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Multitrophic impact of viruses from different realms and hosts on rice health and growth

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Multitrophic impact of viruses from different realms and hosts on rice health and growth

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Abstract

Rice is a major source of food for more than half of the world's population, especially in Asia, and is an important part of the agricultural economy in many countries, providing a source of income for farmers. Firstly, rice viral diseases can lead to a significant decrease in harvest yield. In severe cases, it may even result in total crop failure, posing a threat to global food security.

Rice stripe virus (RSV) is transmitted by the small brown planthopper (SBPH, *Laodelphax striatellus* Fallen) in a persistent and propagative manner. In this study, RNA-Seq was used to analyze the transcriptomic differences between resistant and susceptible rice varieties at different times post RSV infection. Through Gene Ontology (GO) annotation, differentially expressed genes (DEGs) related to transcription factors, peroxidases, and kinases of 2 varieties at 3 time points were identified. Comparing these 2 varieties, the DEGs associated with these 3 GOs were numerically less in the resistant variety than in the susceptible variety. Eighty-seven DEGs involved in some pathways that have a contribution to disease resistance including plant hormone signal transduction and plant–pathogen interactions were identified. Resistance responses regulated by abscisic acid (ABA) and brassinosteroids (BR) were found to be similar for 2 varieties. Fifty-five DEGs in resistant and susceptible varieties at the 3 time points were identified in both PAMP-triggered immunity (PTI) and effector protein triggered immunity (ETI). These findings will provide valuable information for further research on the interactions between rice and RSV, particularly on molecular mechanisms of rice resistance to RSV infection.

Secondly, phages that infect nitrogen-fixing bacteria can contribute to plant nutrition, thus impacting rice yield and quality. Phages act to regulate soil microbial community structure. Here, low relative abundance of nitrogen-fixing bacteria was found but *Enterobacter*-infecting phages were highly present in paddy soil where rice plants showed nitrogen deficiency. A novel virulent phage (named here *Apdecimavirus* NJ2) was identified infecting several species of *Enterobacter.* It has the morphology of the *Autographiviridae* family, with a dsDNA genome of 39,605 bp, 47 predicted open reading frames and 52.64 % GC content. Based on comparative genomics and phylogenetic analysis and ICTV demarcation criteria, *Apdecimavirus* NJ2 could be considered as a novel species in the genus *Apdecimavirus*, subfamily *Studiervirinae*. After natural and sterilized field soil were inoculated with this phage, nitrogen-fixing bacteria and phage, respectively, soil nitrogen-fixation capacity and rice growth were impaired abundance of *Enterobacter* decreased, along with the bacterial community composition and biodiversity changed compared with that of the unadded natural soil control and sterilized soil added with nitrogen-fixing bacteria. Our work provides strong evidence that phages can affect the soil nitrogen cycle by changing the bacterial community. Leveraging phages in the soil could be a useful strategy for improving soil nitrogen fixation.

Keywords: Transcriptomics, resistance, susceptibility, rice stripe virus (RSV), infection, phage, *Enterobacter*, phage-bacteria interaction, 16S rRNA amplicon

sequencing, nitrogen cycling

Résumé

Le riz est une source majeure de nourriture pour plus de la moitié de la population mondiale, en particulier en Asie et constitue un élément important de l'économie agricole dans de nombreux pays. Les maladies virales du riz peuvent entraîner une baisse significative du rendement et dans les cas les plus graves, une perte totale des récoltes.. La maladie de la striure du riz, causée par le rice stripe virus (RSV) est transmise par la petite cicadelle brune (SBPH, *Laodelphax striatellus* Fallen) suivant de manière persistante et propagative.. Dans cette étude, le RNA-Seq a été utilisé pour analyser les différences transcriptomiques entre les variétés de riz résistantes et sensibles à différents moments après l'infection par le RSV. Les gènes différentiellement exprimés (DEG) liés aux facteurs de transcription, aux peroxydases et aux kinases de 2 variétés à 3 moments ont été identifiés dans la variété résistante virosée. Les DEG associés à ces 3 GO ont été observés dans les deux variétés.. Ensuite, certaines voies contribuant à la résistance aux maladies, notamment la transduction du signal des hormones végétales et l'interaction plante-pathogène ont été identifiées. Des réponses de résistance par l'acide abscissique (ABA) et les brassinostéroïdes (BR) étaient similaires pour les 2 variétés alors que celles médiées par l'acide salicylique (SA) et l'acide jasmonique (JA)/éthylène (ET) étaient différentes. Les variétés résistantes et sensibles ont présenté une immunité déclenchée par le PAMP (PTI par la protéine effectrice (ETI). La plupart des gènes de la variété sensible étant impliqués dans la PTI, alors que la plupart des gènes de la variété résistante étaient impliqués dans l'ETI. Ces résultats fourniront des informations utiles pour des recherches plus approfondies sur les mécanismes moléculaires de la résistance du riz à l'infection par le RSV.

Le second objectif de cette thèse est d'analyser les effets des phages infectant les bactéries fixatrice d'azote sur la teneur de ce dernier dans le sol et la croissance du riz. Les phagesrégulent la structure de la communauté microbienne du sol. Une faible abondance relative de bactéries fixatrices d'azote mais une forte abondance de phages infectant *Enterobacter* ont été révélées dans le sol de rizière où les plants de riz présentaient une carence en azote. Un nouveau phage virulent (*Apdecimavirus* NJ2) a été identifié . Il infecte plusieurs espèces d'*Enterobacter.* Il présente une morphologie de la famille des *Autographiviridae*, avec un génome d'ADNdb de 39 605 pb. Sur base d'analyses génomiques comparatives et phylogénétiques, l'*Apdecimavirus* NJ2 devrait être une nouvelle espèce du genre *Apdecimavirus*, sous-famille *Studiervirinae*. Après inoculation de sols stériles avec le phage, la capacité de fixation de l'azote et la croissance du riz ont été altérées. L'abondance d'*Enterobacter* a diminué, tandis que la composition de la communauté bactérienne et la biodiversité ont changé par rapport à celles du sol témoin. Nos travaux fournissent des preuves solides que les phages peuvent affecter le cycle de l'azote du sol en modifiant la communauté bactérienne. Le contrôle des phages dans le sol pourrait être une stratégie utile pour améliorer les rendements culturaux avec des effets sur les ravageurs en fonction du statut azoté de la plante cultivée.

Mots-clés: Transcriptomique, résistance, susceptibilité, virus de la striure du riz (RSV), infection, phage, *Enterobacter*, interaction phage-bactérie, séquençage de l'amplicon de l'ARNr 16S, cycle de l'azote

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List of acronyms

ABA	Abscisic acid
AMG	Auxiliary metabolic gene
AUX	Auxin
Avr	Avirulence
BR	Brassinosteroids
CEV	Citrus exocortis viroid
CK	Cytokinin
COG	Clusters of Orthologous Group
DEG	Differentially expressed gene
DPI	Days post-inoculation
ET	Ethylene
ETI	Effector-triggered immunity
FPKM	Fragments Per Kilobase of transcript per Million
GA	Gibberellin
GMO	Genetically modified organism
GO	Gene Ontology
GWAS	Genome-wide association analysis
HR	Hypersensitive response
HTS	High-Throughput Sequencing
ICTV	International Committee on Taxonomy of Viruses
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Luria Bertani
LHCB3	Light-harvesting chlorophyll a/b complex protein
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
MHP	Major head protein
NCBI	National Center for Biotechnology Information
OTU	Operational taxonomic unit

PAMP	Pathogen-associated molecular patterns
PCoA	Principal coordinate analysis
Pfm	Protein families
PFU	Plaque forming unit
PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
R gene	Resistance gene
RNAP	RNA polymerase
RPL	REPLUMLESS
R protein	Resistance protein
ROS	Reactive oxygen species
RSV	Rice stripe virus
RT-qPCR	Real-time quantitative polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SBPH	small brown planthopper
SOD	superoxide dismutase
terL	Terminase large subunit
TF	Transcription factor
TMV	Tobacco mosaic virus
TuMV	Turnip mosaic virus
Xoo	*Xanthomonas oryzae* pv. *oryzae*
ZIP	ZRT/IRT-like protein

Chapter 1

General introduction

1. Plant and viruses

Plants are exposed to various microbials including a large range of viruses with diversified effects, from negative to neutral or beneficial impacts. In one hand, plants are seriously threatened by phytopathogen viruses from different origins and hosts during their growth. They can be transmitted by vectors, wounds, pollen and seeds. In the other hand, soil viruses can also infect bacteria involved in nutrient element cycling. This could modify directly the plant growth but also susceptibility to potential diseases with indirect consequences.

2. Plant viruses

Pathogenic viruses harm host plants including crops and are very difficult to prevent and control. After infecting the host plant, the virus not only competes with the host for nutrients necessary for growth, but also destroys the nutrient transport of the plant, changes certain metabolic balances, inhibits the photosynthesis cause symptoms such as deformity, and yellowing, and finally induce plant death (Savary et al., 2019).

Phytoviruses cannot directly invade the plant epidermis, but need insects, fungi, nematodes, pollen, seeds or wound to enter the plant cells. Around 80% of plant viruses use insects and nematodes as vectors for primary invasion (Adams, 1991; Brown et al., 1995; Fetters and Ashman, 2023; Gutiérrez-Sánchez et al., 2023; Sastry, 2013; Savatin et al., 2014; Xia et al., 2018). Plant virus genome only encodes a few proteins, and they must rely on host plants to complete their own infection and proliferation. Therefore, most plant viruses use their encoded proteins to hijack or use host factors to achieve their own life processes such as replication, transcription, movement and particle assembly (Zhu et al., 2019a).

The mechanisms have been selected instead and can be classified as: (1) resistance to virus vectors; (2) inhibition of virus proliferation; (3) blocking of virus movement; and (4) degradation of the viral genome by RNA silencing (Soosaar et al., 2005; Wang et al., 2021; Ye et al., 2020).

2.1 Rice stripe disease

Rice stripe disease is one of the important viral diseases in rice production in temperate and subtropical East Asian countries (Wang et al., 2008). In China, this disease was first reported in southern Jiangsu Province in 1963 (Li et al., 2016; Zhang et al., 2007). In the past 30 years, rice stripe disease has shown intermittent outbreaks in China. From 1999 to 2004, the annual area of occurrence in Jiangsu Province increased from 1 million acres to 24 million acres, causing 20 to 30 %losses in rice production (Wang et al., 2008; Wei et al., 2009; Zhang et al., 2007).

The pathogen causing rice stripe disease is rice stripe virus (RSV), belonging to *Tenuivirus* genus, *Phenuiviridae* family. In nature, it is transmitted by small brown plant hopper (*Laodelphax striatellus* Fallen, SBPH) in a persistent propagative manner (Chen et al., 2019; Qin et al., 2018). The RSV genome consists of four single-stranded RNA segments, encoding seven proteins (Sun et al., 2016; Wu et al., 2018).

These proteins are involved in multiple biological processes including viral movement, encapsidation, RNA silencing inhibition, transcription and transmission (Xiong et al., 2008; Xiong et al., 2009; Zhou et al., 2012).

In early-stage infected plants, irregular chlorotic mottling or yellow-white stripes parallel to the leaf veins first appear at the base of the heart leaves. The heart leaves of *Oryza sativa* subsp. japonica gradually turn yellow and curl into paper twists and droop. For *Oryza sativa* L. subsp. sativa, the heart leaves do not curl or droop after the disease occurs, only irregular chlorotic stripes parallel to the veins appear (Wang et al., 2015) (Fig. 1-1).

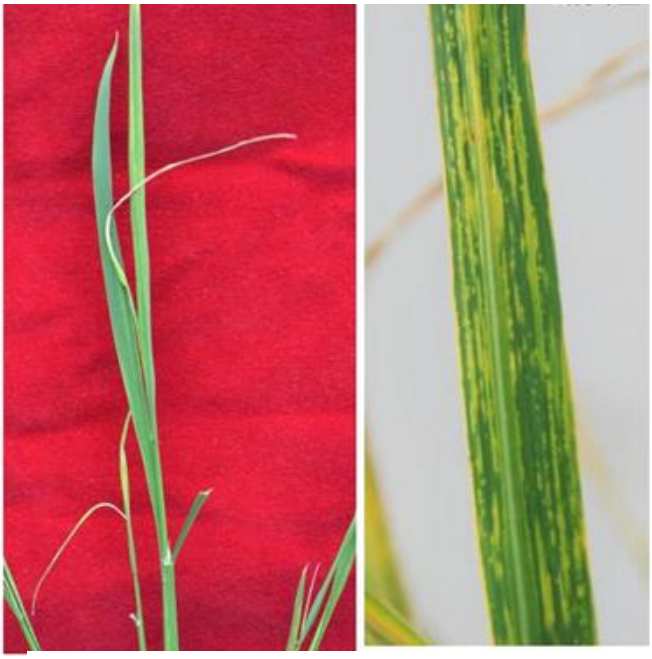


Figure 1-1: Symptom of rice stripe disease.

2.2 Plant disease resistance

Plant disease resistance is a genetic trait that allows a plant to cope with infestation and damage by pathogens. This resistance has been developed by plants over a long period of evolution to resist the invasion of pathogens to a certain extent. Disease resistance in plants is variable and is genetically controlled through the laws of heredity (Savary et al., 2019).

Disease resistance in plants can be categorized into several types, including nonhost -, qualitative -, quantitative - and induced systemic resistances (Hafez et al., 2020; White et al., 2021). Non-host resistance is a broad-spectrum resistance of plant species to non-adapted pathogens, usually a PTI-based defense response activated by the recognition of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) (Asai et al., 2002). Qualitative resistance is usually

controlled by a single or a few primary effector genes and is manifested as complete or near-complete resistance (Xue et al., 2002). Quantitative resistance is associated with multiple genes and is usually broad-spectrum and persistent (French et al., 2016). Induced systemic resistance is resistance produced by plants through activation of basal defenses after exposure to pathogens or other stimuli (Choudhary et al., 2007).

PTI is the first line of defense in the plant defense mechanism, when the pathogen invades the plant, the plant recognizes PAMPs through PRRs, which triggers a broad-spectrum of defense responses to inhibit the initial infection of the pathogen (Zipfel, 2009). After PTI, if the pathogen is well adapted and able to overcome PTI, plants recognize the pathogen's effector proteins through resistance genes (*R* genes), triggering ETI, a high defense response that typically results in programmed cell death at the site of infection to prevent further spread of the pathogen (Laflamme et al., 2020). Following ETI, plants develop systemic acquired resistance (SAR), a long-lasting and broad-spectrum immunity that provides protection to distal tissues (Fu and Dong, 2013). Plants can also be resistant to viruses through RNA silencing mechanisms. The replication intermediates of plant viruses produce double-stranded RNA (dsRNA), and this dsRNA can activate the plant's RNA silencing mechanism to protect the plant (Baulcombe, 2000).

