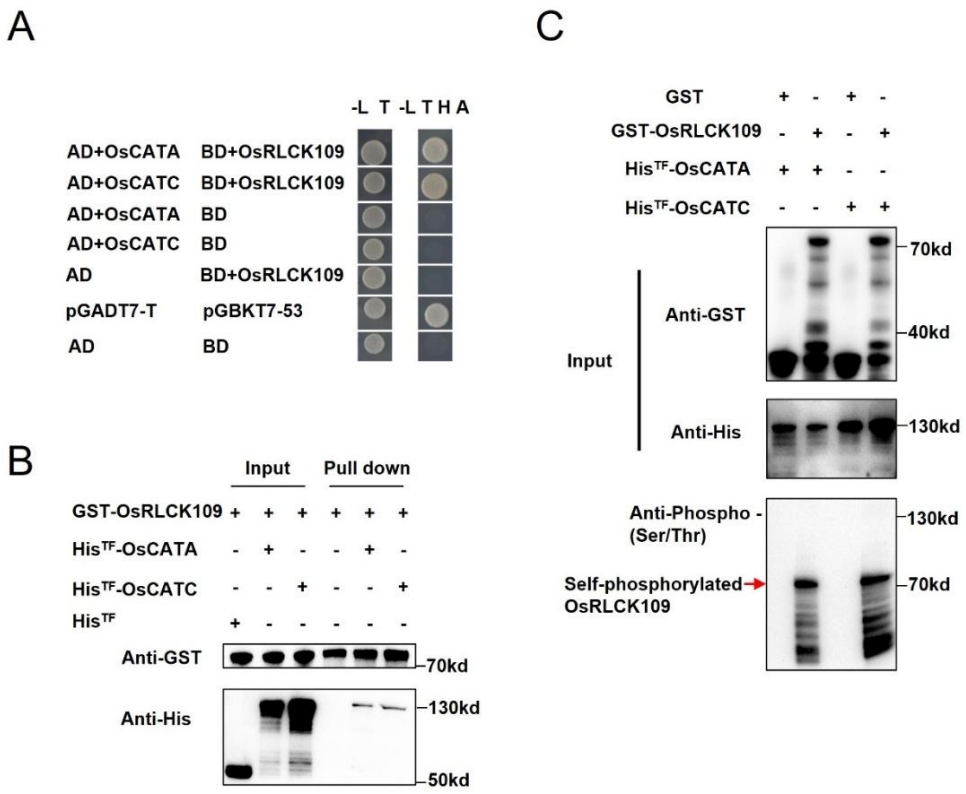
6. Supplementary materials



Supplementary Figure S1: Detection of protein expression levels in the LCI.

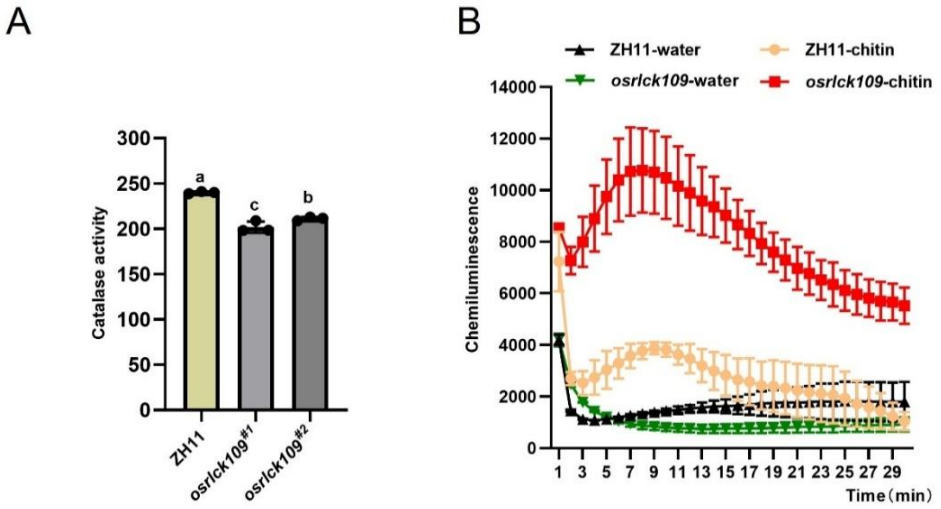


Supplementary Figure S2： LCI experiment analyzed the interaction between OsWNK1 and OsCATC.



Supplementary Figure S3: Analysis of interactions between OsRLCK109 and OsCATA and OsCATC. (A) OsRLCK109 and OsCATA/OsCATC interact in the yeast two-hybrid system. Yeast cells were cultured on SD/-Leu/-Trp (-L T) and SD/-Leu/-Trp/-His/-Ade (-L T H A) media. (B) OsRLCK109 and OsCATA/OsCATC interact in *vitro*. GST-OsRLCK109 and HisTF-OsCATA/OsCATC fusion proteins were expressed and purified in *vitro* and used for the pull-down experiment. (C) Extracellular phosphorylation analysis. An anti-Phospho-(Ser/Thr) Phe antibody was used to identify phosphorylated proteins. The band detected in the figure is considered to be OsRLCK109, which has undergone self-phosphorylation.

Supplementary Figure S4: *oscata* induce cell death phenotypes. Leaves from rice plants grown in the incubator for 40 days were utilized for DAB staining.



Supplementary Figure S5: Determination of catalase activity and chitin-induced H_2O_2 assay. (A) Catalase activity assays were performed in osrlck109. Plants grown in an incubator for 20 days were used for the experiments. ZH11 served as a control. Bars represent means ± SE, n = 3. (B) Chitin-induced H_2O_2 burst in osrlck109 leaves. Water treatment was used as a negative control. Bars are shown as means ± SE, n = 3.

Supplementary Table S1: The primer information in Chapter 1

For mutant sequencing	
Primer	**Sequence of base 5'-3'**
kownk1-F	AGAGCCCAGAGGACCTGGAG
kownk1-R	GGTGCCGGAGGTGAACATC
koCATA-F	AGTTGTGTAGATTCAAGAGAGAGC
koCATA-R	TGCACTCGAAGAAGCCCTTG
For BiFC	
YN+RLCK109-F	TATCATGGCCGGTACCATGCCGCCGAGGCAGTGGAG
YN+RLCK109-R	AGGATCCCCGGGTACCTCAGGGCAGCTTGGCGGAGG
YC+WNK1-F	GCTGTACAAGGGTACCATGATGGGCCCCAAGGCCAA
YC+WNK1-R	AGGATCCCCGGGTACCTCAGGCATCAACAGCATTTA
For Yeast two-hybrid	
AD+WNK3-F	GGAGGCCAGTGAATTCATGATGGGAGCCCTGCAGCA
AD+WNK3-R	CACCCGGGTGGAATTCTCATGGCTGTTCTGAACCTT
BD+CATC-F	CATGGAGGCCGAATTCATGGATCCCTACAAGCACCG
BD+CATC-R	GGATCCCCGGGAATTCTTACATGCTCGGCTTCGCGC
BD+CATA-F	CATGGAGGCCGAATTCATGGATCCTTGCAAGTTCCG
BD+CATA-R	GGATCCCCGGGAATTCTCACATGCTTGGCTTCACGT
AD+WNK1-F	GGAGGCCAGTGAATTCATGATGGGCCCCAAGGCCAA
AD+WNK1-R	CACCCGGGTGGAATTCTCAGGCATCAACAGCATTTA
BD+RLCK109-F	GGAGGCCAGTGAATTCATGCCGCCGAGGCAGTGGAG
BD+RLCK109-R	CACCCGGGTGGAATTCTCAGGGCAGCTTGGCGGAGG
For Subcellular localization	
GFP+WNK1-F	TAGAACTAGTGGATCCATGATGGGCCCCAAGGCCAA
GFP+WNK1-R	GCAGCCCGGGGGATCCGGCATCAACAGCATTTAGGG
GFP+CATA-F	TAGAACTAGTGGATCCATGGATCCTTGCAAGTTCCG
GFP+CATA-R	GCAGCCCGGGGGATCCCATGCTTGGCTTCACGTTGA
For LCI Assay	
nluc-109-F	GGACGAGCTCGGTACCATGCCGCCGAGGCAGTGGAG
nluc-109-R	ACGAGATCTGGTCGACGGGCAGCTTGGCGGAGGCGC
cluc+WNK1-F	GTCCCGGGGCGGTACCATGATGGGCCCCAAGGCCAA
cluc+WNK1-R	TTGGATCCCGGGTACCTCAGGCATCAACAGCATTTA
For qRT-PCR	
q-MoPot2-F	ACGACCCGTCTTTACTTATTTGG

q-MoPot2-R	AAGTAGCGTTGGTTTTGTTGGAT
Actin1-qF	CAGCCACACTGTCCCCATCTA
Actin1-qR	AGCAAGGTCGAGACGAAGGA
PR1a-qF	TGTACTGTCAGCCGTATTTGCT
PR1a-qR	ATGACCATCAGACCATGCATGT
PR1b-qF	TACGCCAGCCAGAGGAGC
PR1b-qR	GCCGAACCCCAGAAGAGG
PR10a-qF	TGCCGAATACGCCTAAGATGAA
PR10a-qR	ACTCAAACGCCACGAGAATTTG
PRL1-qF	GAGAACATCAAGAGCTCCGTCA
PRL1-qR	CCTCGAACTTGGTGATCTTGGA

Chapter 4

OsRLCK109 negatively regulates rice immunity by promoting *OsWRKY76's* suppression of *PR1b*

While Chapter 3 highlights the role of OsRLCK109 in regulating rice immunity through ROS homeostasis and kinase-mediated signaling, these findings raise the question of whether OsRLCK109 also modulates immune responses at the transcriptional level. To address this aspect, Chapter 4 investigates the interaction between OsRLCK109 and transcription factors, with a particular focus on the regulatory role of OsWRKY76.

This chapter is based on the following publication:

Zhang, Y., Kang, Y., Wang, G., Duan, W., Zhang, Y., Wu, W., Chen, D., Hong, Y., Sun, L., Shen, X., Zhan, X., Cheng, S., Ludivine, L., Liu, Q., & Cao, L. (2026). **OsRLCK109 Negatively Regulates Rice Immunity by Promoting OsWRKY76's Suppression of *PR1b*.** *Rice (New York, N.Y.), 19(1), 14.*

1. Abstract

Typically, Receptor-Like Cytoplasmic Kinases (RLCKs) mediate intracellular signal transduction downstream of Pattern Recognition Receptors (PRRs). However, studies on RLCKs directly regulating transcription factors and subsequently influencing Pathogenesis-Related (PR) gene expression are relatively scarce. Our research findings demonstrate that OsRLCK109 interacts with the WRKY transcription factor OsWRKY76 and phosphorylates it. Further investigations have shown that OsWRKY76 can directly bind to the promoter region of *PR1b* and inhibit its expression, thereby negatively regulating rice blast resistance. OsRLCK109 plays a positive role in the regulation of *PR1b* expression by OsWRKY76 and thus functions as a negative regulatory factor in rice blast disease. These findings provide new insights into the role of RLCKs in modulating rice immunity.