The molecular mechanisms of plant disease resistance involve multiple levels, including PRRs on the cell surface and intracellular disease resistance proteins (*R* proteins). PRRs are receptors that can recognize PAMPs to activate PTI. In some cases, *R* proteins are able to directly recognize avirulence (*Avr*) proteins of pathogens based on a direct physical interaction between the *R* protein and *Avr* protein. In more cases, *Avr* proteins indirectly activate *R* proteins through the participation of other proteins, such as in the "defense" and "decoy" models. In the "defense" model, *Avr* proteins attack the plant target, and *R* proteins activate the defense response when they detect that the target has been attacked. In the "decoy" model, the *Avr* protein activates the plant's immune response by affecting the target monitored by *R* proteins (He et al., 2012; Zhang et al., 2021).

In the research on plant disease resistance, scientists are committed to exploring and utilizing new disease-resistant resources, deeply looking for disease resistance mechanisms, and utilizing new technologies such as gene editing and marker-assisted gene polymerization to apply new results in the design and selection of new varieties and the rational layout of disease-resistant varieties (Cao et al., 2022; Wang et al., 2024a). These investigations not only help to breed crop varieties with broad-spectrum and durable resistance but also are of great significance in realizing effective prevention and control of diseases and green prevention and control (Li et al., 2022a).

Breeding and application of plant disease resistance is an important direction of plant genetic breeding to improve crop yield and quality, reduce the use of pesticides and play an important role in the sustainable development of agriculture (Wang et al., 2024b).

2.2.1 Plant hormones and disease resistance

Plant hormones play a crucial role in plant disease resistance. They are natural organic compounds produced that can regulate growth, development and adaptation to the environment (Santner et al., 2009). In the plant immune system, several key

plant hormones play a central role, including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and brassinosteroids (BRs) (Hirayama and Mochida, 2023).

SA is a key signaling molecule in SAR. When plants are infected by pathogens, the synthesis of SA increases sharply, activating the plant's disease resistance response, exogenous SA also induces plant resistance to pathogens and induces SAR. SAR is usually activated after a part of the plant is infected by pathogen and can systemically spread to other parts of the plant, providing protection to uninfected tissues. The early stage of SAR is the endogenous synthesis and accumulation of SA in plant cells. As a signaling molecule, SA is crucial for the initiation and maintenance of SAR. SA is not only involved in the plant's immune response but also regulates plant growth and development as well as various stress responses, such as heat tolerance, drought resistance, seed germination, stomatal closure, photosynthesis, flowering and plant senescence (Ding and Ding, 2020; Kachroo et al., 2020). JA and its derivatives, such as methyl jasmonate (MeJA), are signaling molecules that transmit resistance information in plants. And they play an important role in the stress response and inducing the expression of defense genes in plants. The signaling of ISR is mainly dependent on JA and ET. ISR is induced by the interaction of non-pathogenic microbes, such as some rhizosphere bacteria, with plant roots (Choudhary et al., 2007; Roychowdhury et al., 2024). ET plays a key role in the mechanism of plant defense against herbivorous insects, it can induce plants to produce volatile organic compounds, phenolic compounds, and protease inhibitors, enhancing the plants' defensive capabilities (Ma et al., 2020; Zhao et al., 2017). ABA is also a key regulatory factor in plant defense mechanisms, playing a role in seed germination and responses to abiotic stress and it has distinctly different effects on plant susceptibility to herbivory (Song et al., 2011). BRs are a class of steroid hormones that enhance plants' defense capabilities against abiotic and biotic stresses. In the context of biotic stress, BRs can enhance plant defense against pests and pathogens (Kang et al., 2024; Yao et al., 2023).

The interactions among these plant hormones are very complex and they collectively coordinate the growth, development and response of plants to environmental challenges (Kumari et al., 2024). Phytohormones help plants resist viruses through multiple mechanisms, including directly regulating plant growth and immune response, and enhancing the plant's antiviral ability through interactions with the plant immune system (Islam et al., 2019). These findings provide insights into how plants use hormone systems to defend against viral infection and provide a scientific basis for the development of new antiviral strategies.

2.2.2 Autophagy and disease resistance

Autophagy is an important degradation process in plant cells, which degrades and recycles unwanted proteins or damaged organelles by forming autophagosomes. This process is crucial for plant growth and development, stress responses and plant immune responses (Huang et al., 2023).

In terms of plant immunity, autophagy is involved in the defense mechanisms of plants against pathogens. Inhibition of cellular autophagy was found by regulating the phosphorylation of the key protein ATG18a, thereby affecting the plant resistance to

Botrytis cinerea in *Arabidopsis* (Zhang et al., 2020). Additionally, autophagy is also involved in the resistance of plants to necrotrophic pathogens, such as by regulating the plant's immune response through interactions with plant hormone signaling pathways like SA and JA (Gong et al., 2020; Lai et al., 2011).

Plants with autophagy were shown to be often more sensitive to necrotrophic fungal pathogens, indicating that autophagy plays an active role in plant resistance to pathogens (Lai et al., 2011). Autophagy resist virus infection by promoting programmed cell death and regulating virus-related cell death regulators to limit virus spread (Shi and Kehrl, 2010). However, the role of autophagy in plant immunity may vary depending on the type of pathogen and the physiological state of the plant, and it may sometimes even exhibit negative effects (Lal et al., 2020).

Autophagy plays a complex and diverse role in plant disease resistance, helping plants adapt to and resist external environmental challenges by finely regulating their immune responses and hormone signaling pathways.

2.2.3 Transcription factors, kinases, peroxidases and disease resistance

Disease resistance in plants is a complex physiological process involving multiple biomolecules and signaling pathways (Ding et al., 2022). Transcription factors, kinases and peroxidases play key roles in plant disease resistance (Sopeña-Torres et al., 2018; Wang et al., 2021; Zhao et al., 2019).

Transcription factors are proteins that regulate gene expression by binding to specific sequences on DNA to enhance or repress gene transcription (Strader et al., 2022). In plant disease resistance, transcription factors, such as members of the MYB family, can act as positive or negative regulators of defense by controlling the expression of defense genes, lignin, flavonoid and epidermal wax biosynthesis, polysaccharide signaling, hormone defense signaling and hypersensitive responses (Yu et al., 2023). In addition, transcription factors can enhance plant antiviral ability by activating the RNAi pathway (Wang et al., 2021).

Also, kinases are a class of enzymes capable of transferring phosphate groups to specific target proteins, thereby regulating their activity (Ptacek et al., 2005). In plant immunity, the activation of kinases such as the mitogen-activated protein kinase (MAPK) cascade reaction is an important component of the plant innate immune system. Receptor kinases such as leucine-rich repeat-containing receptor kinases (LRR-RK) act as cell-surface immune receptors by recognizing PAMPs and thereby activating PTI, and they act as the first line of defense in the plant's immune system, recognizing a wide range of pathogens and initiating defense responses (Ortiz-Morea et al., 2022).

Peroxidases are a class of enzymes that catalyze the oxidation of hydrogen peroxide with an electron donor (Ng, 2004). Infection by pathogens can induce an increase in the activity of plant peroxidases and changes in the types of peroxidase isoenzymes. These highly active peroxidases or specific isoenzymes may form physical barriers by enhancing the biosynthesis of lignin and suberin, thereby producing general resistance and non-specialized disease resistance (Tian et al., 2001).

These components interact with each other through a complex signaling network and jointly participate in the regulation of plant recognition, signaling and defense

responses to pathogens. By studying the functions and mechanisms of action of these components, we can provide an important theoretical basis for breeding disease-resist. crop varieties and developing disease prevention and control strategies.

2.1.4 Plant susceptibility genes and breeding disease-resistant varieties

Plant viruses rely on susceptibility factors to successfully infect host plants. The functional loss of susceptibility genes often confers host resistance to viruses. This resistance is determined by recessive alleles and is called recessive resistance (Chen et al., 2022). Using susceptibility genes to breed disease-resistant varieties is an effective strategy. Through gene editing technology, susceptibility genes can be specifically knocked out, thereby enhancing the resistance of crops to viruses (Chen et al., 2022). Mutations in susceptibility genes often confer broad-spectrum and durable disease resistance to plants. This disease resistance is of great significance for preventing and controlling diseases and reducing the use of pesticides and helps achieve green agriculture and sustainable development (Li et al., 2022a). Using gene editing technology such as CRISPR/Cas9, breeding time can be greatly shortened and new varieties with excellent traits can be quickly obtained (Wang et al., 2024a). In summary, the use of susceptibility genes to breed virus-resistant varieties can improve crop disease resistance, shorten the breeding time, precisely manipulate genetic loci, provide broad-spectrum and durable disease resistance, increase yield and quality, and improve food safety.

2. Plant, nitrogen, rhizosphere microbes and phages

Nitrogen is one of the main nutrients necessary for plants, and it has an important impact on growth and development (De Vries et al., 2022). Nitrogen fertilizer is one of the most widely used intrants in agricultural production and can significantly increase crop yields and improve the quality of agricultural products (Govindasamy et al., 2023). Appropriate application of nitrogen fertilizer can promote the growth of plant roots and increase the root's ability to absorb water and nutrients (Wu et al., 2025). Nitrogen deficiency will limit plant growth and development, affect plant physiology and metabolic processes (Mu and Chen, 2021) (Fig. 1-2). Due to the blockage of chlorophyll synthesis, plant leaves will become yellow, affecting the efficiency of photosynthesis, the growth of the plant root system will be restricted, and both root length and biomass may be reduced (Liu et al., 2020; Qi et al., 2023). At the same time, excessive application of nitrogen fertilizer may lead to overgrowth, delayed maturity, susceptibility to pests and diseases and collapse, affecting crop quality and yield (Chen et al., 2021).

Figure 1-2: Rice field showing symptom of nitrogen deficiency.

2.1 Rhizosphere microbes and nitrogen cycling

Rhizosphere microbials have a significant impact on plant growth, directly interact with plant roots and are involved in various processes related to plant growth and health. The activities of rhizosphere microbes help improve the physical structure of the soil, increasing its aeration and water retention. Rhizosphere microbes enhance soil fertility by decomposing organic matter and releasing nutrients (Oluwaseyi et al., 2022; Solomon et al., 2024; Wille et al., 2019).

Nitrogen-fixing bacteria play a vital role in the nitrogen cycle. They provide an essential source of nitrogen for plants and other organisms by converting atmospheric nitrogen (N_2) into ammonia (NH_3). This process is known as biological nitrogen fixation and is one of the key aspects of the nitrogen cycle in nature (Dixon and Kahn, 2004). The nitrogen cycle consists of several steps: atmospheric nitrogen is converted to ammonia through nitrogen fixation, and then ammonia is taken up by plants and synthesized into organic nitrogen, which then becomes a link in the food chain. Next, organic nitrogen is decomposed during ammonification to release ammonia, which is then converted to nitrate by nitrification and finally reduced to nitrogen in denitrification and returned to the atmosphere (Stein and Klotz, 2016).

Nitrogen-fixing bacteria are divided into two main groups: symbiotic and autotrophic nitrogen-fixing bacteria. Symbiotic nitrogen-fixing bacteria, such as rhizobacteria, live in symbiosis with plants, especially legumes, where they convert atmospheric nitrogen into ammonia that can be directly utilized by plants in their rhizomes. Autotrophic nitrogen-fixing bacteria, such as round brown nitrogen-fixing bacteria, are independent of plants and live freely in the soil, where they can also fix nitrogen (Aasfar et al., 2021; Xu and Wang, 2023).

Nitrogen fixation by nitrogen-fixing bacteria is particularly important for agricultural production, as they reduce dependence on chemical nitrogen fertilizers and help to improve soil fertility and crop yields. However, nitrogen-fixing bacteria

are sensitive to environmental conditions such as soil pH, moisture, and temperature, all of which affect nitrogen fixation efficiency and in ecosystems. The activities of nitrogen-fixing bacteria interact with other nitrogen transformation processes such as nitrification, denitrification and anaerobic ammonia oxidation to maintain the balance of the nitrogen cycle (Takai, 2019). These microbial interactions constitute a complex network of nitrogen transformations that have a significant impact on global biogeochemical cycles.

2.2 Rhizosphere microbes and phages

Phages are specialized parasites of bacteria and play an important role in regulating the structure and function of soil microbial communities (Chevallereau et al., 2022). The effects of phages can be understood in both direct and indirect ways. On the one hand, phages directly affect bacteria communities through the lysis phase of their life cycle, leading to death of host bacteria. Phages can act as mediators of gene transfer, delivering genes through transduction in bacterial communities. Phage infection can alter the metabolic pathways of host bacteria, affecting their role and function (Bearson and Brunelle, 2015; Egido et al., 2023). On the other hand, the predatory behavior of phages can alter the structure of bacterial communities and promote the growth of other bacteria, then changing the diversity and stability of the community (Fernández et al., 2018). Phage pressure can promote the selection and spread of bacterial resistance genes, including resistance to antibiotics, and alter bacterial interactions, such as by lysing some bacteria and reducing their competitive pressure on others, or by carrying specific genes that regulate communication and behavior among bacteria (Pfeifer et al., 2022). By affecting the distribution and abundance of bacteria, phages indirectly change the microbial ecological niche in the environment, thereby affecting the overall function of the community (Alseth et al., 2014). In addition, phages can regulate bacterial behavior by affecting sensing systems, such as biofilm formation and virulence expression (Bisht et al., 2021). These indirect effects indicate the complex role of phages in microbial communities, where they are not only predators but also important regulators of microbial community dynamics and functions.

2.3 Plant and phages

The effects of soil phages on plant growth are multifaceted, and they play an important role in plant health and soil ecosystems (Liang et al., 2024). By lysing host bacteria, phages can regulate the abundance of bacterial populations in the soil and drive changes in bacterial community diversity and composition, which in turn affects ecosystem functioning (Braga et al., 2020). Phage therapy reduces the spread of soil-borne diseases and promotes crop growth by lysing host pathogens (Erdrich et al., 2024). For example, it has been found that the occurrence of soil-borne greening blight is closely related to the composition of crop rhizosphere phage communities and the characteristics of phage-host bacteria interactions (Yang et al., 2023). Phage therapy has the potential to biocontrol plant-associated diseases, and soil microbial communities can be manipulated through phage-targeted soil fertilization to increase phage abundance, infectivity, and survival in different soil environments, thereby enhancing soil health and sustainability (Farooq et al., 2022). Phages can reduce soil

organic carbon mineralization by killing soil bacteria, modulate bacterial population size, and thus influence soil organic matter mineralization (Wei et al., 2021). However, phages may target and infect bacteria that are beneficial to plant growth, such as nitrogen-fixing bacteria, indirectly affecting nitrogen-fixing capacity and adversely affecting plant health (Wang et al., 2022).

3. Application of High-Throughput Sequencing

High-Throughput Sequencing (HTS), also known as Next-Generation Sequencing (NGS), is a technology that can sequence and quantitatively analyze hundreds of thousands to millions DNA or RNA molecules at one time, enabling whole-genome sequencing, metagenomic sequencing, transcriptome sequencing, 16S rRNA sequencing and epigenome detection (Levy and Myers, 2016).

3.1 Transcriptome sequencing

Transcriptome sequencing (RNA-Seq) is a method of sequencing cDNA libraries generated by reverse transcription of total RNA in tissue or cell using HTS. The expression of different RNAs is calculated by counting the number of relevant reads, and new transcripts are discovered (Wang et al., 2009).