2. Introduction

Receptor-like cytoplasmic kinases (RLCKs) are essential components of plant signaling pathways, orchestrating various cellular processes associated with biotic and abiotic stress responses, as well as growth, development, and reproduction. The rice genome encompasses numerous OsRLCK members, totaling 379 identified entities (Vij et al. 2008). Notably, members of the RLCK-VII subfamily have been extensively documented to participate in plant immune responses. OsRLCK176 and OsRLCK118 have been reported to interact with the primary NADPH oxidase OsRbohB, which is responsible for ROS production in the rice plasma membrane (Fan et al. 2018). This interaction influences the regulation of reactive oxygen species and the immune response in rice. Additionally, it has been observed that OsCERK1 transmits immune signals from the cell membrane by phosphorylating OsRLCK185. The latter plays a role in the rice immune response triggered by chitin and peptidoglycan through the MAPK signaling cascade involving OsMAPKKKε-OsMAPKK4-OsMAPK3/6 (Wang et al. 2017).

Transcription factors constitute a diverse and complex family of proteins in plants. They bind to the promoter regions of target genes, either activating or repressing their transcriptional activity, thereby regulating the specific expression of target genes across various tissues, cells, and environmental conditions. Furthermore, through the regulatory network of transcription factor cascades, they govern numerous biological processes. With advancements in sequencing technologies, genome sequencing of various plant species has been successfully accomplished, and numerous genes have been predicted to encode transcription factors. Notably, in *Arabidopsis thaliana,* an estimated 1,922 transcription factors are involved in diverse functions, while in sorghum, the number is approximately 2448, in rice, around 1611, and in maize, approximately 3337 (Baillo et al. 2019). Based on variations in the DNA-binding domains of transcription factors, they are further classified into several distinct subfamilies, including DREB, bZIP, WRKY, ABF, MYB, and NAC (Baoxiang et al. 2023).

The WRKY transcription factors are distinguished by their conserved WRKY domain, a 60-amino-acid region that encompasses the conserved amino acid sequence WRKYGQK at the N-terminus and a zinc finger motif at the C-terminus. All identified WRKY proteins contain either one or two WRKY domains. They are categorized based on the number of WRKY domains and the structural characteristics of their zinc finger motifs. Group I comprises proteins possessing two WRKY domains, whereas Group II and Group III include proteins with a single WRKY domain; however, the zinc finger structures differ between these groups. The zinc finger motif in Group II is of the C2H2 type, whereas that in Group III is of the C2HC type. A Group IV of WRKY transcription factors has been identified in Saccharum spontaneum, which includes the conserved WRKYGQK sequence but lacks a zinc finger motif (Javed and Gao 2023). All WRKY transcription factors have been demonstrated to specifically bind to the W-box (TTGAC[T/C]) located within the promoter region of target genes.

The increasingly intricate and variable external environment presents challenges to the survival capabilities of plants. Plants have developed robust stress resistance systems, enabling them to suitably modify their growth and developmental strategies across various stress conditions to adapt effectively to these challenges. WRKY transcription factors play a crucial role in this process, involving molecules such as salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), MAPK, Ca^{2+}-dependent protein kinases (CDPK), and reactive oxygen species (ROS). The WRKY transcription factors identified in rice number 88 are categorised into groups I, II, and III. They are extensively expressed in different rice organs, although they exhibit tissue-specific expression patterns (Li et al. 2025).

Extensive evidence in rice pertains to the regulation of disease resistance by WRKY transcription factors. The two alleles of *OsWRKY45* differ by merely ten amino acids in their respective encoded proteins. Both alleles positively influence rice blast resistance; however, they exert opposite effects on rice's resistance to bacterial diseases. Research indicates that these differential effects are attributable to OsWRKY45-1's capacity to regulate both the SA and JA signaling pathways simultaneously, whereas OsWRKY45-2 influences only the JA pathway (Tao et al. 2009). Overexpression of *OsWRKY67* enhanced the resistance to rice blast disease and bacterial blight disease, while silencing *OsWRKY67* led to an increase in susceptibility to rice blast disease and bacterial blight disease. *OsWRKY67* positively regulates rice immunity by directly activating PR genes *PR1a* and *PR10* (Liu et al. 2018).OsWRKY62 functions as a positive regulator of rice blast disease in the non-Pi9 background; however, it exhibits an antagonistic role in rice blast resistance within the Pi9 background. This suggests that the regulatory mechanisms governing OsWRKY62 in rice immune responses vary between non-Pi9 and Pi9 backgrounds (Shi et al. 2023).

Post-translational modification (PTM) is a fundamental process in the regulation of protein function. WRKY transcription factors have been documented to have their protein activities modulated by PTM. OsWRKY45 undergoes proteasome-mediated

degradation following ubiquitination in the absence of pathogen infection, thereby preventing the premature activation of the defense response (Matsushita et al. 2013). OsWRKY31 can be phosphorylated by OsMPK3, OsMPK4, and OsMPK6, a process that enhances its DNA-binding activity and confers increased resistance to rice blast disease (Wang et al. 2023). OsWRKY76 has been reported to contribute to rice blast resistance as well as to drought and cold stress responses (Xu et al. 2022; Zhang et al. 2023, 2023). However, there are currently no reports indicating that it is subject to PTM.

OsRLCK generally activates the downstream MAPK pathway to regulate immunity by transmitting phosphorylation signals from RLP within the immune signaling cascade. This study has demonstrated that the rice blast disease repressor factor OsRLCK109 interacts with OsWRKY76 and phosphorylates it. The knockout of OsWRKY76 enhances resistance to rice blast, indicating that OsWRKY76 functions as a negative regulatory factor for this disease. Further investigations have shown that OsWRKY76 binds to the promoter region of the PR gene *PR1a* and suppresses its expression, thereby negatively influencing resistance to rice blast disease. Additionally, OsRLCK109 positively influences the transcriptional repression of *PR1b* mediated by OsWRKY76. In summary, this research elucidates the mechanism by which OsRLCK109 exerts negative regulation on rice immunity through the modulation of transcription factor activity, thereby offering new insights into the role of OsRLCK in governing the immune response in rice.

3. Materials and methods

3.1 Plant materials and growth conditions

osrlck109 rice plants were developed utilizing the CRISPR/Cas9 technique within the Z H 11 background. The authors were responsible for vector construction, and the genetic transformation was performed by Biorun BioSciences. *oswrky76* was purchased from BIOGLE GeneTech. The researcher independently conducted reproduction and mutation site examinations. This mutant was produced employing the CRISPR/Cas9 technology, with the vector utilizing BGK03 (Lu et al. 2017), the target sequence being CTCCTCTTCTTGCCCGATAACGG, and the sgRNA sequence being GTCCTCTTCTTGCCCGATAA.

Plants designated for rice blast disease resistance assay and catalase activity tests were grown in sterilized soil inside an incubator under a 14-hour light/10-hour dark cycle at 30°C. All other plant materials were cultivated under natural conditions in the experimental fields of the China National Rice Research Institute, located in Hangzhou or Lingshui.

3.2 RNA-seq

The RNA was extracted from the plants that had grown in the field for 60 days for RNA-seq analysis. Each sample has three biological replicates. The RNA extraction

was carried out by the authors, while the expression level analysis and the differential gene enrichment analysis were performed by Novogene Company.

3.3 Yeast two-hybrid system

The coding sequences (CDS) of *OsRLCK109* and *OsWRKY76* (LOC_Os09g25060) were, respectively, fused to the pGBKT7 and pGADT7 vectors. The vectors were then introduced into the Y2HGold strain. The selective culture medium for yeast was prepared using products from Takara Company (Cat. Nos. 630417 and 630419). Yeast monoclonal cells exhibiting uniform growth after cultivation at 28°C were subsequently employed for spotting.

3.4 Co-immunoprecipitation assay

The OsRLCK109-Myc vector is regulated by the ubiquitin promoter, with OsRLCK109 fused to the N-terminal of 4*Myc. The OsWRKY76-GFP vector is driven by the 35S promoter, with OsWRKY76 linked to the N-terminal of EGFP. After a 20-hour transformation of the vector into rice protoplasts, total proteins were extracted for subsequent analyses. Immunoprecipitation was performed using Anti-Myc magnetic beads (Cat.No. B26301) obtained from Bimake Company.

3.5 Pull-down assay

The His-tagged antibody is produced using the pCold™ TF DNA vector, while the GST-tagged vector utilizes pGEX-4T-1. Upon introduction of the fusion vector into the BL21 strain, induction was performed at 16°C with agitation at 120 rpm for 16 hours. The protein was subsequently purified and employed in subsequent experiments. The mixture of protein and Glutathione High-Capacity Magnetic Agarose Beads (Sigma G0924) was incubated at room temperature for 30 minutes, followed by Western blot analysis.

3.6 Extracellular phosphorylation experiment

The expression vector OsRLCK109-GST employs pGEX-4T-1, while the vector OsWRKY76-His utilizes pET-28a (+). The purified protein was incubated in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM ATP, and 10 mM MgCl2 at 30°C for 1 hour. Detection of input proteins was performed using Anti-His Mouse Monoclonal Antibody and Anti-GST Mouse Monoclonal Antibody (TransGen HT601-01, HT501-01). In contrast, the Anti-Phospho-(Ser/Thr) Phe antibody (Abcam ab17464) was used to identify phosphorylated proteins.

3.7 Yeast one-hybrid assay

The EGY48-LacZ Yeast One-Hybrid interaction-proving kit is used to conduct Y1H experiments (Coolaber YH3010-10T). The CDS of OsWRKY76 was cloned into the pB42AD vector, and the promoter sequence of the PR gene was cloned into the pLACZi plasmid. Two fusion plasmids were introduced into EGY48 yeast-competent cells and cultured on SD/-Trp/-Ura medium at 28-30°C for 3-5 days. Subsequently,

the cells were selected on SD/-Ura/-Trp plates containing 2% galactose, 1% raffinose, 1× BU salts, and 80 mg/L X-Gal. Upon interaction, the LacZ reporter gene is expressed, resulting in the production of β-galactosidase. This enzyme cleaves X-gal into galactose and the dark-blue substrate 5-bromo-4-chloroindigo. The interaction was validated by the appearance of blue colonies on the medium, indicating successful interaction.

3.8 Quantitative RT-PCR analysis

The rice leaves, which had grown for 30 days, were used to extract total RNA. Quantitative analysis of cDNA was performed using TB Green Premix Ex Taq II (Takara RR820A) on the CFX96 Touch real-time fluorescence quantitative PCR system (Bio-Rad).

3.9 Dual-Luciferase Reporter assay

The CDSs of *OsRLCK109* and *OsWRKY76* were respectively cloned into the "None" vector as effectors, and the promoter region of the PR1b-p2 was cloned into the 190LUC vector as a reporter gene (Supplementary Figure S11). Following the introduction of the fusion vector into rice protoplasts for a period of 20 hours, total protein extraction was performed in order to determine enzyme activity. Initially, the luciferase activity of firefly luciferase (Luc) was measured, followed by the measurement of Renilla luciferase (Ren). For each sample, three independent replicates were conducted, and the relative luciferase activity was calculated as the ratio of Luc to Ren (Luc/Ren).

3.10 Electrophoretic Mobility Shift Assay

The probe was synthesized by Beijing Tsinke Biotechnology Company and labeled with an EMSA Probe Biotin Labeling Kit (Beyotime, Cat No. GS008). Protein binds to DNA using the Chemiluminescent EMSA Kit (Beyotime, Cat No. GS009). The reaction conditions are 23°C for 20 minutes, and electrophoresis utilizes a 6% polyacrylamide gel. Subsequent experiments were conducted in accordance with the kit's instructions.

3.11 Pathogen inoculation and quantification

The *Magnaporthe oryzae* strain RB22 was cultivated on oat tomato medium in darkness for 5 days at 25 °C, then exposed to light for 7 days. Spores were washed with sterile water and adjusted to a final concentration of 5×10^5 spores per mL. For inoculation, a 2 mm diameter hole was punched in the leaf, and 10 μL of spore suspension was applied. The wound was sealed with tape to maintain humidity. Post-inoculation, plants remained in darkness for 24 hours, then were transferred to a 12-hour light/12-hour dark cycle at 25 °C for 14 days. Lesion areas were measured using ImageJ. Total DNA from the inoculation site was extracted via the CTAB method and used to estimate fungal biomass through DNA-based qRT-PCR.

3.12 Gene ID

OsRLCK109 (LOC_Os03g24930), *OsWRKY76* (LOC_Os09g25060), *WRKY24* (LOC_Os01g61080), *WRKY45* (LOC_Os05g25770), *WRKY71* (LOC_Os02g08440), *WRKY62* (LOC_Os09g25070), *PR1b*(LOC_Os07g03600), *PR1a*(LOC_Os07g03710), *PR10a*(LOC_Os12g36880), *RSOsPR10*(LOC_Os12g36830), *DREB1E*(LOC_Os04g48350)

4. Result

4.1 Differential expression of WRKY transcription factors regulating rice blast resistance in osrlck109

Based on previous research findings, it has been established that the OsRLCK109-OsWNK1-OsCATA module regulates hydrogen peroxide homeostasis and, consequently, influences rice immunity. However, when assessing the resistance performances of *osrlck109*, *oswnk1*, and *oscata*, *osrlck109* demonstrated enhanced resistance to rice blast in comparison to *oswnk1* and *oscata*. It is hypothesized that RLCK109 may also modulate rice immunity through alternative pathways. To verify this hypothesis, RNA-seq analysis was conducted on *osrlck109* and the wild-type ZH11. The analysis identified 3642 differentially expressed genes, with *osrlck109* exhibiting 1774 genes with decreased expression and 1868 genes with increased expression relative to the wild-type ZH11(Figure 22A). These genes are predominantly involved in pathways such as Phenylpropanoid biosynthesis, Oxidative phosphorylation, and Plant hormone signal transduction (Figure 22B). Given the significant role of WRKY transcription factors in the regulation of rice resistance, particular attention was given to the differential expression of WRKY transcription factors.

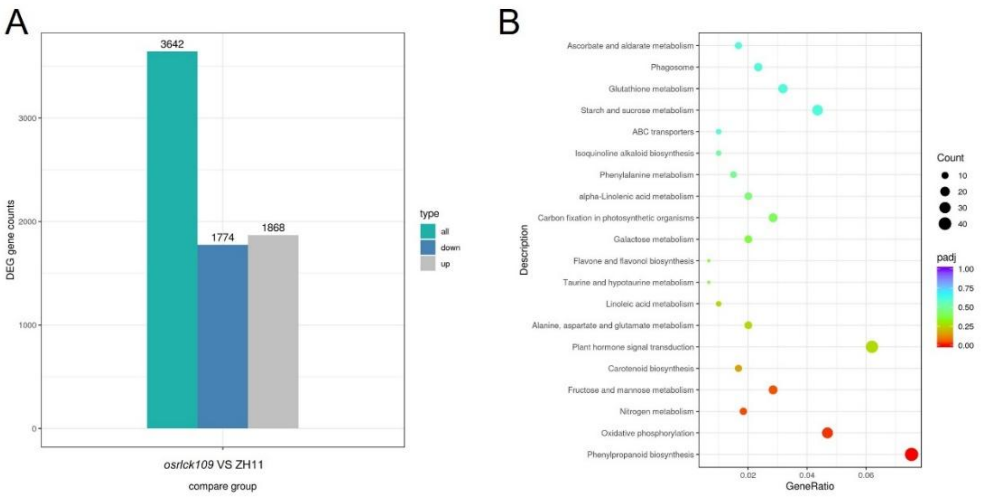


Figure 22: RNA-Seq was employed to analyze the differentially expressed genes in *osrlck109* and ZH11. (A) Statistical bar chart illustrating the differentially expressed genes. (B) KEGG enrichment scatter plot, wherein the horizontal axis indicates the ratio of the number of differentially expressed genes annotated to the KEGG pathways relative to the total number of differentially expressed genes, and the vertical axis denotes the KEGG pathways.

The expression of twelve WRKY transcription factors related to biological stress was altered. Among these, nine WRKY transcription factors exhibited increased expression levels, whereas three showed a decrease (Table 3). Notably, there are well-documented reports indicating that the expression levels of all WRKY transcription factors that positively regulate rice blast resistance have risen. This observation aligns with the phenotypic effect of *osrlck109* in enhancing rice blast resistance. *OsWRKY76*, which negatively regulates rice blast resistance, also demonstrates an increase in expression levels (Supplementary Figure S6). This appears to be inconsistent with the previously stated information concerning the resistance to rice blast disease, *osrlck109*. This naturally prompts speculation regarding a potential relationship between *OsRLCK109* and *OsWRKY76*. To test this hypothesis, subsequent research will investigate the interaction between *OsRLCK109* and *OsWRKY76*.

Table 3: Differentially expressed WRKY transcription factors in *osrlck109*VSZH11

Gene name	Locus	Up or down	Biological stress response	References
OsWRKY24	LOC_Os01g61080	Up	Positive regulation of rice blast disease	**(Yokotani et al. 2018)**
OsWRKY46	LOC_Os11g02480	Up	Positive regulation of rice blast disease	**(Gao et al. 2020)**
OsWRKY45	LOC_Os05g2577	Up	Positive regulation of rice blast disease	**(Shimono et al. 2012)**
OsWRKY6	LOC_Os03g58420	Up	Positive regulation of rice blast disease	**(Gao et al. 2020)**
OsWRKY62	LOC_Os09g25070	Up	Positive regulation of rice blast disease	**(Shi et al. 2023)**
OsWRKY71	LOC_Os02g08440	Up	Positive regulation of bacterial blight resistance	**(X et al. 2007)**
OsWRKY70	LOC_Os05g39720	Up	Negative regulation of brown planthopper resistance	**(Zhang et al. 2015)**
OsWRKY54	LOC_Os05g40080	Up	Positive regulation of salt tolerance	**(Huang et al. 2022)**
OsWRKY76	**LOC_Os09g25060**	**Up**	**Negative regulation of rice blast resistance**	**(Yokotani et al. 2013)**
OsWRKY11	LOC_Os01g43650	Down	Positive regulation of bacterial blight resistance	**(Lee et al. 2018)**
OsWRKY12	LOC_Os01g43550	Down	Positive regulation of the PR gene	**(Liu et al. 2005)**
OsWRKY13	LOC_Os01g54600	Down	Positive regulation of Fusarium head blight resistance	**(John Lilly and Subramanian 2019)**

4.2 OsRLCK109 and OsWRKY76 physically interact

First, we measured the expression levels of *OsWRKY76* in ZH11 and *osrlck109*. As previously stated, the expression level of OsWRKY76 in *osrlck109* was significantly higher than that in ZH11(Figure 23A). Subsequently, an interaction test between OsRLCK109 and OsWRKY76 was conducted using a yeast system. The results demonstrated that yeast cells carrying the AD-OsWRKY76 and BD-OsRLCK109 fusion plasmids were able to grow on the selective medium, which indicates that OsRLCK109 and OsWRKY76 can interact within the yeast system (Figure 23B). Other OsWRKY transcription factors do not interact with OsRLCK109 (Supplementary Figure S7). Furthermore, the Co-IP method was employed to investigate whether this interaction occurs in rice cells. After co-expressing OsWRKY76-GFP and OsRLCK109-Myc proteins in rice protoplasts, immunoprecipitation was performed using anti-Myc magnetic beads. The results indicated that OsWRKY76-GFP could be immunoprecipitated along with OsRLCK109-Myc, whereas GFP alone could not, suggesting an interaction between OsRLCK109 and OsWRKY76 within rice cells (Figure 23C). Additionally, in *vitro* GST Pull-down assays showed that GST-OsRLCK109 could precipitate His^{cold}-OsWRKY76, while the control did not (Figure 23D), confirming an in *vitro* interaction between OsWRKY76 and OsRLCK109. In conclusion, OsWRKY76 and OsRLCK109 directly and physically interact.

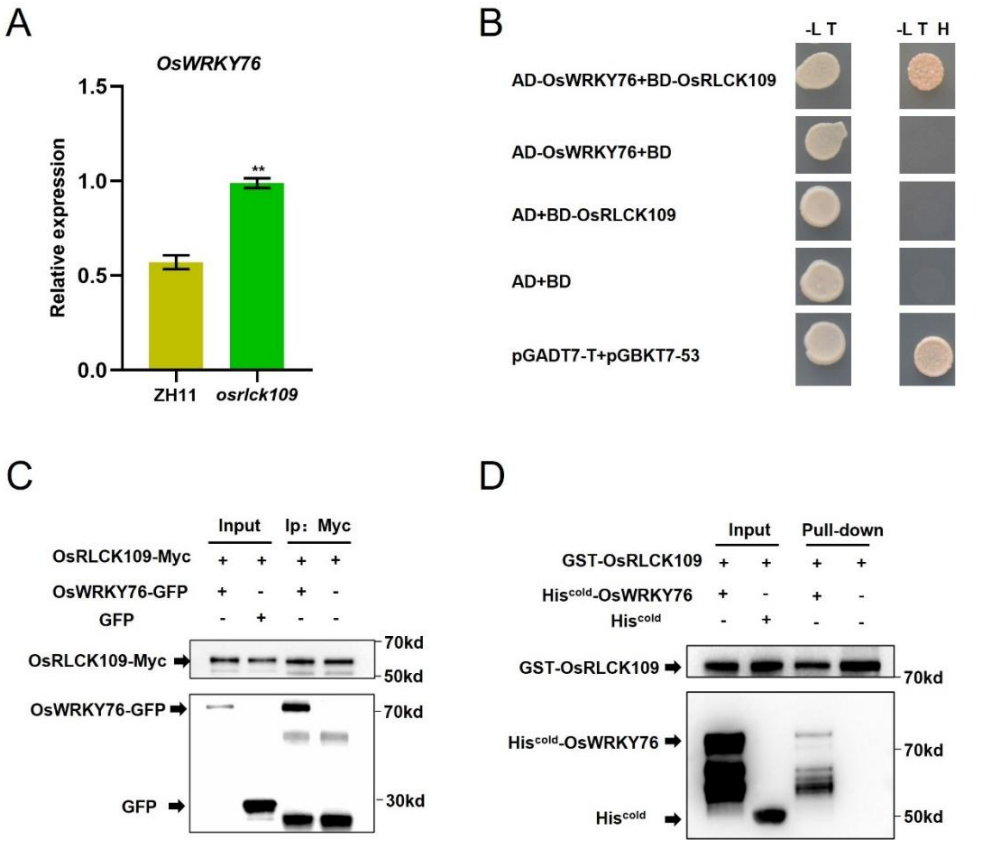


Figure 23: OsRLCK109 and OsWRKY76 interact directly. (A) The expression levels of *OsWRKY76* in the wild-type ZH11 and *osrlck109*. Data are presented as mean ± SD, n=3. Statistical significance was determined using Student's t-test, with **p < 0.01. (B)OsRLCK109 and OsWRKY76 interact with each other in the yeast system. Yeast cells were cultured on SD/-Leu/-Trp (-L T) and SD/-Leu/-Trp/-His (-L T H) media. pGADT7-T+pGBKT7-53 as a positive control. (C) OsRLCK109 and OsWRKY76 interact within rice protoplasts. Plasmids encoding OsRLCK109-Myc and OsWRKY76-GFP fusion proteins were introduced into rice protoplasts. After a 20-hour incubation period, total proteins were extracted for the Co-IP assay. GFP protein served as a control. (D) OsRLCK109 and OsWRKY76 interact with each other in *vitro*. The GST-OsRLCK109 and His^{cold}-OsWRKY76 fusion proteins were expressed and purified in *vitro*, and then used for the Pull-down experiment.