In the study of plant virus resistance, RNA-Seq is widely used to understand the gene expression changes in plants after virus infection, reveal how plants respond to virus infection by regulating gene expression, and identify key genes and signaling pathways involved in antiviral responses in plants, thereby revealing the molecular mechanisms of plant antiviral resistance (Anjanappa et al., 2018; Tan et al., 2022). RNA-Seq provides a powerful tool for the study of plant virus resistance and provides a theoretical basis and genetic resources for cultivating new disease-resistant varieties.

3.2 Metagenomic sequencing

Metagenome refers to the sum of the genetic material of all microbes (including viruses) in a specific environment, including culturable microbes and those that cannot currently be obtained through traditional culture methods. Metagenomic sequencing analyzes the genomes of all microbes in a specific environment without prior culturing (Wooley and Ye, 2009).

Metagenomic sequencing provides insights into the important microbial relationship between soil and plant growth. Beneficial rhizosphere microbes that can improve crop growth and enhance crop resistance to environmental stress, and potential pathogenic bacteria or fungi are detected. By analyzing soil samples, microbial communities that have a critical impact on soil ecosystem are identified, and their roles in nutrient cycling and disease resistance are explored (Mahmud et al., 2021). Soil viruses play a key role in soil ecosystems by infecting soil microbes (especially bacteria and archaea). Metagenomic sequencing is an effective tool to understand the diversity and abundance of viruses, as well as the roles of soil viruses, including the impact on the structure, function and dynamics changes of microbial community (Graham et al., 2024; Ma et al., 2024).

3.3 16S rRNA sequencing

16S rRNA (16S ribosomal RNA) is a component of the 30S small subunit of the ribosome of prokaryotes (bacteria and archaea). 16S rRNA sequencing is a technique that uses differences in the 16S rRNA gene sequences of bacteria and archaea to classify and identify them (Woo et al., 2011).

16S rRNA sequencing has become an important tool for studying the composition and distribution of microbial communities. In the study of soil nitrogen cycling, the abundance of soil bacteria involved in the nitrogen cycle, including nitrogen fixation, nitrification, anaerobic ammonia oxidation and denitrification, was investigated by 16S rRNA sequencing to evaluate the stability of the soil nitrogen cycle function. A significant change in microbial community structure or a decrease in the abundance and activity of key microorganisms involved in the nitrogen cycle may indicate that the soil nitrogen cycle function is affected (Zheng et al., 2019). In addition, 16S rRNA sequencing can monitor changes in the structure of bacterial communities before and after phage infection. When phages infect and lyse bacteria in a community, the composition and diversity of the bacterial community changes (Braga et al., 2020). 16S rRNA sequencing is often combined with metagenomic sequencing to study phages. Metagenomic sequencing can obtain genomic information of all microbes in a sample, including phage genomes, while 16S rRNA sequencing focuses on bacterial composition. By combining these two technologies, a more comprehensive understanding of the role of phages in microbial ecosystems can be obtained. When studying soil microbial communities, metagenomic sequencing can help identify phage gene sequences in the soil, such as phage-encoded virulence genes, lysozyme genes, etc. At the same time, 16S rRNA sequencing can determine the bacterial host species infected by the phage, thereby constructing a complete network of phage-bacteria interactions (Huang et al., 2021).

Chapter 2

Objectives and thesis structure

Rice is the staple food for more than half of the world's population. It provides 21 % of the calories consumed by humans worldwide, especially in East Asia, South Asia, the Middle East, the West Indies and Latin America, and plays a decisive role in food security. However, rice stripe disease has posed a serious threat to the production of rice. Most of the diseased plants cannot head normally or produce abnormal panicles and poor fruit, resulting in severe yield reduction. Generally, the yield loss of diseased fields is 20 to 30 %, and serious fields almost cause no harvest. The cultivation of disease-resistant varieties is one of the most cost-effective measures to prevent and control this viral disease. Also, nitrogen has an important impact on rice growth, it is the basis for the formation of rice yield and quality, and also the most demanded element during rice growth. Nitrogen deficiency will lead to slow growth of rice, reduction of plant height, tillering and chlorophyll synthesis, gradual yellowing of leaves. It can also affect root growth and morphology, as well as the plant's ability to absorb water and nutrients. Bacteria play an important role in the soil nitrogen cycle and participate in multiple processes of nitrogen cycle, including nitrogen-fixation, ammonification, nitrification, and denitrification, affecting the availability and cycling of nitrogen in the soil. Phages are involved in regulating the structure and function of soil microbial communities by lysing bacteria in the soil. They can alter the species and number of bacteria in the soil, thereby affecting soil biological activity and nutrient cycling.

Therefore, the objectives of this thesis are: (1) to use RNA-Seq technology to analyze the transcriptome differences between resistant and susceptible rice varieties after RSV infection, specifically, to screen out related genes that may be involved in the disease resistance process through bioinformatics analysis (Chapter 3); (2) to isolate and identify a novel virulent phage that infects various *Enterobacter* species (nitrogen-fixing bacteria) and characterize its impact on nitrogen fixation in the soil and in plants (Chapter 4).

This document started with an introduction on the general context of viral threats on plants during growth from different realms and hosts. We first introduce the harm of plant viruses to plants and plant disease resistance, then the effects of soil phages on the community structure and function of rhizosphere microbes, the cycling and transformation of nitrogen in soil and the growth of plants (Chapter 1).

Differences at transcription level between resistant and susceptible varieties of rice after RSV infestation were analyzed by sequencing their transcriptomes. Differentially expressed genes related to transcription factors, kinases, peroxidases, phytohormone signaling, plant-pathogen interactions, and autophagy were annotated, which may play important roles in host disease resistance responses. In the transcriptome data, we screened two candidate genes, constructed knockout vectors and overexpression vectors through CRISPR/Cas9 gene editing and overexpression technology, and transformed the vectors into rice varieties Zhendao 88 and Wuyujing 3 through *Agrobacterium*-mediated transformation. T0 knockout and overexpression mutant rice plants were successfully obtained, laying the foundation for further verification of the function of candidate genes (Chapter 3).

A novel phage from the soil where rice plants showed typical symptoms of nitrogen deficiency in Nanjing, Jiangsu Province, China was isolated and identified. The phage can affect nitrogen fixation in the soil and the nitrogen content and growth of rice plants in several experiments in which the soils were amended with the novel phage. (Chapter 4).

Finally, a conclusion and perspectives for the results above are proposed (Chapter 5).

Chapter 3

Transcriptome analysis reveals different response of resistant and susceptible rice varieties to rice stripe virus infection

In this study, RNA-Seq was used to analyze the transcriptomic differences between the resistant and susceptible rice varieties at different times post RSV infection. Through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations, the differentially expressed genes (DEGs) related to disease resistance of 2 varieties at 3 time points were identified.

From Liu Y, Liu W W, Li L, Frederic F, Wang X F. Transcriptome analysis reveals different response of resistant and susceptible rice varieties to rice stripe virus infection. Journal of Integrative Agriculture, 2023, 22(6): 2–14.

Abstract: Rice stripe disease, caused by rice stripe virus (RSV) which is transmitted by small brown planthopper (SBPH, *Laodelphax striatellus* Fallen), resulted in serious losses to rice production during the last 2 decades. Research on the molecular differences between resistant and susceptible rice varieties and the interaction between rice and RSV remains inadequate. In this study, RNA-Seq was used to analyze the transcriptomic differences between the resistant and susceptible rice varieties at different times post RSV infection. Through Gene Ontology (GO) annotation, the differentially expressed genes (DEGs) related to transcription factors, peroxidases, and kinases of 2 varieties at 3 time points were identified. Comparing these 2 varieties, the DEGs associated with these 3 GOs were numerically less in the resistant variety than in the susceptible variety, but the expression showed a significant up- or down-regulation trend under the conditions of $|\log_2(\text{Fold change})|>0$ & $P_{adj}<0.05$ by significance analysis. Then through Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation, DEGs involved in some pathways that have a contribution to disease resistance including plant hormone signal transduction and plant–pathogen interaction were found. The results showed that resistance responses regulated by abscisic acid (ABA) and brassinosteroids (BR) were the same for 2 varieties, but that mediated by salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) were different. The DEGs in resistant and susceptible varieties at the 3 time points were identified in both PAMP-triggered immunity (PTI) and Effector proteintriggered immunity (ETI), with that most of the unigenes of the susceptible variety were involved in PTI, whereas most of the unigenes of the resistant variety were involved in ETI. These results revealed the different responses of resistant and susceptible varieties in the transcription level to RSV infection.

Keywords: transcriptomics, resistance, susceptibility, rice stripe virus (RSV), infection

1. Introduction

Rice stripe disease is responsible for significant yield losses in rice production in temperate and subtropical east Asian countries (Wang et al., 2008). It occurs in more than 20 provinces and cities in China (Zhang et al., 2007; Li et al., 2016). From 2000 to 2005, 1,700,000 hectares of rice fields were affected by this virus in Jiangsu Province, including 1,000,000 hectares where yield losses exceeded 50 %, and some places without any rice harvest (Zhang et al., 2007; Wang et al., 2008; Wei et al., 2009). The disease is caused by RSV, which is transmitted by SBPH in a persistent propagative manner (Qin et al., 2018; Chen et al., 2019). RSV belongs to the genus *Tenuivirus*, and its genome consists of 4 single-stranded RNA segments which can encode 7 proteins (Sun et al., 2016; Wu et al., 2018). These proteins are involved in many biological processes including virus movement, viral encapsidation, RNA silencing inhibition, viral transcription and virus transmission (Xiong et al., 2008, 2009; Zhou et al., 2012). Symptoms of rice stripe disease typically include irregular chlorosis mottlings or yellow-white stripes at the base of the heart leaves parallel to the veins. For japonica rice, the heart leaves gradually turn yellow and curl up into papertwisting drooping, while for indica rice, only irregular chlorosis mottlings appear on the leaves (Wang et al., 2015).

Plant disease resistance means the ability of plants to avoid, prevent or block the invasion and expansion of pathogens (Savary et al., 2019). During long-term coevolution, disease resistance is the result of mutual adaptation and selection between plant and pathogen (Chisholm et al., 2006). The purpose of investigating plant disease resistance is to understand the regularity of disease resistance and how to utilize the resistance to protect plants (Jones et al., 2006; Peng et al., 2020).

Plant disease resistance mainly depends on the signal pathway network of innate immunity and the resistance gene (*R* gene) (He et al., 2007). Plant defense systems include basic defense systems and specific defense systems. Basic defense systems detect pathogens invading plants and provide immunity when infection occurs; specific defense mechanisms limit pathogen growth and disease progression through hypersensitive response (HR) resulting from programmed cell death around the site of infection (Jones et al., 2006; Yin et al., 2022). In addition, many biological processes and cellular compounds are involved in the immunity process of plants in the interaction between plant and pathogen (Bailey-Serres and Mittler 2006; Bari and Jones 2009; Tao et al., 2009; Zhang et al., 2010; Derrien et al., 2012). It is reported that cytokinin and SA signal transduction enhances rice resistance to biotrophic pathogens in a synergistic way (Jiang et al., 2013). Besides, autophagy leads to programmed cell death (PCD) at the sites of TMV infection, uninfected adjacent tissue and the systemic leaves in N gene-containing plants to limit the spread of pathogens (Liu et al., 2005). Moreover, Os*WRKY45-2* (a kind of transcription factor) shows resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resulting in the increasing accumulation of jasmonic acid (JA) (Tao et al., 2009). Previous research found that plants produced reactive oxygen species (ROS) to limit the invasion of pathogens by strengthening cell walls and cellulose deposition to enhance the innate immune response (Tripathy and Oelmüller 2012). Kinases form the largest gene family of receptors in plants and play an important role in recognizing pathogen-associated

molecular patterns and regulating plant immunity to pathogens (Zhang et al., 2010). However, research on the molecular differences between resistant and susceptible rice varieties and the interaction between rice and RSV remains inadequate.

As a transcriptome research method, RNA-Seq helps to provide more information about the transcription level of organisms (Mortazavi et al., 2008). Recently, RNA-Seq has been used to research the structure of protein-coding genes, obtain new protein-coding genes and quantify and compare gene expression (Wang et al., 2009; Garber et al., 2011; Rao et al., 2019). RNA-Seq also works in the study of the overall changes in host-gene expression during plant–virus interaction (Cho et al., 2015; Sun et al., 2016; Liu et al., 2020). In this study, we analyzed the transcriptome differences between resistant and susceptible rice varieties after RSV infection by RNA-Seq. We found that the DEGs associated with disease resistance, including transcription factors, peroxidases, kinases, plant hormone signal transduction and plant–pathogen interaction of the resistant variety were more significantly up- or down-regulated than that of the susceptible variety. These findings will provide valuable information for further research on the interaction between rice and RSV, and the molecular mechanisms of rice resistance to RSV infection.

Based on the transcriptome data, we screened one susceptibility gene and two autophagy related genes for knockout and overexpression mutations. Susceptibility genes are some genes that exist in the plant genome, the expression of which may make the plant more susceptible to infection by pathogens. By editing or modifying the susceptibility genes, it is possible to precisely target the susceptibility of the plant for modification, thus realizing targeted enhancement of disease resistance (Li et al., 2022a). Compared to traditional breeding methods, this precision avoids the need for many random combinations and screening processes and greatly improves breeding efficiency. Using gene editing technology, such as CRISPR-Cas9, disease susceptibility genes can be directly edited to create plants with disease resistance traits in a relatively short period of time.

2. Materials and methods

2.1. Rice seedlings inoculated with RSV

Rice seedlings of resistant variety (Zhendao 88) and susceptible variety (Wuyujing 3) were planted in plastic pots containing nutrient soil in the greenhouse. When seedlings grow at the stage of 3 leaves, both varieties were inoculated with 5 third instar viruliferous SBPHs in a plastic pot and healthy insects as the control. At 2 days post-inoculation (dpi), these insects were removed from the plants and rice seedlings continue to grow in the greenhouse. The insect vector (SBPH) was reared in a 1-L glass beaker in a climate chamber at 26°C with a photoperiod of 14 h light and 10 h dark.

2.2. RNA extraction and Illumina sequencing

Heart leaves of each rice plant were both collected from susceptible and resistant lines in RSV inoculated and mock plants at 2, 10 and 20 dpi. Then samples were immediately immersed in liquid nitrogen and stored at –80°C until RNA extraction.

We recorded phenotypes of rice seedlings at 3 time points, and typical symptoms showed at 20 dpi (Fig. 3-1). Total RNA was extracted by Trizol reagent (Invitrogen Trading, Shanghai, China) according to the protocols. Total RNA concentration in different samples was measured using Agilent 2100 Bioanalyzer (Plant RNA Nano Chip, Agilent, USA). The library construction and sequencing were done by Novogene (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). Using the structural feature that most eukaryotic mRNAs have a polyA tail, mRNA with polyA tail was enriched by Oligo (dT) magnetic beads. The obtained mRNA was randomly fragmented using divalent cations in NEB Fragmentation Buffer. Using fragmented mRNA as the template and random oligonucleotides as primer, the first strand of cDNA was synthesized in the M-MuLV reverse transcriptase system. The RNA strand was then degraded with RNase H, and the second strand of cDNA was synthesized using DNA polymerase I with dNTPs as substrates. The purified double-stranded cDNA was end repaired, A-tailed, and ligated to sequencing adapters. cDNA around 250-300 bp was selected using AMPure XP beads, PCR amplification was performed, and the PCR products were purified using AMPure XP beads, and the cDNA library was finally constructed. The basic principle of sequencing is sequencing by synthesis. Four types of fluorescently labeled dNTP, DNA polymerase, and adapter primer were added to the sequencing flow cell for amplification. When extending the complementary strand of each sequencing cluster, each fluorescently labeled dNTP added released corresponding fluorescence, and the sequencer captured the fluorescence signals and converted the light signals into sequencing peaks through computer software to obtain the sequence information of the fragment to be tested.