4.3 In vitro phosphorylation of OsWRKY76 by OsRLCK109

In *Arabidopsis thaliana*, the RLCK BIK1 can phosphorylate WRKY transcription factors, thereby modulating defense responses(Lal et al. 2018). An in *vitro* phosphorylation experiment was performed to determine whether OsRLCK109 can

phosphorylate OsWRKY76. GST-OsRLCK109 and His-OsWRKY76 were expressed in Escherichia coli, and after purification, in *vitro* phosphorylation experiments were conducted. The results showed that GST-OsRLCK109 could phosphorylate His-OsWRKY76, while GST alone could not (Figure 24). Additionally, it was observed that OsRLCK109 is capable of auto-phosphorylation (Supplementary Figure S8).

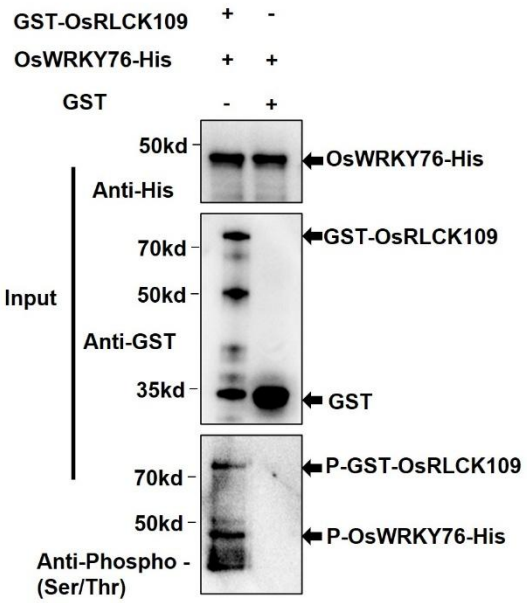


Figure 24: OsRLCK109 phosphorylates OsWRKY76 in *vitro*. Anti-Phospho - (Ser/Thr) antibody is used for detecting phosphorylated proteins.

4.4 OsWRKY76 negatively regulates rice blast resistance

Additionally, we generated the functionally deficient mutant *oswrky76* of *OsWRKY76* via CRISPR/Cas9 technology and subsequently assessed its resistance to rice blast disease. The results from inoculation assays indicated that the lesion area and the number of pathogenic bacteria in *oswrky76* were significantly lower than those in ZH11. The knockout of *OsWRKY76* markedly increased rice's resistance to blast disease. (Figure 25A, B, C). We measured the expression levels of PR genes in the mutants and found that *PR1a*, *PR1b*, and *PR10a* were significantly elevated in the mutants (Figure 25D). This might be the reason for the increase in mutant resistance. In conclusion, OsWRKY76, similar to OsRLCK109, functions as a negative regulatory factor in rice blast resistance.

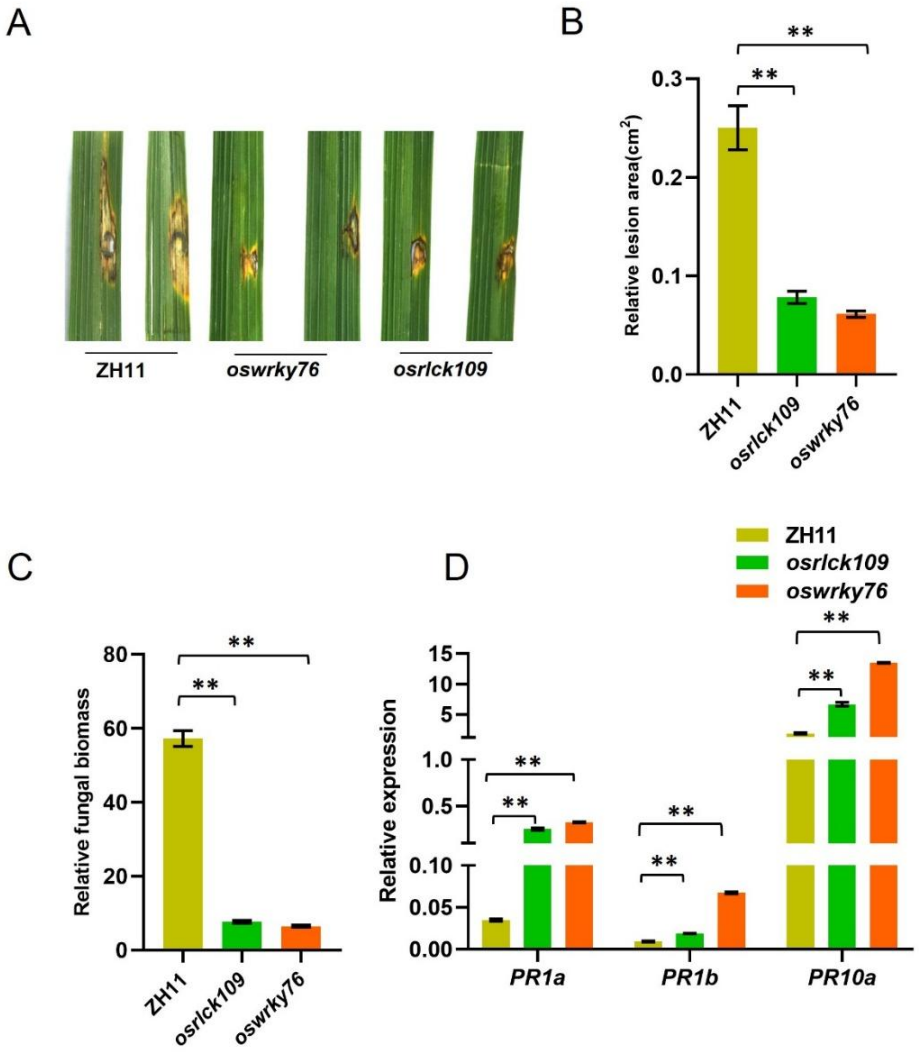


Figure 25: *OsWRKY76* negatively regulates rice blast resistance. (A)The performance of ZH11, *osrlck109*, and *oswrky76* after being inoculated with rice blast disease for 7 days. (B) and (C) Analysis of relative lesion area and relative fungal biomass post-inoculation. (D)The expression levels of pathogen-related genes in *osrlck109* and *oswrky76*. Wild-type ZH11 served as the control. Data shown as mean±SD, n=3, **p < 0.01, Student's t-test.

4.5 OsWRKY76 binds to the PR1b promoter and negatively regulates its expression

It has been reported that WRKY transcription factors possess the capacity to bind to the W-box within the promoter region of PR genes (Liu et al. 2018), thereby modulating their expression. Based on the observed increase in PR gene expression levels in *oswrky76* and the utilization of the PlantCARE online tool for promoter analysis, it was revealed that all these PR genes contain the W-box (Supplementary Figure S9). We hypothesize that OsWRKY76 functions as a transcriptional negative regulatory factor, directly governing the expression of *PR1a*, *PR1b*, and *PR10a*. First, the yeast one-hybrid experiment (Y1H) was used to verify our hypothesis. The results demonstrated that OsWRKY76 directly binds to the promoter of *PR1b* (Figure 26A, B), whereas it did not bind to the *PR1a* and *PR10a* promoters (Supplementary Figure S10). In addition, we also conducted a transient transcriptional activity assay of luciferase in the protoplasts of rice. The results showed that OsWRKY76 significantly reduced the activity of PR1b-p2-190Luc (Figure 26C). PR1b-p2 contains two W-boxes. Subsequently, we conducted an electrophoretic mobility shift assay (EMSA) to test the in *vitro* binding. The results showed that both W-boxes could bind to OsWRKY76 in *vitro* (Figure 26D). In summary, OsWRKY76 directly binds to the two W-boxes in the promoter of *PR1b* and inhibits the expression of *PR1b*.

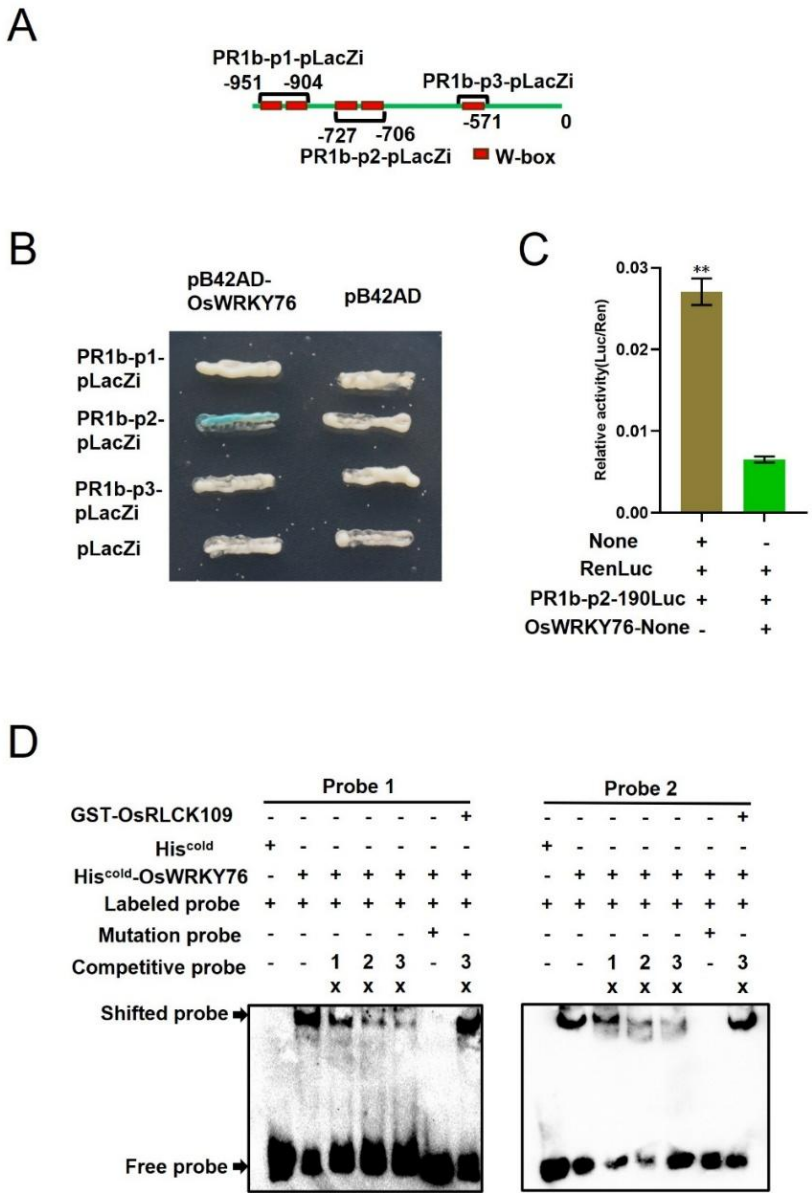


Figure 26: OsWRKY76 binds to the promoter of *PR1b* and inhibits its transcription. (A) Segmentation rules of the *PR1b* promoter. (B) Y1H assay of OsWRKY76 to the promoter of *PR1b*. (C) Luc transient transcriptional activity assay in rice protoplast. Data shown as mean±SD, n=3, **p < 0.01, Student's t-test. (D) The EMSA analysis demonstrated that OsWRKY76 interacts with the W-box of PR1b-p2. Additionally, OsRLCK109 can augment the binding affinity when a competing probe is introduced.

4.6 OsRLCK109 positively regulates the inhibitory effect of OsWRKY76 on PR1b

To evaluate whether OsRLCK109 affects the transcriptional activity of OsWRKY76, a dual-luciferase reporter assay was utilized to determine whether OsRLCK109 can enhance the transcriptional inhibitory effect of OsWRKY76 to *PR1b*. The results demonstrated that, within rice protoplasts, the transcriptional activity of *PR1b* in the absence of OsRLCK109 exceeds that of the wild-type ZH11. Subsequently, upon the manual addition of OsRLCK109, the transcriptional activity of *PR1b* was diminished relative to the control group lacking OsRLCK109 (Figure 27A). Similarly, in the EMSA experiment, when the reaction system was additionally supplemented with OsRLCK109 and contained a competitive probe, the binding of OsWRKY76 to the labeled probe was enhanced compared to not adding OsRLCK109 (Figure 26D lane 7 vs. lane 6). This indicates that OsRLCK109 can enhance OsWRKY76's binding to the *PR1b* promoter. Previous studies have shown that OsWRKY76 acts as a transcriptional activator of *OsDREB1E* (Zhang et al. 2023) and as a repressor of *RSOsPR10* (Yamamoto et al. 2018). We further examined the expression levels of these genes in the *osrlck109* mutant. The findings revealed that *OsDREB1E* levels decreased, while *RSOsPR10* levels increased in *osrlck109* (Figure 27B and C). Interestingly, it was previously observed that the transcriptional level of OsWRKY76 in *osrlck109* was elevated (Figure 23A); however, in *osrlck109*, the expression of these target genes did not correspond to the anticipated results of *OsWRKY76* overexpression; instead, it aligned with the phenotype indicative of *OsWRKY76* being functionally deficient. This indicates that the absence of OsRLCK109, despite its capacity to augment OsWRKY76 expression, indeed reduces OsWRKY76's transcriptional ability, encompassing its functions in both repression and activation of target genes. Based on the above evidence, the interaction between OsRLCK109 and OsWRKY76 enhances OsWRKY76's transcriptional regulatory capacity. In other words, the expression of target genes regulated by OsWRKY76 requires OsRLCK109.

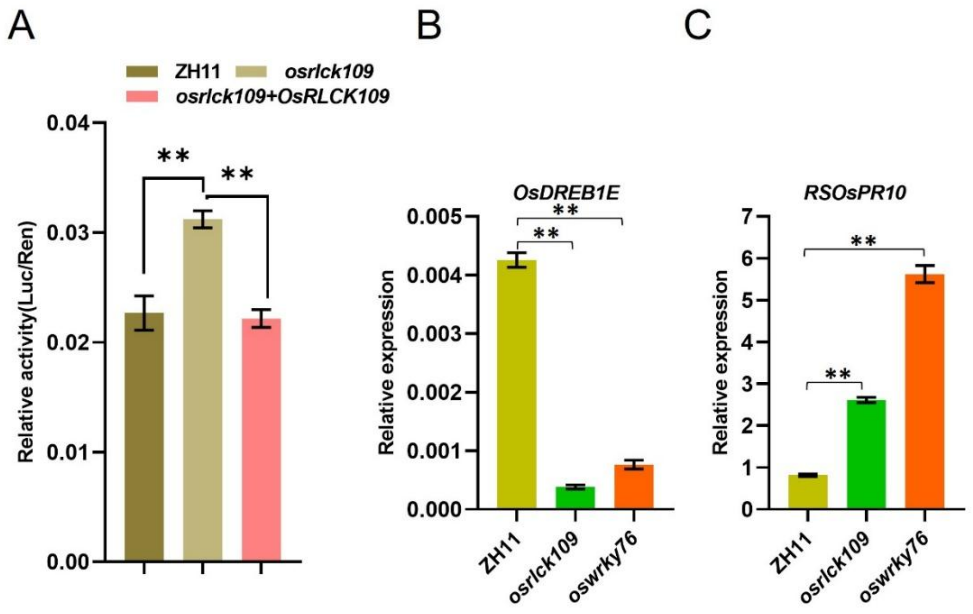


Figure 27: OsRLCK109 positively regulates the inhibitory effect of OsWRKY76 on *PR1b*.(A) The inhibitory effect of OsWRKY76 on the luciferase transcriptional activity of *PR1b* was observed in the protoplasts of ZH11 and *osrlck109*, respectively. Additionally, the OsRLCK19-none protein was expressed in the *osrlck109* background to compensate for the absence of OsRLCK109. Data shown as mean±SD, n=3, **p < 0.01, Student's t-test. (B) and (C) The expression levels of *OsDREB1E* and *RSOsPR10* in *osrlck109* and *oswrky76*. Wild-type ZH11 was employed as the control.

5. Discussion

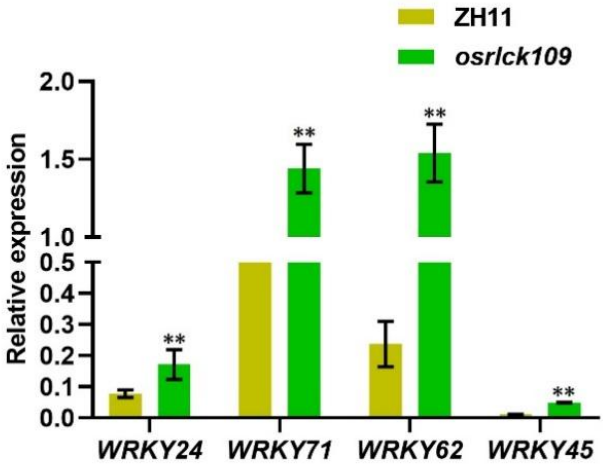
In the rice immune pathway, RLCK has been extensively documented to mediate phosphorylation signals originating from RLP, thereby regulating multiple signaling nodes, including the MAPK cascade, NADPH oxidase, calcium ion channels, and heterotrimeric G proteins, to coordinate diverse immune responses. There are relatively few reports concerning RLCK's direct regulation of WRKY transcription factors and, consequently, the modulation of downstream PR genes to influence immunity. In this study, we identified that OsRLCK109 interacts with OsWRKY76, thereby regulating the inhibitory effect of OsWRKY76 on downstream *PR1b* and modulating the immune signaling pathway. This finding enhances our understanding of RLCK's role in regulating immune responses.

In an organism, phosphorylation represents the most prevalent form of covalent modification in PTM and is also the paramount regulatory modification in both prokaryotic and eukaryotic systems. Phosphorylation plays an essential regulatory role in maintaining the normal functionality of proteins. This process is mediated by

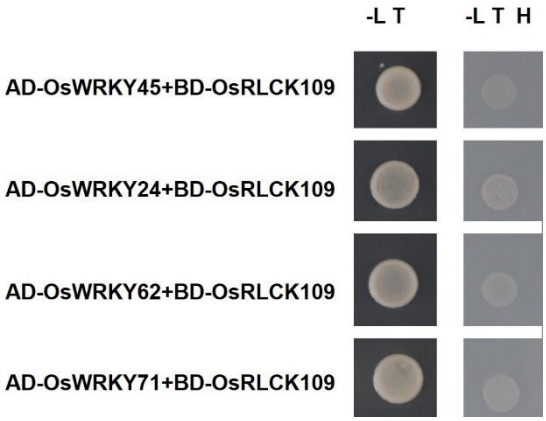
protein kinases, which transfer the γ-phosphate group from ATP or GTP to specific amino acid residues—including serine, threonine, and tyrosine—in the substrate protein. Conversely, protein phosphatases facilitate the removal of these phosphate groups. The opposing actions of these enzymes, coupled with the associated energy consumption and release, underpin phosphorylation as the preferred mechanism for regulating numerous physiological processes. Our research demonstrates that OsWRKY76 can be phosphorylated by OsRLCK109 in *vitro*, and that loss of OsRLCK109 function directly impacts OsWRKY76 transcription factor activity. This observation suggests that phosphorylation may directly influence OsWRKY76 protein function; however, further experimental data are required to substantiate this hypothesis.

OsWRKY76 has the capacity to directly bind to the promoter region of *OsDREB1E*, thereby activating its expression under conditions of drought stress (Zhang et al. 2023). The knockout of *OsWRKY76* results in a diminution of drought resistance in rice seedlings. Moreover, OsWRKY76 interacts with OsbHLH148 and collaboratively promotes the expression of *OsDREB1B*, which enhances the cold tolerance of rice. Notably, the cold tolerance in the *OsWRKY76* knockout strain is markedly decreased (Zhang et al. 2022). Although the targeted knockout of *OsWRKY76* increases resistance to rice blast disease, it simultaneously reduces tolerance to drought and cold stress. This study indicates that OsRLCK109 might influence the transcriptional activity of OsWRKY76 by modulating its interaction with OsWRKY76, rather than causing a direct loss of OsWRKY76 function, thereby opening new avenues for understanding OsWRKY76's role in balancing biotic and abiotic stress responses.

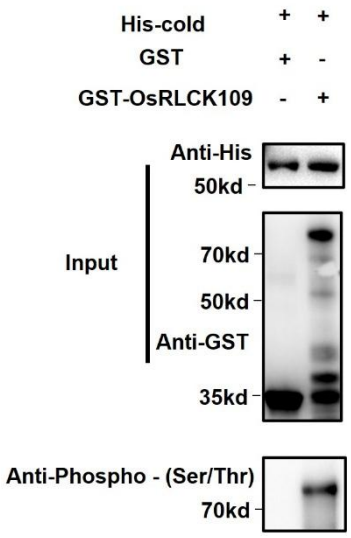
6. Supplementary materials



Supplementary Figure S6: Analysis of the expression levels of some WRKY transcription factors in ZH11 and *osrlck109*.Data shown as mean±SD, n=3, **p < 0.01, Student's t-test.