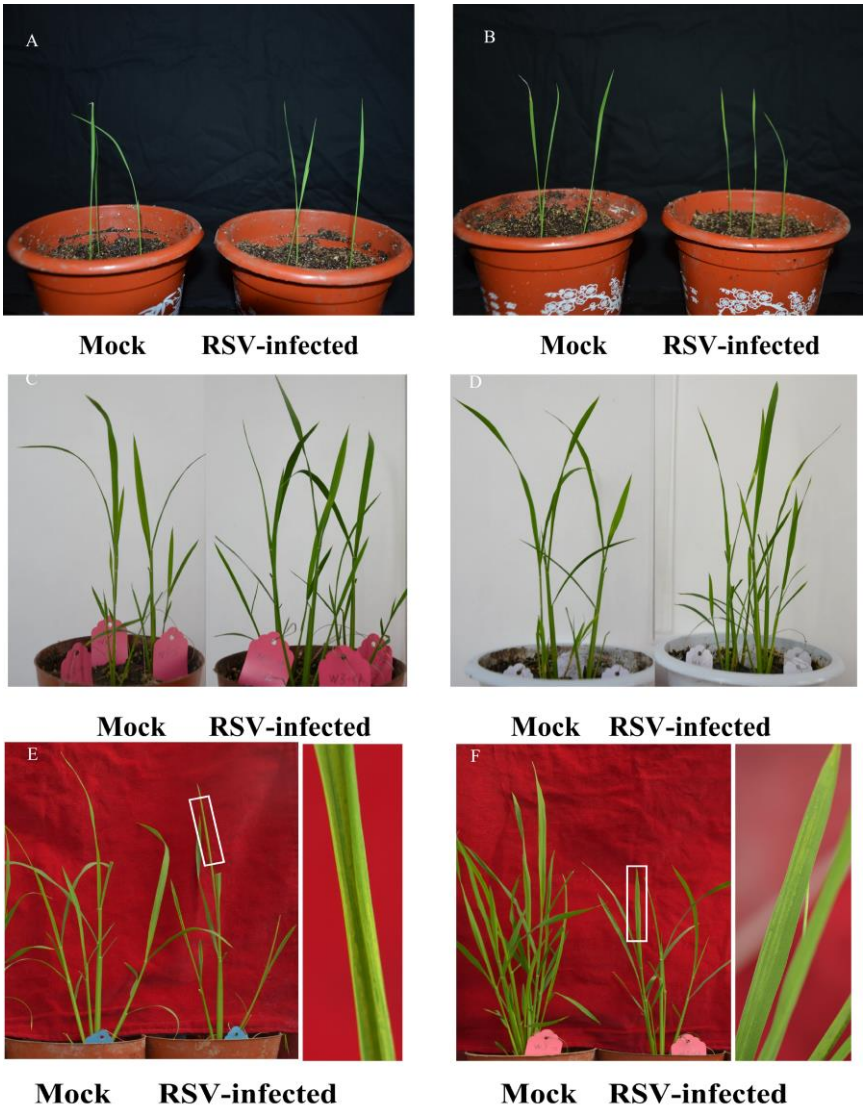


Figure 3-1: Phenotypes of rice seedlings of WYJ3 and ZD88 inoculated with rice stripe virus (RSV) at 3 time points. A, C, and E, phenotypes of WYJ3 at 2, 10, and 20 days post-inoculation (dpi), respectively. B, D, and F, phenotypes of ZD88 at 2, 10, and 20 dpi, respectively. Mock, mock-treated rice plant; RSV-infected, RSV-inoculated rice plant.

2.3. Analysis of the RNA-Seq data

Raw reads were produced via the fastQC application (Anders and Huber, 2010). Clean data (clean reads) were obtained by removing reads containing adapters, reads containing ploy-N and low-quality reads from raw data. Index of the reference genome was built and paired-end clean reads (200–300 bp) were aligned to the reference genome using HISAT2 (2.0.5) (http://ccb.jhu.edu/software/hisat2/faq.shtml). FeatureCounts (1.5.0-p3) was used to count the reads numbers mapped to each gene. And then number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and reads count mapped to this gene. The differential expression analysis between RSV-inoculated and mock samples was performed using the DESeq2 R package (1.16.1). Genes with $|\log_2(\text{Fold change})|>0$ and $P_{adj}<0.05$ were considered as DEGs in comparative analysis.

2.4. Bioinformatics analysis

For a clearer understanding of the DEGs, the GO annotation for functional analysis and KEGG for the complex biological processes were done using the clusterProfiler Software. For GO annotation, these DEGs were classified based on the molecular function, biological process, and cellular components, and GO term with corrected *P*-value < 0.05 was considered significantly enriched by DEGs. And for pathway analysis, we obtained all the pathway items that all the DEGs were involved in, and then screened the significantly enriched pathway items, taking corrected *P*-value < 0.05 as a threshold.

2.5. Validation by RT-qPCR

The transcriptional level of identified genes was detected by RT-qPCR. The reverse transcription was done by the TRUEscript 1st Strand cDNA Synthesis Kit (Aidlab, Beijing) according to the manufacturer's instructions. Then RT-qPCR was performed using the SYBR TransStart® Green qPCR SuperMix (Transgen, Beijing) with the ABI 7500 Real-Time PCR Thermal Cycler (Applied Biosystems, USA). The following cycle program: 94°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Data for the melt curve were collected at 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. UBC was set as the reference gene. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. All reactions were performed in 3 technical and biological replicates. All the primers were designed by Vector NTI (Thermo Fisher Scientific, USA). Detailed information on primers showed in Table S3-1.

2.6 Creation of knockout and overexpression mutants

To further clarify the functions of the candidate genes during RSV infection, it is necessary to obtain the corresponding mutants. The mutant vectors were constructed by CRISPR/Cas9 system and overexpression technique, the mutants were successfully created by *Agrobacterium*-mediated genetic transformation, and the positive T0 generation of transgenic rice was obtained by PCR and RT-qPCR detection, which provided good experimental materials for further research on the functions of the candidate genes in rice. Rice varieties used in this study were Wuyujing 3 and

Zhendao 88.

2.6.1 Constructs for gene overexpression and genome editing

To overexpress candidate genes in rice, the fulling-length coding regions were amplified from cDNA, and cloned into pROX vector. For rice genome editing, plasmid g4 and pBY02-OsCas9-ccdB were used. The sgRNA primers were designed using the CRISPR-GE website (http://skl.scau.edu.cn/targetdesign/), the appropriate PAM was selected according to the sequence of the gene coding region. The synthesized single-stranded primers were ligated to the enzymatically cleaved g4 vector by terminal phosphorylation and recombination to form a double-strand. The plasmid obtained was ligated to the enzymatically digested pBY02-OsCas9-ccdB vector through Gateway LR reaction to obtain the final CRISPR vector. All plasmid vectors were provided by Prof. Huanbin Zhou, Plant Genome Targeted Editing Research Group, Institute of Plant Protection, Chinese Academy of Agricultural Sciences. Sequences of PCR primers and other oligonucleotides used for construction are listed in Table S3-6.

2.6.2 Genetic transformation of rice

Recombinant plasmids were delivered into *Agrobacterium* competent cells using heat shock method. Soaking rice callus in *Agrobacterium* suspension enabled *Agrobacterium* to infect rice cells, co-cultivating for 2-3 days under dark conditions at 25-28°C. The materials after co-cultivation were transferred to selection medium containing antibiotics to screen for successfully transformed calli. Then, the resistant calli were transferred to differential medium to induce the differentiation of buds and roots under light conditions, T0 transgenic plants were obtained and transferred to soil and grown in the greenhouse.

2.6.3 Identification of T0 transgenic plants

To identify knockout mutations in the candidate genes, gene-specific primers were designed around the target site (Table 3-6). The DNA of the transgenic rice leaves was extracted by CTAB, and was used as template for PCR amplification. The PCR products were genotyped by Sanger sequencing.

Single plant sampling was performed on the overexpressed transgenic plants, and total RNA was extracted by Trizol. The reverse transcribed cDNA was used as template. Different transgenic rice varieties used the corresponding Wuyujing 3 or Zhendao 88 rice cDNA as control. The relative expression level of candidate genes was detected using specific primers (Table S3-6) through RT-qPCR, calculated using the $2^{-\Delta\Delta CT}$ method, and significance was tested by t-test.

2.6.4 RSV infection assay

All rice mutants and wild types were planted in greenhouse at 28°C with 16 h of light. When seedlings grew at the stage of three leaves, all rice plants were inoculated with 5 third instar viruliferous SBPHs per plant in plastic pots and healthy insects as the control. After 48 hours of inoculation, all SBPH were removed, and the rice seedlings continued to grow in the greenhouse. Symptoms were observed at 20 dpi, and the number of diseased plants was recorded.

3. Results

3.1 Sequencing and de novo assembly of transcriptome

To obtain a global view of the transcriptome changes of the rice plants in response to RSV infection, the expression profiles of RSV-infected rice samples (2W1, 2W2, 2W3, 10W1, 10W2, 10W3, 20W1, 20W2, 20W3, 2Z1, 2Z2, Z23, 10Z1, 10Z2, 10Z3, 20Z1, 20Z2 and 20Z3) were compared to mock-inoculated control plants (2WC1, 2WC2, 2WC3, 10WC1, 10WC2, 10WC3, 20WC1, 20WC2, 20WC3, 2ZC1, 2ZC2, 10ZC1, 10ZC2, 10ZC3, 20ZC1, 20ZC2 and 20ZC3) by high-throughput sequencing. RNA-seq yielded 41,894,242 to 58,786,946 and 43,519,758 to 68,782,166 raw reads for the RSV-infected plant group and the control group, respectively. After the low-quality reads and adapter sequences were removed, the clean reads ranged from 39,578,458 to 57,615,764 for the RSV-infected group, and 40,498,626 to 67,380,462 for the control group, respectively (Table S3-2). To show technical reproducibility among all the samples, we used hierarchical clustering analysis for gene expression and homogenized the rows of expression data. Samples with similar expression patterns are clustered together (Fig. S3-1). The number of DEGs obtained is shown in Fig. 3-2.

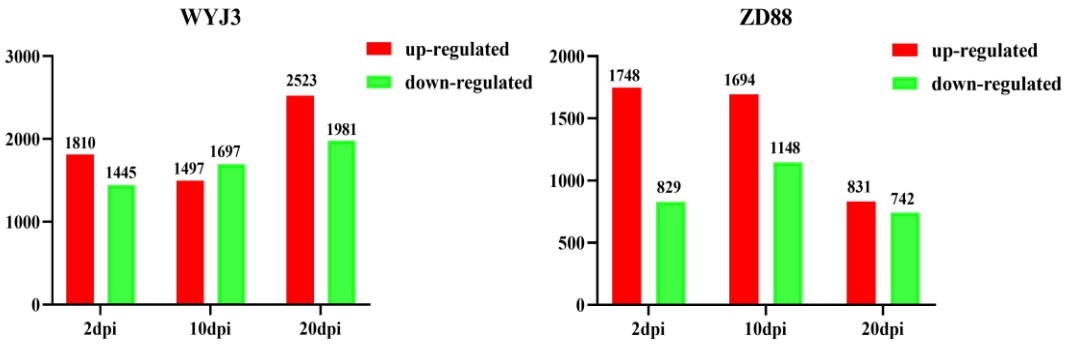
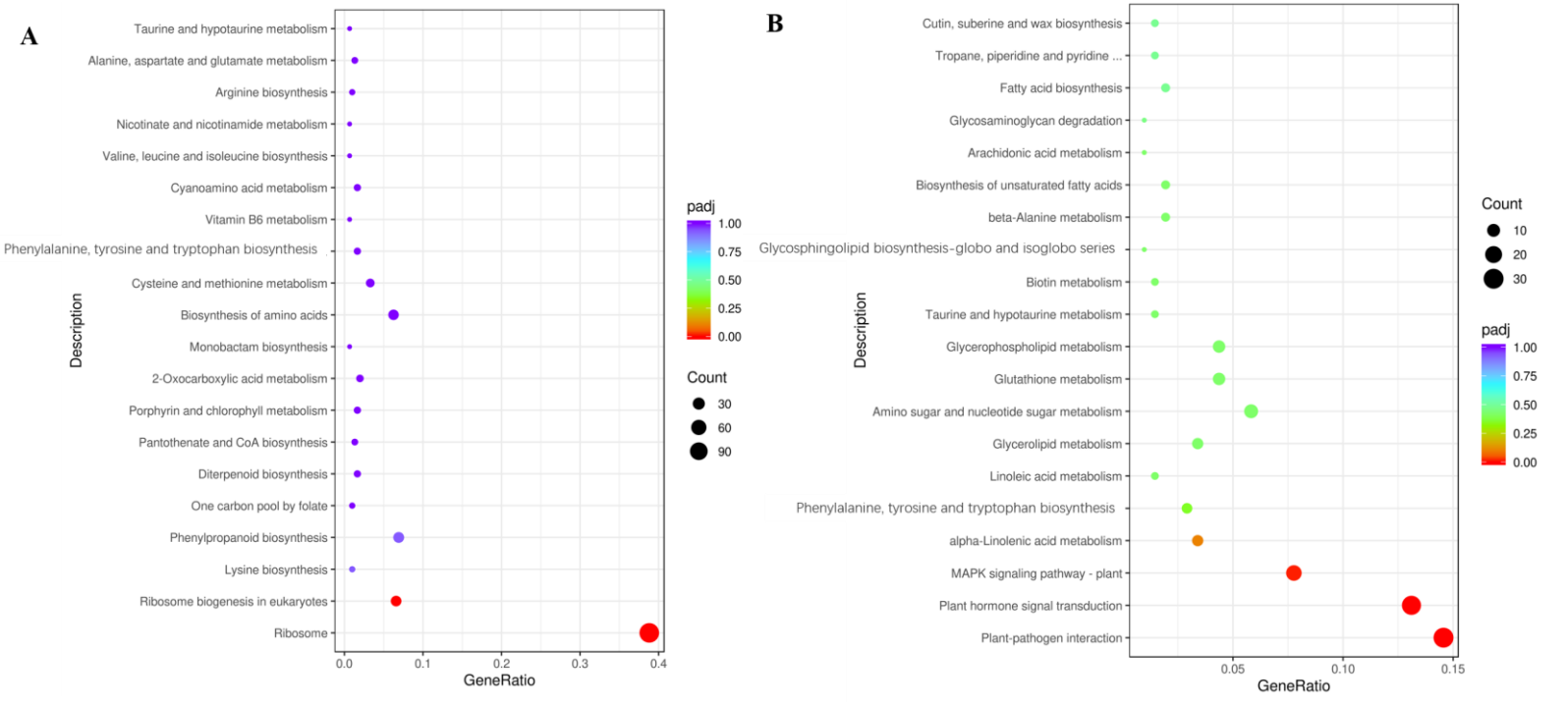


Figure 3-2: The number of differently expressed genes of WYJ3 (A) and ZD88 (B) rice varieties at 2, 10 and 20 days post-inoculation (dpi).