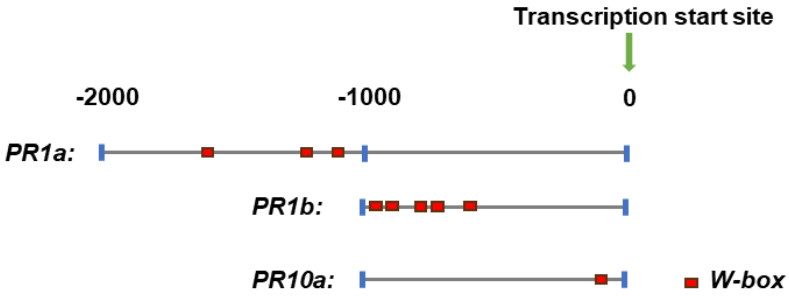


Supplementary Figure S7: The interaction scenario between OsRLCK109 and various transcription factors within the yeast two-hybrid system. (A) OsRLCK109 does not interact with specific transcription factors in the yeast system. Yeast cells were cultured on SD/-Leu/-Trp (-L T) and SD/-Leu/-Trp/-His (-L T H) media. pGADT7-T+pGBKT7-53 as a positive control.

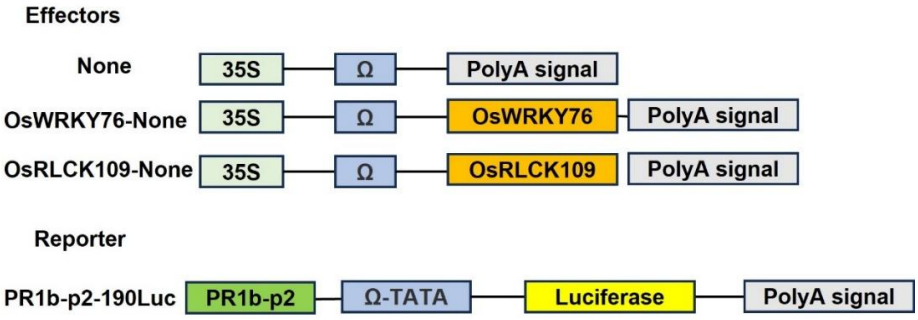


Supplementary Figure S8: OsRLCK109 is capable of auto-phosphorylation.

Supplementary Figure S9: Analysis of the promoter region of the PR gene, the red rectangle indicates the W-box.



Supplementary Figure S10: OsWRKY76 did not bind to the promoters of PR1a and PR10a in the yeast one-hybrid system.



Supplementary Figure S11: Diagram of the various constructs used in the Luc assay.

Supplementary Table S2: The primers used in Chapter 3

Primers for prokaryotic expression	
HIS-WRKY76-F	CAAATGGGTCGCGGATCCATGGACGCGGCGTGGCGCGG
HIS-WRKY76-R	TCGACGGAGCTCGAATTCGAATTCGGGCAGCTTCTGGA
HIScold+WRKY76-F	CTCGAGGGATCCGAATTCATGGACGCGGCGTGGCGCGG
HIScold+WRKY76-R	GTCGACAAGCTTGAATTCCTAGAATTCGGGCAGCTTCT
GST+RLCK109-F	TGGATCCCCGGAATTCATGCCGCCGAGGCAGTGGAG
GST+RLCK109-R	GTCGACCCGGGAATTCTCAGGGCAGCTTGGCGGAGG
For yeast two-hybrid primers	
BD+RLCK109-F	GGAGGCCAGTGAATTCATGCCGCCGAGGCAGTGGAG
BD+RLCK109-R	CACCCGGGTGGAATTCTCAGGGCAGCTTGGCGGAGG
AD+WRKY76-F	GGAGGCCAGTGAATTCATGGACGCGGCGTGGCGCGG
AD+WRKY76-R	CACCCGGGTGGAATTCCTAGAATTCGGGCAGCTTCT
For co-immunoprecipitation primers	
1132GFP+WRKY76-F	TAGAACTAGTGGATCCATGGACGCGGCGTGGCGCGG
1132GFP+WRKY76-R	GCAGCCCGGGGGATCCGAATTCGGGCAGCTTCTGGA
RLCK109-MYC-F	CGGAGCTAGCTCTAGAATGCCGCCGAGGCAGTGGAG
RLCK109-MYC-R	TCGAGACGTCTCTAGAGGGCAGCTTGGCGGAGGCGC
For the transcriptional activity experiment, primers	
none+RLCK109-F	TCTAGAACTAGTGGATCCATGCCGCCGAGGCAGTGGAG
none+RLCK109-R	CTGCAGCCCGGGGGATCCGGGCAGCTTGGCGGAGGCGC
none+WRKY76-F	TCTAGAACTAGTGGATCCATGGACGCGGCGTGGCGCGG

none+WRKY76-R	CTGCAGCCCGGGGGATCCGAATTCGGGCAGCTTC TGGA
190LUC+PR1b-p2-F	GAGAGGACACGCTGGATCCAGGAAAATAATTGGA AAGTT
190LUC+PR1b-p2-R	TTGTTGGTAATTGTGGATCCTTTAGGTCATATCATTT GAT
For q-RTPCR primers	
Q-RSOsPR10-F	ATGAAGCTCAACCCTGCTGT
Q-RSOsPR10-R	TGAGCTTGCCCACCTTACTT
Q-DREB1E-F	ACTTCCCTTGCTACCCGATG
Q-DREB1E-R	GGCTCGATGAGCATTCCCTG
Q-RLCK109-F	CTGGTGAAGCTGCTGGGCTA
Q-RLCK109-R	CACCGTCGCCGATATCCTCTT
Q-WRKY76-F	TTCCGAATGCTTTTCTGCTG
Q-WRKY76-R	ATCGTGAGGCCCGATAGAAG
Q-Actin1-F	CAGCCACACTGTCCCCATCTA
Q-Actin1-R	AGCAAGGTCGAGACGAAGGA
Q-WRKY45-F	GACACGGGCCGGGTAAA
Q-WRKY45-R	TTTCTGTACACACGCGTGGAA
Q-WRKY24-F	GACTTTGACTGGAGGCCGGA
Q-WRKY24-R	GCGCCGTCTGAAACGAGAAG
Q-WRKY71-F	AGAAGGTCACCAAGGACAACC
Q-WRKY71-R	AACCTTCTTCTTGACAGGGCA
Q-WRKY62-F	ACCCCTACCCTAGAGCTTACTTCC
Q-WRKY62-R	GACCTATCCTCCGCACATCTTT
Q-PR1b-F	TACGCCAGCCAGAGGAGC
Q-PR1b-R	GCCGAACCCCAGAAGAGG
Q-PR1a-F	TGTACTGTCAGCCGTATTTGCT
Q-PR1a-R	ATGACCATCAGACCATGCATGT
Q-PR10a-F	TGGCATGCTCAAGATGATCGAGGA
Q-PR10a-R	TTACTCTCACGGACTCAAACGCCA
For EMSA	
Probe-PR1b-1F	TTTGACACCGTTGACTTTTTAGCACGTGTT
Probe-PR1b-1R	AACACGTGCTAAAAAGTCAACGGTGTCAAA

Probe-PR1b-2F	TTTAGCACGTGTTTGACCGTTCACCTTATT
Probe-PR1b-2R	AATAAGGTGAACGGTCAAACACGTGCTAAA
Mutation probe-PR1b-1F	TTTGACACCGTCGACTTTTTAGCACGTGTT
Mutation probe-PR1b-1R	AACACGTGCTAAAAAGTCGACGGTGTCAAA
Mutation probe-PR1b-2F	TTTAGCACGTGTTCGACCGTTCACCTTATT
Mutation probe-PR1b-2F	AATAAGGTGAACGGTCGAACACGTGCTAAA
For Y1H primers	
PR1b-p1-pLacZi-F	ATCTGTCGACCTCGAGCATGGTATTAAAGTAATTTG
PR1b-p1-pLacZi-R	GAGCACATGCCTCGAGTTTCCTGATCGATCTTTACT
PR1b-p2-pLacZi-F	ATCTGTCGACCTCGAGAGGAAAATAATTGGAAAGT T
PR1b-p2-pLacZi-R	GAGCACATGCCTCGAGTTTAGGTCATATCATTTGAT
PR1b-p3-pLacZi-F	ATCTGTCGACCTCGAGATAAGACGAATTGTCAAAC A
PR1b-p3-pLacZi-R	GAGCACATGCCTCGAGCCAGCAGCACGAGGGCAA AG
PB42AD-WRKY76-F	TGCCTCTCCCGAATTCATGGACGCGGCGTGGCGC GG
PB42ADWRKY76-R	CGAGTCGGCCGAATTCCTAGAATTCGGGCAGCTT CT

Chapter 5

Conclusion and future prospects

1. General conclusion and discussion

Rice is characterized by low fat and cholesterol content, while being abundant in carbohydrates, proteins, vitamins, and minerals. Specifically, the carbohydrates present in rice serve as a vital energy source for the human body. Globally, rice ranks among the most important food crops, with over 3.5 billion individuals relying on it as their primary dietary staple. In particular, more than 60% of the population in Asia depends on rice as their main nutritional source (Shi et al. 2023). Moreover, rice is not only a fundamental agricultural commodity but also an economic pillar in numerous countries and regions. The annual global production value of rice surpasses 230 billion USD, accounting for more than 10% of the total global agricultural output. Rice blast, caused by *Magnaporthe oryzae*, can occur at any stage of rice development. Annually, rice blast results in global production losses estimated at approximately 10% to 15%, posing a significant threat to food security (Kou et al. 2024). Due to the complex pathogenic agents involved in rice blast, effective and precise prevention and control remain a persistent challenge in rice cultivation. Consequently, the identification and deployment of broad-spectrum and durable resistance genes against rice blast are essential for the advancement of rice disease resistance breeding.

In addition to R genes, numerous other genes have been reported to be associated with rice blast resistance. These include transcription factors, ubiquitin-binding proteins, MAPK, phytoalexin synthesis, microRNAs, ROS production and scavenging, PR proteins, and hormones (Kou et al. 2024). RLCK plays an important role in the regulation of rice blast. During rice blast infection, OsRLCK185 receives phosphorylation signals from OsCERK1 and further phosphorylates OsMAPKKKε by interacting with the C-terminal domain of OsMAPKKKε. Subsequently, the downstream phosphorylation of OsMKK4 and OsMAPK3/6 through the MAPK cascade activates the expression of resistance-related genes and positively regulates rice blast resistance (Wang et al. 2017). The rice blast positive regulator SDS2 can phosphorylate OsRLCK118 and activate the latter to NADPH oxidase OsRbohB to stimulate ROS production and positively regulate immunity (Fan et al. 2018). OsBSK1-2 interacts with OsHLH46 and stabilizes its protein. OsHLH46 inhibits the function of OsbHLH6 by blocking its DNA binding and transcriptional inhibitory activity, thereby alleviating the transcriptional repression of the target gene OsWRKY45 by OsbHLH6 and positively regulating rice blast resistance (Wang et al. 2024).