3.2 Metabolic pathway ' s difference between resistant and susceptible varieties

Through KEGG analysis, the pathways that the DEGs of the resistant and susceptible varieties at 3 time points involved in are shown in Figs. 3-3 and 3-4. As for the pathways that contribute to plant growth and development, including "photosynthesis", "chlorophyll biosynthesis", "carbon metabolism", "amino acid biosynthesis" and "ribosome", the DEGs of the 2 varieties were both down-regulated.


A
Taurine and hypotaurine metabolism
Alanine, aspartate and glutamate metabolism
Arginine biosynthesis
Nicotinate and nicotinamide metabolism
Valine, leucine and isoleucine biosynthesis
Cyanoamino acid metabolism
Vitamin B6 metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Cysteine and methionine metabolism
Biosynthesis of amino acids
Monobactam biosynthesis
2-Oxocarboxylic acid metabolism
Porphyrin and chlorophyll metabolism
Pantothenate and CoA biosynthesis
Diterpenoid biosynthesis
One carbon pool by folate
Phenylpropanoid biosynthesis
Lysine biosynthesis
Ribosome biogenesis in eukaryotes
Ribosome
Description
0.0
0.1
0.2
0.3
0.4
GeneRatio
padj
1.00
0.75
0.50
0.25
0.00
Count
30
60
90
B
Cutin, suberine and wax biosynthesis
Tropane, piperidine and pyridine ...
Fatty acid biosynthesis
Glycosaminoglycan degradation
Arachidonic acid metabolism
Biosynthesis of unsaturated fatty acids
beta-Alanine metabolism
Glycosphingolipid biosynthesis-globo and isoglobo series
Biotin metabolism
Taurine and hypotaurine metabolism
Glycerophospholipid metabolism
Glutathione metabolism
Amino sugar and nucleotide sugar metabolism
Glycerolipid metabolism
Linoleic acid metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
alpha-Linolenic acid metabolism
MAPK signaling pathway - plant
Plant hormone signal transduction
Plant-pathogen interaction
Description
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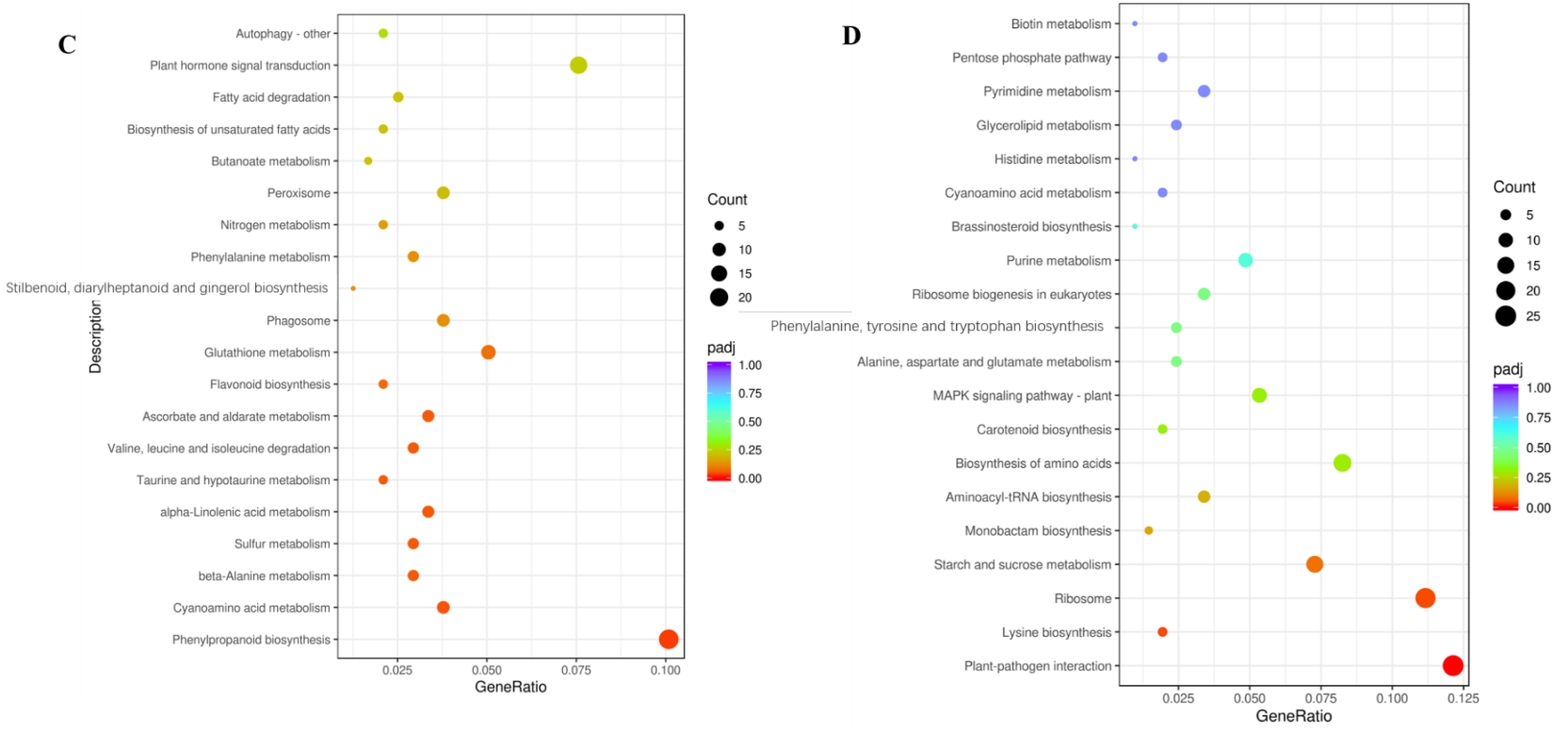
0.10
0.15
GeneRatio
Count
10
20
30
padj
1.00
0.75
0.50
0.25
0.00


C
Autophagy - other
Plant hormone signal transduction
Fatty acid degradation
Biosynthesis of unsaturated fatty acids
Butanoate metabolism
Peroxisome
Nitrogen metabolism
Phenylalanine metabolism
Stilbenoid, diarylheptanoid and gingerol biosynthesis
Phagosome
Glutathione metabolism
Flavonoid biosynthesis
Ascorbate and aldarate metabolism
Valine, leucine and isoleucine degradation
Taurine and hypotaurine metabolism
alpha-Linolenic acid metabolism
Sulfur metabolism
beta-Alanine metabolism
Cyanoamino acid metabolism
Phenylpropanoid biosynthesis
Description
0.025
0.050
0.075
0.100
GeneRatio
Count
5
10
15
20
padj
1.00
0.75
0.50
0.25
0.00
D
Biotin metabolism
Pentose phosphate pathway
Pyrimidine metabolism
Glycerolipid metabolism
Histidine metabolism
Cyanoamino acid metabolism
Brassinosteroid biosynthesis
Purine metabolism
Ribosome biogenesis in eukaryotes
Phenylalanine, tyrosine and tryptophan biosynthesis
Alanine, aspartate and glutamate metabolism
MAPK signaling pathway - plant
Carotenoid biosynthesis
Biosynthesis of amino acids
Aminoacyl-tRNA biosynthesis
Monobactam biosynthesis
Starch and sucrose metabolism
Ribosome
Lysine biosynthesis
Plant-pathogen interaction
0.025
0.050
0.075
0.100
0.125
GeneRatio
Count
5
10
15
20
25
padj
1.00
0.75
0.50
0.25
0.00

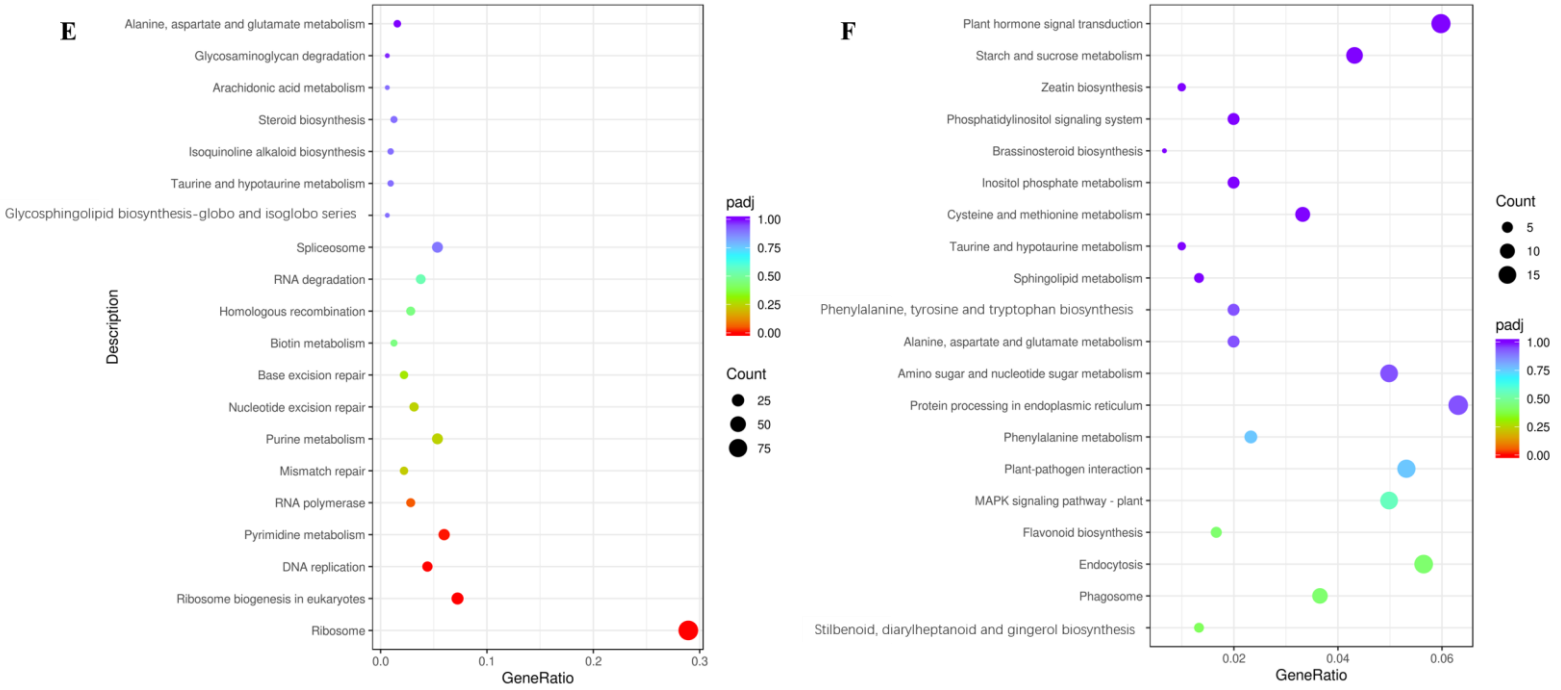
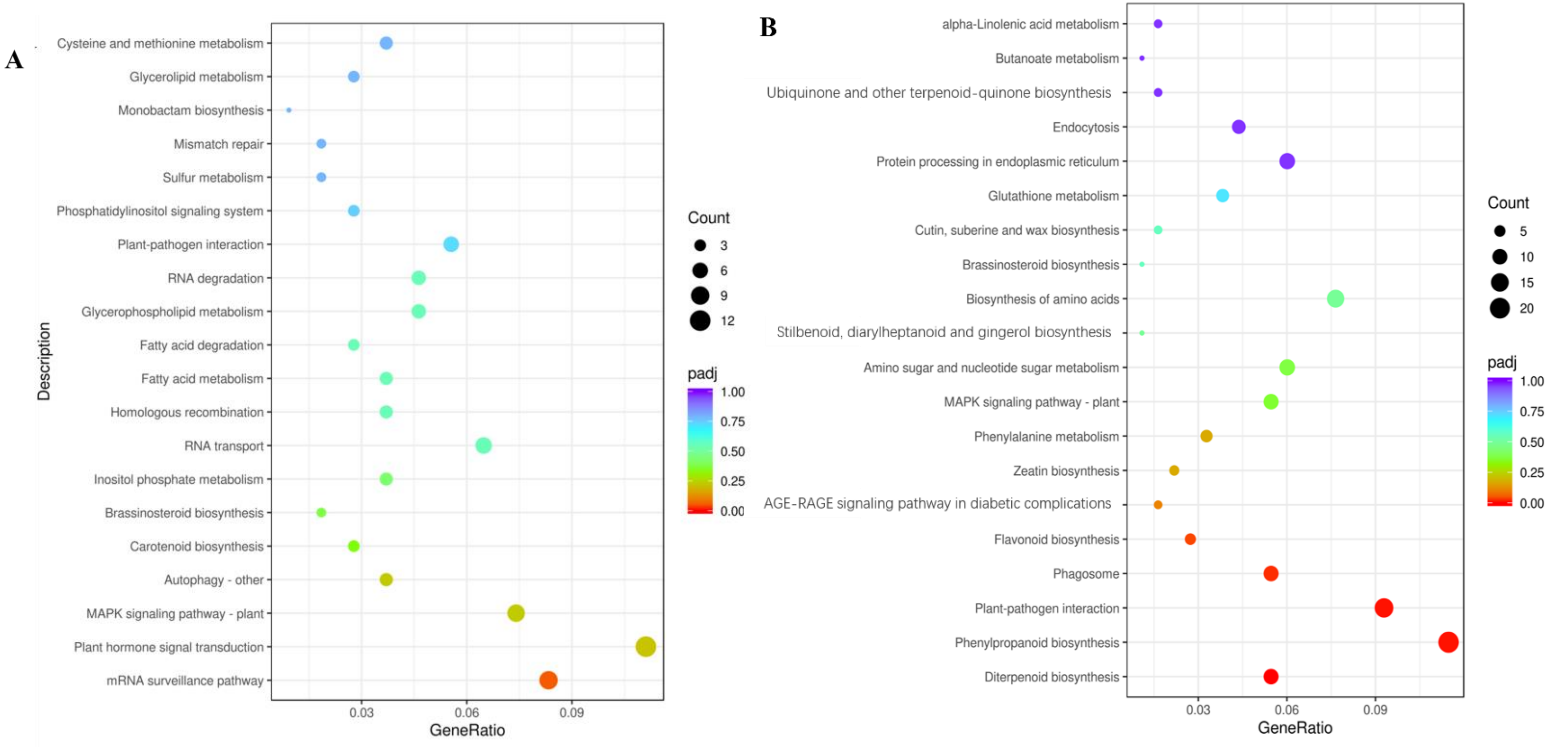
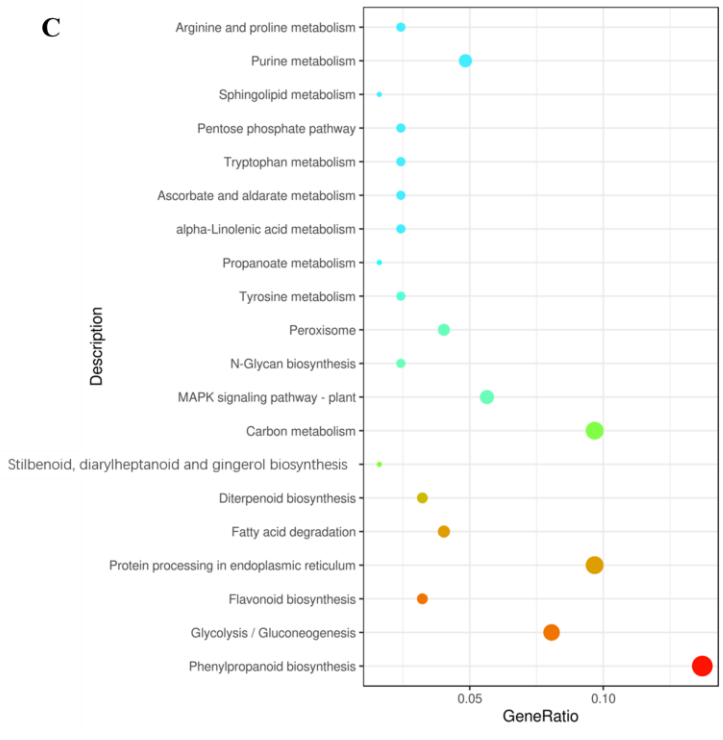


Figure 3-3: Top 20 KEGG pathways enriched of differently expressed genes (DEGs) for WYJ3. The size of each circle represents the number of DEGs and the rich factor was calculated using the number of enriched genes divided by the total number of background genes in the corresponding pathway. A, C, and E, down-regulated DEGs of WYJ3 at 2, 10, and 20 days post-inoculation (dpi). B, D, and F, up-regulated DEGs of WYJ3 at 2, 10, and 20 dpi. P-value was calculated using the Benjamini-Hochberg correction; $P<0.05$ is considered significantly enriched.


A
Cysteine and methionine metabolism
Glycerolipid metabolism
Monobactam biosynthesis
Mismatch repair
Sulfur metabolism
Phosphatidylinositol signaling system
Plant-pathogen interaction
RNA degradation
Glycerophospholipid metabolism
Fatty acid degradation
Fatty acid metabolism
Homologous recombination
RNA transport
Inositol phosphate metabolism
Brassinosteroid biosynthesis
Carotenoid biosynthesis
Autophagy - other
MAPK signaling pathway - plant
Plant hormone signal transduction
mRNA surveillance pathway
Description
0.03
0.06
0.09
GeneRatio
Count
3
6
9
12
padj
1.00
0.75
0.50
0.25
0.00
B
alpha-Linolenic acid metabolism
Butanoate metabolism
Ubiquinone and other terpenoid-quinone biosynthesis
Endocytosis
Protein processing in endoplasmic reticulum
Glutathione metabolism
Cutin, suberine and wax biosynthesis
Brassinosteroid biosynthesis
Biosynthesis of amino acids
Stilbenoid, diarylheptanoid and gingerol biosynthesis
Amino sugar and nucleotide sugar metabolism
MAPK signaling pathway - plant
Phenylalanine metabolism
Zeatin biosynthesis
AGE-RAGE signaling pathway in diabetic complications
Flavonoid biosynthesis
Phagosome
Plant-pathogen interaction
Phenylpropanoid biosynthesis
Diterpenoid biosynthesis
0.03
0.06
0.09
GeneRatio
Count
5
10
15
20
padj
1.00
0.75
0.50
0.25
0.00


C
Arginine and proline metabolism
Purine metabolism
Sphingolipid metabolism
Pentose phosphate pathway
Tryptophan metabolism
Ascorbate and aldarate metabolism
alpha-Linolenic acid metabolism
Propanoate metabolism
Tyrosine metabolism
Peroxisome
N-Glycan biosynthesis
MAPK signaling pathway - plant
Carbon metabolism
Stilbenoid, diarylheptanoid and gingerol biosynthesis
Diterpenoid biosynthesis
Fatty acid degradation
Protein processing in endoplasmic reticulum
Flavonoid biosynthesis
Glycolysis / Gluconeogenesis
Phenylpropanoid biosynthesis
Description
0.05
0.10
GeneRatio
padj
1.00
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0.50
0.25
0.00
Count
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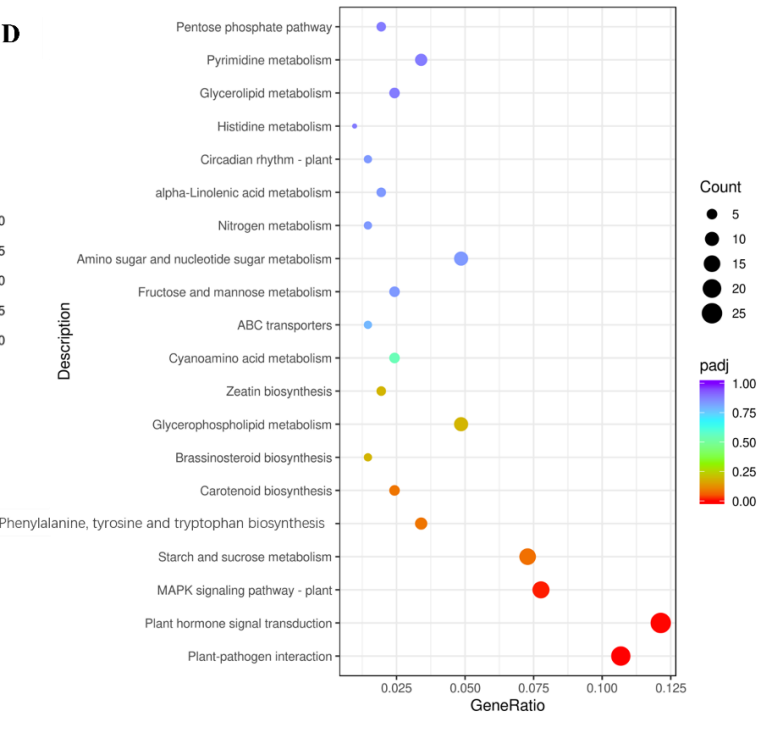
8
12
16


D
Pentose phosphate pathway
Pyrimidine metabolism
Glycerolipid metabolism
Histidine metabolism
Circadian rhythm - plant
alpha-Linolenic acid metabolism
Nitrogen metabolism
Amino sugar and nucleotide sugar metabolism
Fructose and mannose metabolism
ABC transporters
Cyanoamino acid metabolism
Zeatin biosynthesis
Glycerophospholipid metabolism
Brassinosteroid biosynthesis
Carotenoid biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Starch and sucrose metabolism
MAPK signaling pathway - plant
Plant hormone signal transduction
Plant-pathogen interaction
Description
0.025
0.050
0.075
0.100
0.125
GeneRatio
Count
5
10
15
20
25
padj
1.00
0.75
0.50
0.25
0.00

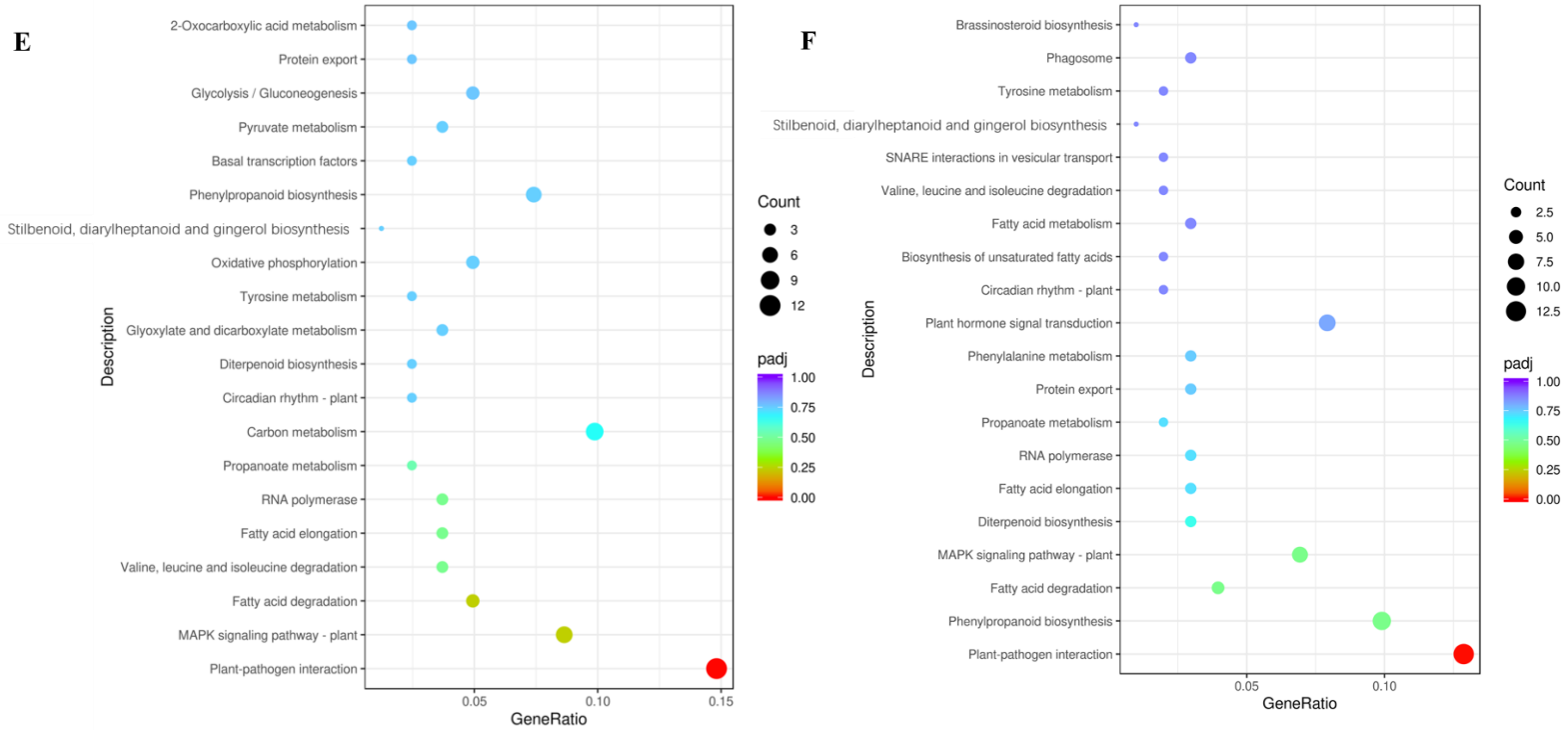


Figure 3-4: Top 20 KEGG pathways enriched of differently expressed genes (DEGs) for ZD88. The size of each circle represents the number of DEGs and the rich factor was calculated using the number of enriched genes divided by the total number of background genes in the corresponding pathway. A, C, and E, down-regulated DEGs of ZD88 at 2, 10, and 20 days post-inoculation (dpi). B, D, and F, up-regulated DEGs of ZD88 at 2, 10, and 20 dpi. P-value was calculated using the Benjamini-Hochberg correction; $P<0.05$ is considered significantly enriched.

For the “plant-pathogen interaction” pathway, which is related to plant disease resistance, the DEGs of these 2 varieties were both up-regulated at 3 time points. In our experiment, the DEGs related to PTI (PAMP-triggered immunity) and ETI (Effector-triggered immunity) of the 2 varieties at 3 time points were identified to be up-regulated. However, comparing these 2 varieties, at 2 dpi, the unique genes of the susceptible variety were all involved in PTI; but for the resistant variety, the unique DEGs were mainly related to ETI, and just one gene encoding WRKY22 was involved in PTI. At 10 dpi, for the susceptible variety, 2 genes related to WRKY33 which was involved in PTI and 3 related to HSP90 which was involved in ETI were observed; but for the resistant variety, the unique DEGs were only involved in ETI. At 20 dpi, the unique genes of the susceptible variety were all involved in PTI; while for the resistant variety, the unique DEGs were mainly related to ETI, and just one gene encoding MEKK1 was involved in PTI. As time went by, the up-regulated genes involved in this pathway were always significantly enriched for the resistant variety, but for the susceptible variety, the number of DEGs involved in this pathway gradually decreased at 3 time points, suggesting that RSV invasion may have an effect on this pathway (Fig. 3-5A).

Another pathway that is related to disease resistance is “plant hormone signal transduction”. Plant hormones play an important role in plant growth and development, including auxin (Aux), cytokinins (CK), gibberellins (GA), BR, ET, ABA, SA and JA, among them, JA, ET, BR, SA and ABA signaling pathway are related to disease resistance (Yang et al., 2013). Comparing these 2 varieties, no DEGs involved in the ABA signaling pathway were identified in the susceptible variety at 20 dpi, but down-regulated genes involved in this pathway were found at 2 dpi and 10 dpi; the DEGs involved in this pathway were identified at 3 time points for the resistant variety and showed down-regulation. The up-regulated genes involved in the BR signaling pathway were only identified in the resistant variety at 10 dpi, while the DEGs involved in this pathway were identified in the susceptible variety at 3 time points and were up-regulated. The SA-mediated resistance is antagonistic to JA/ET, the resistance response may be mediated by SA in the resistant variety, while the resistance response may be mainly mediated by JA/ET in the susceptible variety (Fig. 3-5B).

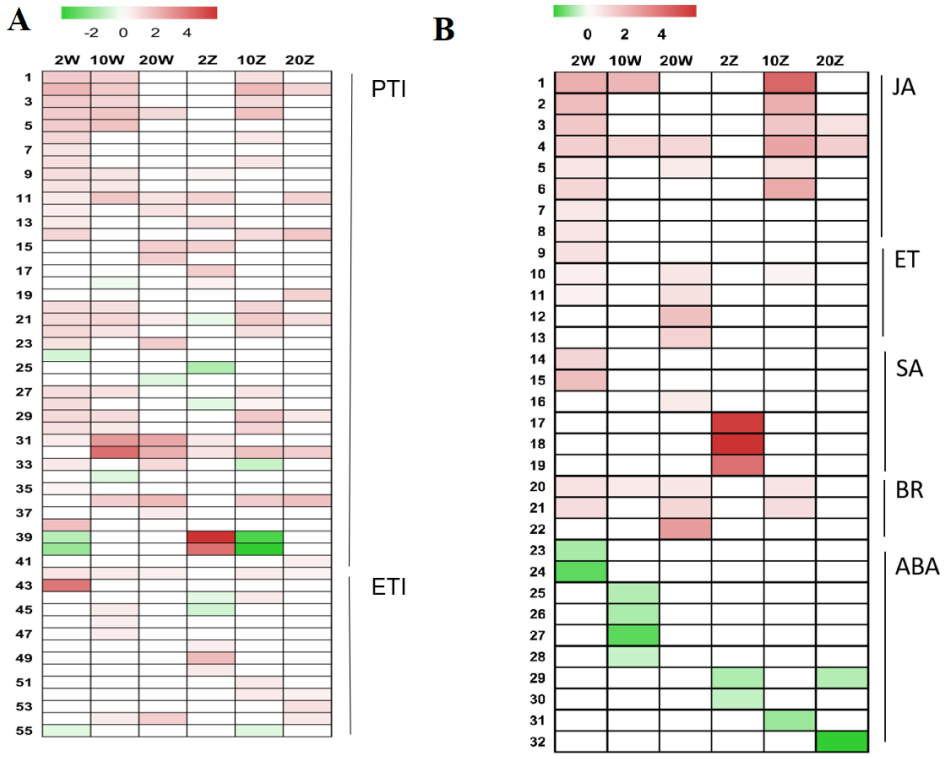


Figure 3-5: Heatmap of differential expression of genes involved in plant hormone signal transduction (A), plant–pathogen interaction (B) of two varieties (W, WYJ3; Z, ZD88) at 2, 10, and 20 days post-inoculation. Each gene involved in biotic stress pathway is depicted by color signal where red signifies the genes expressed highly and green indicates the genes down-regulated after rice stripe virus (RSV) infection. The intensity of the color is representing the level of expression. PTI, PAMP-triggered immunity; ETI, Effector protein-triggered immunity; JA, jasmonic acid; ET, ethylene; SA, salicylic acid; BR, brassinosteroids; ABA, abscisic acid.