In our previous work, we discovered that OsRLCK109 acts as a negative regulator of rice blast resistance. The objective of this thesis is to identify the proteins that interact with OsRLCK109, with the aim of elucidating the molecular mechanism through which OsRLCK109 modulates immunity. Based on our experimental findings, we have established the following conclusions.

1.1 OsRLCK109-OsWNK1-OsCATA module regulates rice immunity

To identify the interacting proteins of OsRLCK109, OsRLCK109 was employed as bait in a yeast two-hybrid library screening. Among the candidate interactors identified, OsWNK1—encoding a member of the serine/threonine protein kinase OsWNK family—was selected as the research subject. We used the methods of Y2H, LCI, Co-IP, and BiFC to demonstrate that there is a direct interaction between OsRLCK109 and OsWNK1. Then, using CRISPR-Cas9, a mutant lacking the function of OsWNK1 was created to verify the role of the OsWNK1 protein in regulating rice blast resistance. The results of the rice blast pathogen inoculation experiment showed that the loss of OsWNK1 function enhanced the plant's resistance to rice blast disease, indicating that OsWNK1 negatively regulates rice blast resistance. Excessive accumulation of H_2O_2 and cell death were observed in the leaves of OsRLCK109 and OsWNK1 mutants. It is hypothesized that the loss of function of both OsRLCK109 and OsWNK1 leads to an imbalance in the H_2O_2 homeostasis within the plant, and that the ROS signaling. Additional research shows that OsWNK1 interacts with the catalase OsCATA, and OsWNK1 can phosphorylate OsCATA. This suggests that OsWNK1 might regulate OsCATA's ability to remove hydrogen peroxide through phosphorylation. In plants lacking OsRLCK109, OsWNK1, and OsCATA, their catalase activity decreases, and the reactive oxygen species burst caused by chitin increases, supporting this idea. Loss of OsCATA function also conferred increased resistance to rice blast disease. In conclusion, in wild-type plants, OsRLCK109 interacts with OsWNK1 to facilitate phosphorylation of OsCATA, ensuring optimal catalase activity and maintaining ROS homeostasis. When the OsRLCK109 function is compromised, OsWNK1 activity is also affected, resulting in reduced OsCATA catalase activity, disrupted ROS balance, increased H_2O_2 accumulation, and activation of disease resistance mechanisms, such as the induction of pathogenesis-related genes, thereby conferring resistance to rice blast disease.

OsRLCKs have been extensively reported to participate in both biological and non-biological stress responses (Kanda et al. 2023; Zhou et al. 2023). These molecules frequently act as downstream signaling nodes linking the RLK pathway to the MAPK pathway (Cui, Sun, and Kong 2018). Among the OsRLCK mutants documented in rice that regulate immune responses, several exhibit disruptions in ROS homeostasis within the plants (Fan et al. 2018; Zeng et al. 2018; Mou et al. 2024; Wang et al. 2025). Although the molecular mechanisms through which these OsRLCKs regulate reactive ROS homeostasis have not been explicitly elucidated, these findings suggest that OsRLCKs may play a role in maintaining ROS homeostasis. Our research indicates that in the plants of the OsRLCK109 mutant with a loss of function, there is an excessive accumulation of ROS, and the activity of catalase decreases. Chitin can induce ROS bursts, all of which suggest that OsRLCK109 plays a significant role in regulating ROS homeostasis. Subsequent studies have demonstrated that OsRLCK109 interacts with ROS clearance-related enzymes CATA and CATC, although it does not phosphorylate them. This suggests that OsRLCK109 does not directly control plant ROS homeostasis through phosphorylation of OsCATA and OsCATC. Further research has revealed that one of the interacting partners of OsRLCK109, the kinase OsWNK1, can interact with the ROS clearance-related

enzyme OsCATA, and OsCATA can be phosphorylated by OsWNK1. Our findings imply that OsRLCK109 may modulate the enzymatic activity of OsCATA via OsWNK1, thereby influencing H_2O_2 clearance in plants. This differs from the mechanism whereby RLCKs in various plant species undergo phosphorylation to activate RBOHs and modulate ROS production during PTI (Kadota et al. 2014; Fan et al. 2018; Chu et al. 2023). Our findings suggest a novel mechanism through which RLCKs manage ROS homeostasis, thereby impacting immune responses. This advancement enhances our understanding of the role of RLCK in plant immunity. This provides substantial evidence for elucidating the regulatory mechanism of RLCK in ROS homeostasis and its subsequent impact on immune responses. However, it is important to acknowledge the limitations of our study. For instance, it remains unclear whether OsRLCK109 directly confers kinase activity to OsWNK1 through phosphorylation, influencing the phosphorylation of OsCATA by OsWNK1. Further investigations are required to clarify these aspects.

Although the OsWNK family has fewer members in rice, specifically OsWNK1-OsWNK9, our current understanding of OsWNKs is limited. Presently, there are relatively few research reports concerning OsWNKs. It has been observed that one study demonstrated that OsWNK1 exhibits different expression patterns under conditions of cold, high temperature, salt, and drought stress, and that the expression of the OsWNK1 gene is regulated by the circadian rhythm. Additionally, two reports suggest that overexpression of OsWNK9 in Arabidopsis enhances its tolerance to salt, drought, and arsenite stress (Manuka, Saddhe, and Kumar 2015; (Negi and Kumar 2025). Recent research has demonstrated that under conditions of salinity stress, OsWNK9 interacts with ABA-8-hydroxylase, RACK1A, and V-type ATPase. By meticulously regulating the ABA and IAA-mediated salinity stress response, it facilitates seed germination, enhances ROS detoxification, and promotes stomatal closure (Negi and Kumar 2025). Among the ten WNK family members in the model organism *Arabidopsis thaliana*, T-DNA knockout mutants of *wnk2*, *wnk5*, and *wnk8* induce early flowering, whereas the *wnk1* mutation results in delayed flowering (Wang et al. 2008). Additionally, the *wnk8* mutant exhibits increased antioxidant activity and confers enhanced tolerance to salt and osmotic stress (Zhang et al. 2013). These findings suggest that the WNK family may be involved in regulating flowering and responses to abiotic stress in plants. Our research indicates that the loss of OsWNK1 function does not significantly affect flowering time in rice. This implies that, contrary to observations in *Arabidopsis*, OsWNK1 may not play a major role in flowering regulation in rice. There are an additional eight OsWNKs in rice, and some functions among them may be overlapping. It is also possible that other OsWNKs could influence the flowering process in rice. Results from cold and drought stress experiments show that OsWNK1 deficiency decreases rice tolerance to these stresses, supporting the hypothesis that OsWNK1 contributes positively to abiotic stress resistance. Considering that the expression of WNKs in various crops is upregulated in response to abiotic stress and that members of the WNK family have been documented to modulate abiotic stress across multiple plant species (Wei et al. 2024; Zhang et al. 2025), we hypothesize that the capacity of WNKs to regulate abiotic stress

is conserved throughout the plant kingdom. Further, infection studies with rice blast disease reveal that OsWNK1 loss enhances resistance to this pathogen, indicating a role for OsWNK1 in biological stress responses, a function not previously reported. The involvement of the OsWNK family in plant immunity presents a new perspective for understanding its functional roles in plants.

Catalase (CAT), an enzymatic entity, is extensively distributed within plant tissues and constitutes a vital component of the protective enzymatic repertoire. Its primary function is to catalyze the decomposition of H_2O_2 generated during metabolic processes, thereby averting oxidative damage to cellular structures caused by H_2O_2 accumulation. Consequently, the activity level of catalase correlates with the stress tolerance exhibited by plants. Recent investigations have demonstrated that CAT participates in the plant's disease resistance mechanisms. Specifically, tobacco enzymes CAT1 and CAT3 facilitate resistance to viral infections through interactions between SA and auxin signaling pathways (Huang et al. 2023). Furthermore, the rice E3 ubiquitin ligase APIP6 directly interacts with the peroxidase OsCATC, targeting it for degradation, which diminishes its capacity to eliminate hydrogen peroxide, a principal reactive oxygen species. Mutants lacking OsCATC accumulate elevated levels of hydrogen peroxide, thereby inducing cell death and augmenting resistance to rice blast fungus (You et al. 2022). Our research findings suggest that the absence of OsCATA function results in impaired H_2O_2 clearance, activation of immune signaling pathways, and, consequently, heightened resistance to rice blast disease. Additionally, OsCATA can undergo phosphorylation by the kinase OsWNK1, a modification that appears essential for preserving its full enzymatic activity. This represents the inaugural evidence that PTM of OsCATA influences its functional capacity and modulates rice immunity.

1.2 OsRLCK109 boosts OsWRKY76's inhibition of PR1b, negatively affecting immunity

The WRKY transcription factors are integral to the immune regulation pathway in rice (Wei et al. 2013). Analysis of RNA-seq data from *osrlck109* and the wild-type ZH11 revealed that WRKY transcription factors, which positively regulate rice blast resistance, exhibited higher expression levels in *osrlck109* than in the wild-type ZH11. Notably, the expression level of OsWRKY76—a WRKY transcription factor previously reported to diminish rice blast resistance when overexpressed—increased as well (Yokotani et al. 2013). This observation contradicts the enhanced rice blast resistance observed in *osrlck109*, thereby implying a potential interaction between OsRLCK109 and OsWRKY76. Subsequently, experiments utilizing a yeast system demonstrated an interaction between OsRLCK109 and OsWRKY76, which was further validated by in *vivo* Co-IP and in *vitro* pull-down assays.

To ascertain the role of OsWRKY76 in rice blast resistance, we employed CRISPR-Cas9 technology to generate a loss-of-function mutant of OsWRKY76. Following inoculation with the rice blast pathogen, it was observed that the mutant strain *oswrky7676* exhibited enhanced resistance compared to the wild type. This suggests

that OsWRKY76 functions as a negative regulator of rice blast resistance. In the *oswrky7676* mutant, the expression levels of PR genes were assessed, revealing significant upregulation and activation of immune responses. Promoter analysis indicated the presence of W-box elements within these PR gene promoters, which are conducive to binding by WRKY transcription factors. Consequently, we hypothesize that OsWRKY76 can bind these promoters to regulate gene transcription. Yeast one-hybrid, Dual-Luciferase Reporter assays, and EMSA experiments demonstrated that OsWRKY76 binds to the PR1b promoter and represses its transcription, potentially explaining the enhanced disease resistance observed in *oswrky76*.