3.3 Function annotation's difference between resistant and susceptible varieties

Through GO annotation, we described the molecular function, the cellular component and the biological process of the DEGs of the resistant and susceptible varieties at 3 time points (Figs. S3-2, 3). Comparing the 2 varieties, the DEGs annotated "ubiquitin-protein transferase activity" and "transcription factor activity" are both significantly up-regulated. We identified DEGs related to "transcription factor", "kinase" and "peroxidase", the changes of expression in different stages for resistant and susceptible varieties are shown in Fig. 3-6. Transcription factors of rice are classified into 56 families (http://planttfdb.gao-lab.org/). In this study, the DEGs belonging to17 TF families (AP2, HSF, WRKY, bZIP, HTH, EIN3, GATA, SRF, Med11, Med7, E2F, Med14, Med12, SCAI, ZF-TAC, HD-ZIP, Med22) were identified (Table S3-3). The number of DEGs related to AP2 TF and WRKY TF was the largest, and these 2 TFs are also the most studied TFs related to disease resistance (Zhao et al., 2012; Hwang et al., 2016). At 2 dpi, the DEGs related to WRKY TF were up-regulated in the resistant variety, while the up-regulated genes of the susceptible variety were mainly related to AP2 TF. At 10 dpi and 20 dpi, the DEGs belonging to AP2 TF were up-regulated in resistant and susceptible varieties; the DEGs related to WRKY TF were significantly down-regulated in the susceptible variety, and up-regulated in the resistant variety.

For the kinase of susceptible variety, 4 DEGs encoding histidine kinase, phosphotransferase, phosphate kinase and SRK5 protein were identified to be 4 up-regulated. At 10 dpi, one gene related to pyruvate kinase was down-regulated, whereas, 4 up-regulated DEGs encoding histidine kinase, phosphotransferase, phosphate kinase and protein kinase were found. Moreover, at 20 dpi, 3 DEGs including 2 protein kinase and one phosphofructokinase were retrieved to be down-regulated, while among 3 up-regulated DEGs, 2 genes related to phosphotransferase and one phosphate kinase. And for the resistant variety, at 2 dpi, 4 genes were identified to be up-regulated, including one SRK5 protein, one protein kinase, one pyruvate kinase and one phosphofructokinase. At 10 dpi, 2 down-regulated DEGs encoding phosphofructokinase and pyruvate kinase were found; however, 4 kinase-related genes (2 phosphotransferase, one phosphate kinase and one protein kinase) were down-regulated. While, at 20 dpi, 2 genes (one down-regulated and one up-regulated) encoding phosphofructokinase and phosphotransferase were identified to be differently expressed, respectively (Table S3-4). More kinase-related DEGs were found in the susceptible variety, and the downregulation trend was more significant.

In our study, for the susceptible variety, 23 significantly differently expressed responsive peroxidase genes were identified at 2 dpi, including 15 down-regulated and 8 up-regulated. At 10 dpi, 14 down-regulated and 6 up-regulated DEGs were identified after RSV infection. Nevertheless, at 20 dpi, 6 DEGs were identified to be down-regulated and 12 DEGs were up-regulated. As for the resistant variety, at 2 dpi, only 2 down-regulated and 13 up-regulated genes were observed in RSV-infected vs mock. At 10 dpi, 16 DEGs were down-regulated and 7 were up-regulated. Moreover, at 20 dpi, only one gene was down-regulated and 3 genes were up-regulated in RSV inoculated leaves (Table S3-5). Most of the POD-related DEGs in these 2 varieties

were down-regulated, but the DEGs of the susceptible variety were significantly up-regulated at the later stage, while the DEGs in the resistant variety were more significantly up-regulated at the early stage.

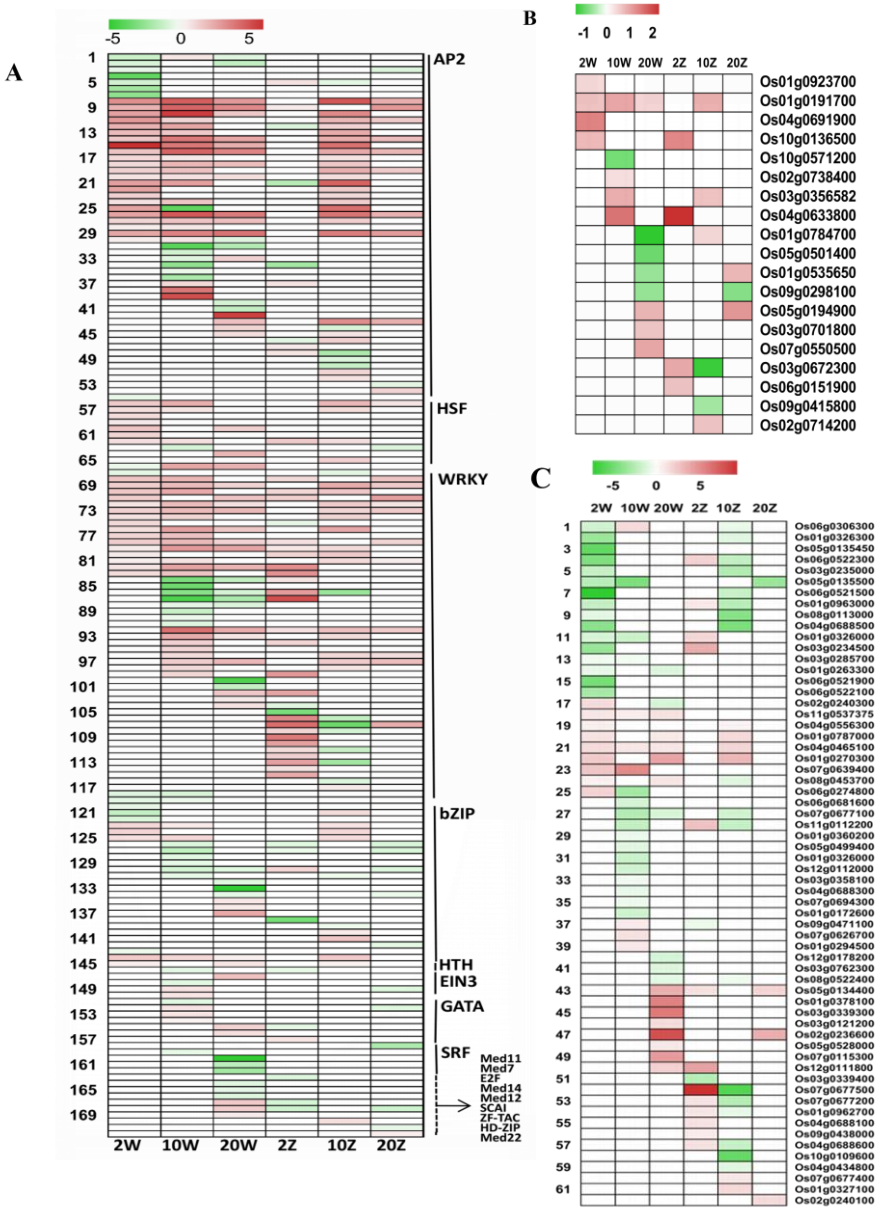


Figure 3-6: Heatmap of differential expression of genes related to transcription factor (A), kinase (B) and peroxidase (C) of two varieties (W, WYJ3; Z, ZD88) at 2, 10 and 20 days post-inoculation. Each gene involved in biotic stress pathway is depicted by color signal where red signifies the genes expressed highly and green indicates the genes down-regulated after rice stripe virus (RSV) infection. The intensity of the color is representing the level of expression.

3.4 Validation of transcriptomics data by RT-qPCR

Five genes that were differentially expressed in 2 varieties at 3 time points were assessed for their expression quantity to validate the transcriptome data, besides, UBC was selected to be the reference gene (Fig. 3-7). *Os01g0905200* encodes "Exocyst subunit Exo70 family protein", *Os08g0452500* encodes "Auxin-induced protein-related-like protein", *Os01g0622600* encodes "Calcium-dependent protein kinase 1", *Os08g0386200* encodes "Putative transcription factor WRKY5" and *Os09g0486500* encodes "Zinc finger A20 and AN1 domain-containing stress-associated protein 1". The variation trend of these selected genes was consistent with the transcriptomics data. It suggests that RNA-Seq is an accurate and reliable method to study the rice transcriptome changes after RSV infection.

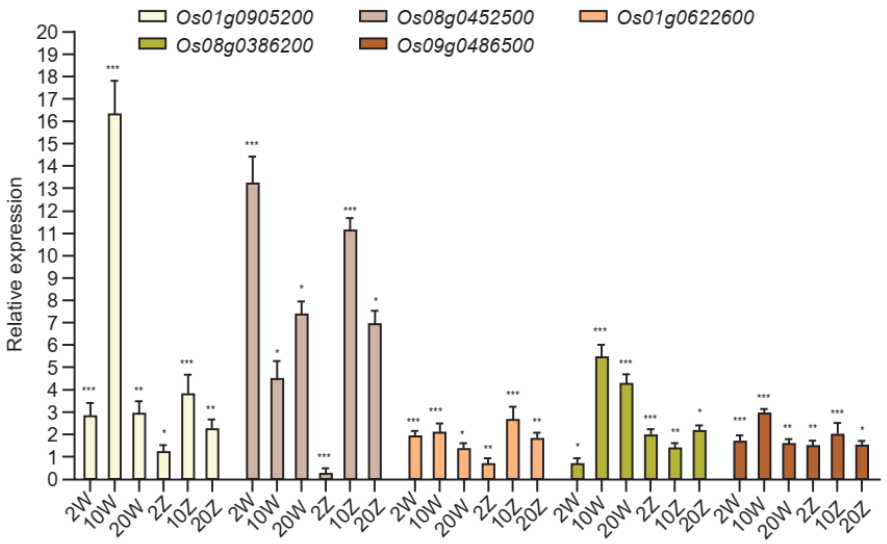


Figure 3-7: Validation of transcriptome results by RT-qPCR. Five genes that differentially expressed in 2 varieties (W, WYJ3; Z, ZD88) at 3 time points (2, 10, and 20 days post-inoculation) were selected from the RNA-Seq data for RT-qPCR. The error bars represent standard deviations of the means (n=3). Asterisks mean significant difference: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

3.5 Identification of T0 knockout mutants

Comparing the sequencing results with the wild-type sequence, seven *Os06g0267600* knockout mutant plants of Wuyujing 3 were obtained, as shown in Fig. 3-8A, including six mutant types; and one *Os06g0267600* knockout mutant plant of Zhendao 88 was obtained, as shown in Fig. 3-8B. Three *Os09g0538800* knockout mutant plants of Wuyujing 3 were obtained, as shown in Fig. 3-8C, including three mutant types; and nine *Os09g0538800* knockout mutant plant of Zhendao 88 were obtained, including seven mutant types, as shown in Fig. 3-8D.

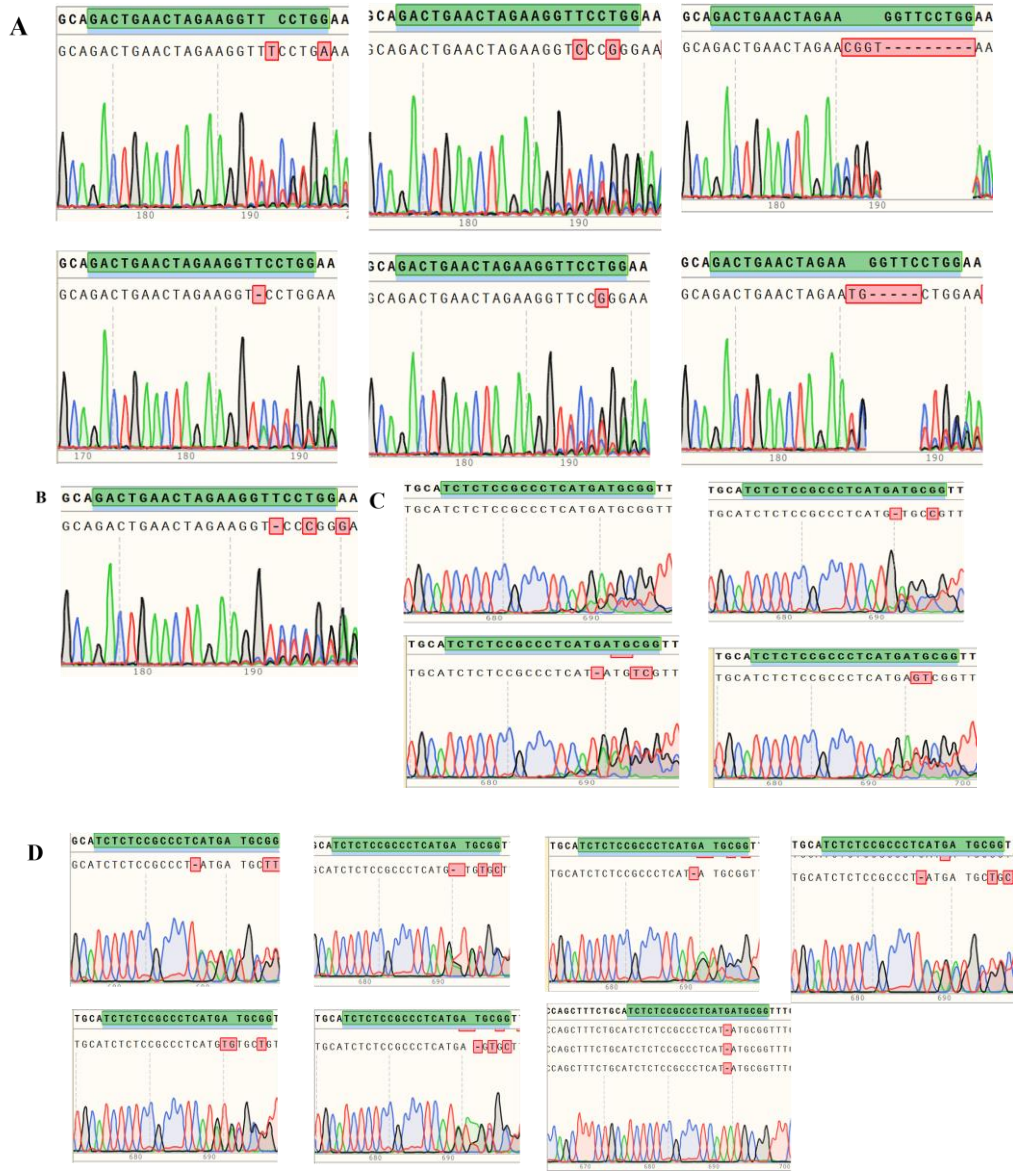


Figure 3-8: Mutation type of T0 generation knockout transgenic rice plants. A, WYJ3-pBY02-A2; B, ZD88-pBY02-A2; C, WYJ3-pBY02-*OS09G0538800*; D, ZD88-pBY02-*OS09G0538800*.