As a negative regulator of rice blast resistance, OsWRKY76 expression levels increase; intuitively, this should diminish disease resistance. Notably, in *osrlck109*, where OsWRKY76 expression is markedly elevated, resistance is nonetheless enhanced. We hypothesize that OsRLCK109 may modulate OsWRKY76 activity. Transcriptional activity assays revealed that, absent OsRLCK109, OsWRKY76's repression of PR1b is significantly diminished, while the addition of OsRLCK109 restores repression. In *vitro* EMSA experiments confirmed that higher OsRLCK109 levels enhance OsWRKY76's binding to the PR1b promoter. Additionally, the expression of other OsWRKY76 target genes in the *osrlck109* background displayed characteristics indicative of impaired OsWRKY76 function. These findings collectively suggest that OsRLCK109 positively regulates OsWRKY76 activity. It has been reported in *Arabidopsis* that the receptor-like cytoplasmic kinase BIK1 can target the WRKY transcription factors WRKY33 and WRKY50/57, thereby regulating the immunity of *Arabidopsis* (Lal et al. 2018). Our discovery also indicates that in rice, there exists a mechanism where RLCK regulates immunity by targeting WRKY transcription factors.

Kinases often modulate the activity of their interacting proteins via phosphorylation. Does OsRLCK109, as a kinase, regulate OsWRKY76 through phosphorylation? In *vitro* phosphorylation assays demonstrated that OsRLCK109 can phosphorylate OsWRKY76. However, further research is required to determine whether this phosphorylation is the mechanism through which OsRLCK109 actively influences OsWRKY76 function.

1.3 Analysis of using genetically edited plants from the thesis for breeding

In crop breeding, breeders typically enhance desirable agricultural traits by creating and integrating superior genetic variations (Meyer and Purugganan 2013). However, passively awaiting beneficial mutations to occur naturally is an exceedingly time-consuming process. In this regard, the importance of rapid artificial mutagenesis techniques is progressively increasing. Traditional physical and chemical mutagenesis methods are limited to generating random mutations and often necessitate extensive screening to identify advantageous mutations (Holme, Gregersen, and Brinch-Pedersen 2019). Gene editing technology allows for the precise modification of specific genomic targets within organisms, enabling efficient gene insertion, deletion,

or substitution, thus altering genetic information and phenotypic traits. The application of gene editing technology in crop breeding has the potential to markedly improve breeding efficiency and is emerging as a disruptive innovation in the field. This advancement is poised to have a significant impact on future agricultural development and food security (Li et al. 2024).

This study utilized CRISPR/Cas9 gene editing technology to develop multiple rice mutant lines (*osrlck109, oswnk1, oscata, oswrky76*). Resistance testing confirmed that targeted gene editing can significantly enhance the plants' resistance to rice blast disease under laboratory conditions, thereby providing valuable targets and germplasm resources for molecular improvement of rice resistance. However, regarding agronomic traits, these mutants demonstrated several adverse phenotypes, including stunted growth, reduced tillering, and decreased yield. These characteristics exemplify the growth-defense trade-off, a common biological phenomenon. Based on a comprehensive assessment of the core objectives in rice high-yield and disease resistance collaborative breeding, the immediate field breeding and commercial application prospects of this batch of gene-edited mutants are limited.

The current focus of crop breeding is directed towards achieving a balance between high yield and disease resistance. However, breeding objectives are often hindered by the trade-off between growth and defense mechanisms, which considerably restricts the utilization of R genes, as the introduction of certain resistance genes may diminish other agronomic traits and result in a decrease in yield (Ning, Liu, and Wang 2017). Although numerous R genes and regulatory components associated with yield have been effectively characterized in various crops, their incorporation into a cohesive genetic framework remains a significant challenge. Future research targeting an understanding of the growth-defense trade-off on a whole-genome scale is expected to resolve this breeding bottleneck (Gao et al. 2024).

Certainly, the successful example of breeding strategies that achieve an optimal balance between disease resistance and yield in rice exemplifies the potential for applying the findings of this study in practical breeding efforts. Li et al. cloned the "*RBL1*" broad-spectrum disease-resistant gene. The mutant of this gene exhibited broad-spectrum disease resistance but caused significant yield reduction. Through targeted gene editing on *RBL1*, a 12-base-pair deletion mutant, designated $rbl1^{\Delta 12}$, was obtained. After multiple years of field trials, it was established that this mutant conferred broad-spectrum disease resistance without compromising yield (Sha et al. 2023). The *LRD6-6* gene encodes an AAA-type ATPase (Zhu et al. 2016). The constitutive expression of the dominant-negative mutant $LRD6\text{-}6^{E315Q}$ markedly enhances rice resistance to rice blast, sheath blight, and bacterial blight. However, this approach also results in decreased plant height, reduced thousand-grain weight, lower seed setting rate, and deterioration of rice quality. To mitigate these effects, the researchers employed the pathogen-inducible promoter MIG6P to drive the expression of $LRD6\text{-}6^{E315Q}$, ensuring that it is only activated upon pathogen infection to initiate an immune response. Under normal growth conditions, the gene remains

minimally expressed or inactive, thereby avoiding adverse effects on growth and yield (Zhu et al. 2026).

MicroRNAs (miRNAs) constitute a class of non-coding RNAs that regulate gene expression through the degradation of target mRNAs or the inhibition of translation (Zhang et al. 2006). They participate in a variety of biological processes, including plant immunity (Baldrich and Segundo 2016). More than 700 miRNAs have been identified within the rice genome, with over 100 types induced upon infection by *M. oryzae* (Li et al. 2019). Although research regarding the role of miRNAs in rice blast resistance is limited, evidence indicates their involvement at various stages of the immune response, such as PTI, ETI, and ETS (Bhutto et al. 2025). This involvement encompasses multiple signaling pathways, including ROS homeostasis, the induction of disease-resistant genes, DNA methylation, and plant hormone regulation (Jia et al. 2025). Given the potent functions of miRNAs, artificial regulation of these molecules to maintain a balance between growth and defense represents an important breeding strategy. The *IPA1* gene encodes a class of Squamosa promoter binding proteins; miR156 can inhibit IPA1 transcription (Jiao et al. 2010). Removing this inhibition can reduce rice tillering, increase grain number per spike, and the weight of a thousand grains, while also thickening stems and improving lodging resistance, thereby increasing yield. The rice blast fungus can induce phosphorylation of IPA1, enhancing its affinity for the promoter of the disease-resistant gene *WRKY45*, promoting its expression, and strengthening the immune response (Wang et al. 2018). Researchers achieved this by introducing a target mimic of miR156fhl-3p, MIM156-3p, which decreased the accumulation of miR156fhl-3p and miR156-5p. This resulted in increased *IPA1* expression, subsequently promoting WRKY45 expression and enhancing resistance to *M. oryzae* without compromising yield, thus effectively balancing growth and defense (Zhang et al. 2020).

The above cases collectively demonstrate that gene editing technology constitutes a significant means of disrupting the balance between growth and defense. Nevertheless, the practical application of genetically edited crops in production remains subject to legal oversight. On January 24, 2022, the Ministry of Agriculture of China issued the "Guidelines for Safety Evaluation of Agricultural Genetically Engineered Plants (Trial)"(https://www.moa.gov.cn/ztzl/zjyqwgz/sbzn/202201/t20220124_6387561.htm), marking the first formal distinction between genetically modified crops and genetically edited crops. It explicitly states that genetically edited plants devoid of exogenous gene introduction should adhere to these guidelines, whereas genetically edited plants incorporating exogenous genes should comply with the "Guidelines for Safety Evaluation of Transgenic Plants." To some extent, this reflects the Chinese government's understanding and prioritization of gene editing technology. However, unlike countries such as the United States and Argentina, which explicitly specify that genetically edited crops meeting certain conditions are exempt from transgenic regulation, China's regulation of genetically edited crops continues to operate within the framework of transgenic laws, with only specific provisions made for the safety evaluation system applicable to genetically edited crops.

In conclusion, the disease-resistant gene-editing mutants obtained are constrained by the trade-off relationship between gene pleiotropy and growth-defense capabilities, thereby limiting their direct selection and application. Nonetheless, these mutants constitute valuable genetic resources for disease resistance. Moving forward, it is essential to circumvent direct utilization methods and instead employ precise allelic editing to achieve a balanced enhancement of both disease resistance and yield traits, thereby facilitating the integration of these disease-resistant genes into the breeding programs for new high-yield, disease-resistant rice varieties.

2. Perspectives

This study extends previous research by identifying the interacting proteins of OsRLCK109, thereby elucidating two pathways through which OsRLCK109 modulates immunity. Firstly, OsRLCK109 interacts with OsWNK1, affecting its regulation of catalase CATA and thereby maintaining ROS homeostasis within the plant. Secondly, OsRLCK109 interacts with the transcription factor OsWRKY76, augmenting its inhibitory effect on the downstream target gene PR, which prevents abnormal activation of the defense response. The absence of OsRLCK109 directly impairs the plant's capacity to clear hydrogen peroxide, resulting in an imbalance of ROS homeostasis—a critical immune signaling molecule—and subsequently activating the immune response. Additionally, the suppression of the PR gene is diminished, leading to its activation and the initiation of the immune response. This research clarifies the role of OsRLCK109 within the rice immune pathway and enriches our understanding of the intricate immune regulatory mechanisms in rice. Nonetheless, certain unresolved issues remain, warranting further investigation in future studies.

1. The phosphorylation relationship between OsRLCK109 and OsWNK1. In our experiment, the inability of OsWNK1 to be expressed in *vitro* as a prokaryote was the greatest obstacle in exploring the phosphorylation relationship between OsRLCK109 and OsWNK1, even though the genetic codons of WNK1 do not contain rare codons. The next step could involve using eukaryotic systems for expression, such as yeast expression systems, insect cell systems, etc.

2. The enzymatic activity of OsCATA is explicitly demonstrated by the phosphorylation effect of OsWNK1 upon it. In our research, we established that OsWNK1 directly phosphorylates OsCATA, and the knockout of OsWNK1 resulted in impaired catalase activity in the plants. The peroxidase enzymes found in rice, namely OsCATA, OsCATB, and OsCATC, exhibit complex interrelationships and mutual influences. By expressing OsWNK1 and OsCATA exogenously and analyzing their interaction independently, we can gather direct evidence that the enzymatic activity of OsCATA is modulated by the phosphorylation of OsWNK1.

3. Explore the phosphorylation sites of OsWRKY76 by OsRLCK109, which are of significant interest. Phosphorylation modification is a crucial mechanism for regulating protein functions. The activity of many transcription factors is modulated by kinases. We have demonstrated that OsRLCK109 is capable of phosphorylating

OsWRKY76. However, the specific phosphorylation sites remain unidentified. Future analysis of these sites can be conducted using mass spectrometry, which will provide a foundation for modulating OsWRKY76's function through amino acid sequence alterations, rather than solely via OsRLCK109.

4. The potential applications of OsRLCK109 in breeding have been explored. The loss of OsRLCK109 function confers substantial resistance to rice blast on the plants; however, it also results in adverse effects such as plant dwarfing, impaired photosynthesis, and reduced yield. This suggests that OsRLCK109 plays a dual role, regulating both immunity and growth and development. Understanding the mechanisms by which OsRLCK109 influences these processes may involve targeting specific nodes within the pathway. Such an approach could enable the development of plants with enhanced immune capabilities while preserving optimal growth, thereby achieving a balance between immunity and development. This research aims to provide genetic materials for the development of rice varieties that are both highly resistant and high-yielding.

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