3.6 Identification of T0 overexpression mutants

The relative expression levels of target genes in overexpressed materials were detected by RT-qPCR, as shown in Figure 3-9. Compared with the wild-type rice Wuyujing 3, the relative expression of *Os06g0267600* was significantly increased by 4-40 fold (Fig. 3-9A) and *Os09g0538800* by 75-330 fold (Fig. 3-9B); compared with the wild-type rice Zhendao 88, the relative expression of *Os06g0267600* was significantly increased by 10-32 fold (Fig. 3-9C), and the relative expression of *Os09g0538800* was significantly increased by 35-140 fold (Fig. 3-9D).

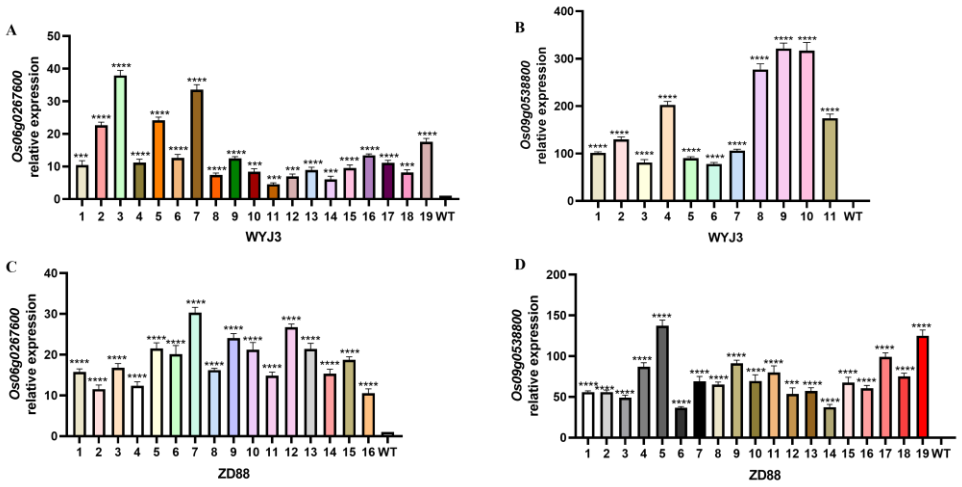


Figure 3-9: Relative expression level of in T0 generation overexpressed transgenic rice plants. A, WYJ3-pROX-*Os06g0267600*; B, WYJ3-pROX-*OS09G0538800*; C, ZD88-pROX-*Os06g0267600*; D, ZD88-pROX-*OS09G0538800*. The error bars represent standard deviations of the means (n=3). Asterisks mean significant difference: ***, $P<0.001$; ****, $P<0.0001$.

3.7 Statistics on the incidence of mutants after RSV infection

The number of diseased plants was recorded at 20 dpi. In Table 3-1, the knockout and overexpression mutants of the *Os06g0267600* showed no significant difference in the incidence of the disease compared with the wild types. For the *Os09g0538800*, the knockout mutant showed reduced incidence compared with the wild-type susceptible variety Wuyujing 3, and the overexpression mutant showed increased incidence compared with the wild-type resistant variety Zhendao 88. This result suggests that *Os09g0538800* may be a susceptibility gene, which needs to be verified by subsequent molecular biology experiments.

Table 3-1: Incidence Rate Statistics

	Total	Diseased plants	Incidence Rate
WYJ3-WT	34	21	61.8%
ZD88-WT	45	2	4.4%
OS06G0267600-WYJ3-CRISPR	45	26	57.8%
OS06G0267600-WYJ3-OE	54	28	51.9%
OS06G0267600-ZD88-CRISPR	46	3	6.5%
OS06G0267600-ZD88-OE	44	2	4.5%
OS09G0538800-WYJ3-CRISPR	43	8	18.6%
OS09G0538800-WYJ3-OE	36	23	63.9%
OS09G0538800-ZD88-CRISPR	47	2	4.3%
OS09G0538800-ZD88-OE	40	6	15.0%

4.Discussion

In nature, plants are attacked by different pathogens, as a result, various metabolic processes are interfered with to varying degrees. Accordingly, plants have evolved kinds of physiological, cellular and molecular mechanisms to cope with these pathogens (Jones et al., 2006).

4.1 Transcription factors (TFs)

Plant immune receptors activate several genes in response to biotic stresses and coordinate their stress response with growth to maximize their fitness. Transcription factors interact with cis-regulatory elements in the promoter regions of stress-related genes and up-regulate the expression of many genes to activate biotic stress tolerance (Agarwal et al., 2010). The AP2/ERF TFs are plant-specific and play a crucial role throughout the plant life cycle, such as involving in nutrition signaling pathways and response to various biotic and abiotic stresses (Zhang et al., 2020). Under different biotic stress conditions, AP2/ERF TFs can activate the defense-related genes, namely PR (pathogenesis-related proteins), osmolyte, β-1,3-glucanase and chitinase response genes in *Arabidopsis* (Moffat et al., 2012; Zarei et al., 2011). WRKY TFs play a very important role in regulating the expression of plant defense-related genes. *Arabidopsis Thaliana* gene RRS1, which is resistant to *Ralstonia solanacearum*, has a typical *R* protein structural domain of TIR-NBS-LRR at the N-terminal and a typical WRKY52

protein at the C-terminal; the R protein recognizes the avirulence (*Avr*) protein signal and subsequently activates the expression of defense-related genes through the functional domain of WRKY TFs (Deslande et al., 2003). In addition, the expression product of the resistance gene TIZZ, induced by TMV, also has a WRKY structural domain and a C2H2 zinc finger structural domain (Yoda et al., 2002). A previous study found that a group I WRKY transcription factor, NbWRKY1, can regulate mulberry mosaic dwarf-associated virus (MMDaV) -triggered cell death in *Nicotiana benthamiana* to improve plant defense (Sun et al., 2022). In the present study, AP2/ERF TF-related genes were differentially expressed in both varieties, and most of them were up-regulated. In terms of expression quantity, for ZD88, AP2/E2F TF-related DEGs were mostly down-regulated at the early stage, and up-regulated at the middle and late stages; while for WYJ3, the DEGs were mostly significantly up-regulated expressed at the 3 stages, indicating that AP2/ERF TFs may have regulated PR response genes after RSV infection in both varieties. Unlike AP2/ERF TFs, WRKY TFs were significantly more up-regulated in ZD88 than in WYJ3, suggesting that WRKY TFs have more influence on the expression of disease-resistance-related genes in resistant variety.

4.2 Peroxidase

Peroxidase is one of the key enzymes in the plant defense system under adverse conditions, scavenging excess free radicals and maintaining them at a normal level inside the plants, thus interacting synergistically with superoxide dismutase (SOD) and catalase to improve the stress tolerance of plants. It has been shown that several species of plants infected with citrus exocortis viroid (CEV) induce the accumulation or expression of several peroxidases at the transcriptional level (Gadea et al., 1996). Peroxidase mediates ROS production in the plant (Khokon et al., 2010), and previous research reported that downregulation of the light-harvesting chlorophyll a/b complex protein 3 (LHCB3) of photosystem II is involved in defense against turnip mosaic virus (TuMV) by inducing ROS production in *Nicotiana benthamiana* (Qiu et al., 2021). Peroxidase is required for lignin biosynthesis (Liu et al., 2018a), and increasing peroxidase activity can promote lignification of the infected tissues and play a role in limiting the movement of the virus in the plant. In our study, peroxidase-related DEGs were mostly down-regulated, and in WYJ3, the DEGs showed significantly up-regulated expression in the late stage, indicating that peroxidase-related genes may regulate the lignin biosynthesis process and promote lignification in susceptible variety, thus playing a defensive role.

4.3 Plant-pathogen interaction

The plant's innate immune system consists of 2 main immune responses, namely, PTI and ETI. In our experiment, analysis of transcriptome data revealed that both varieties triggered PTI after RSV invasion, in which kinase and related signal-mediated genes acted as pattern recognition receptors to activate the plant defense system (Abramovitch et al., 2006). In terms of expression quantity, the kinase-related DEGs identified in ZD88 showed significant up- or down-regulation, indicating that resistant variety activates the defense system more actively. Moreover, several DEGs involved in ETI were identified in ZD88 at all 3 time points, and relatively few in

WYJ3, suggesting that the susceptible variety lacks ETI and shows susceptibility.

4.4 Plant hormone signal transduction

Plant hormones play a critical role in almost every aspect of plant biological processes, including growth, development and pathogen defense. Disruption of normal development physiology is usually associated with changes in phytohormone accumulation and signaling during viral infection. As mentioned previously, our study analyzed DEGs related to SA, JA, ET, BR and ABA signaling. SA and JA/ET respond to adversity in an antagonistic manner (Koornneef et al., 2008). SA pathway mainly targets biotrophic pathogens, while responses induced by necrotrophic pathogens are synergistically regulated by the JA and ET pathways. SA is required for systemic acquired resistance (SAR) (Gaffney et al., 1993). Ding et al (2018) reported that SA may play an important role in the amplification of PTI and ETI responses through the induction of the expression of related genes. BR is involved in the regulation of innate immunity based on BAK1-dependent and BAK1-independent defense responses. On the other hand, ABA is usually associated with tolerance of abiotic stress and is a negative regulator of biotic stress responses. In our study, DEGs of the resistant variety were mainly involved in SA-mediated resistance response, while DEGs of the susceptible variety were mainly involved in JA/ET synergistic resistance; DEGs associated with BR-mediated signaling pathways showed up-regulation in both varieties, and DEGs associated with ABA signaling pathways showed down-regulation in both varieties, indicating that there are similarities and differences in effects of hormones on the resistance response of both varieties.

4.5 Breeding disease-resistant varieties with susceptibility genes

Susceptibility genes refer to plant genes which make plants more susceptible to pathogen infection. Loss-of-function mutations in susceptibility genes can lead to the acquisition of broad-spectrum disease resistance in plants (Li et al., 2022a). Susceptibility genes are also important genetic resources, through in-depth study and rational utilization, we can better explore the plant's own disease resistance potential and enrich the database of disease resistance gene resources (Li et al., 2022a). Compared with traditional breeding methods, the manipulation of susceptibility genes through technologies such as gene editing can achieve precise and targeted improvement of plant disease resistance. Known susceptibility genes can be directly knocked out, avoiding a large amount of hybridization and screening work in traditional breeding, and greatly improving breeding efficiency and accuracy. And gene editing technology can accurately edit susceptibility genes without affecting other desirable agronomic traits of plants (Manzoor et al., 2024). In summary, we can effectively utilize susceptibility genes to enhance crop disease resistance while maintaining or increasing yields, which is important for global food security and sustainable development.

5. Conclusion

In this study, RNA-Seq was used to analyze the transcriptomic differences between the resistant and susceptible rice varieties at different times after RSV infection.

Through GO annotation, the DEGs related to transcription factors, peroxidases, and kinases of 2 varieties at 3 time points were identified. Comparing these 2 varieties, the DEGs associated with these 3 proteins were numerically less in the resistant variety than in the susceptible variety, but the expression showed a significant up- or down-regulation trend by significance analysis. Then through KEGG annotation, DEGs involved in some pathways that have a contribution to disease resistance including plant hormone signal transduction and plant-pathogen interaction were found. The results showed that resistance responses regulated by ABA and BR were the same for the 2 varieties, but that mediated by SA and JA/ ET were different. The DEGs in resistant and susceptible varieties at the 3 time points were identified in both PTI and ETI, but most of the unigenes of the susceptible variety were involved in PTI, whereas most of the unigenes of the resistant variety were involved in ETI. These results revealed the different responses of resistant and susceptible varieties in the transcription level to RSV infection. Our study provides valuable and novel information to obtain putative resistance-related genes by bioinformatics analysis. In the transcriptome data's, we screened two candidate genes, constructed knockout vectors and overexpression vectors through CRISPR/Cas9 gene editing and overexpression technology, and transformed the vectors into rice varieties Zhendao 88 and Wuyujing 3 through *Agrobacterium*-mediated transformation. T0 knockout and overexpression mutant rice plants were successfully obtained, laying the foundation for further verification of the function of candidate genes.

In addition to RSV affecting the growth of rice plants in the above-ground part, the growth of plants is also influenced by viruses from soil. In the next section, we elaborate on how the phage affects the nitrogen-fixing efficiency of nitrogen-fixing bacteria, thereby influencing rice growth.

Acknowledgements

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Supplementary Materials

Table S3-1: Primers for RT-qPCR

Primer name	Primer sequence (5′-3′)	Remark
UBC-F	CCGTTTGTAGAGCCATAATTGCA	Ubiquitin-conjugating enzyme E2
UBC-R	AGGTTGCCTGAGTCACAGTTAAGTG	
Os01g0905200-F	GCTCATTTACATCCTTGGCATG	Exocyst subunit Exo70 family protein
Os01g0905200-R	ACCGTCTCGTACCTTCGCTAT	
Os08g0452500-F	GCAAGCTGAGGTGCATGATC	Auxin-induced protein-related-like protein
Os08g0452500-R	TCGACGAGGTTCTGGAACAG	
Os01g0622600-F	GACTTCGGTCTTTCCGTTTTC	Calcium-dependent protein kinase 1
Os01g0622600-R	AGCCTCTGCTCCATAGTTTCG	
Os08g0386200-F	GACCACCAACGCCAAGAAGA	Putative transcription factor WRKY5
Os08g0386200-R	TTCTGCCCGTACTTCCTCCA	
Os09g0486500-F	CGCGACAAGAAGGATCAGGA	Zinc finger A20 and AN1 domain-containing stress-associated protein 1
Os09g0486500-R	AAAGAAGACGGCGACGAGGT	

Table S3-2: Summary of mapping reads of the RNA-seq

Sample	Raw reads	Clean Reads	Mapped Reads	Mapping Rate (%)	GC Percent (%)	Unique Mapping	Unique Mapping Rate (%)
2W1	53,106,402	49,865,320	47,684,694	95.63	53.48	46,760,923	93.77
W2	49,498,334	45,417,764	43,083,515	94.86	53.54	42,317,414	93.17
2W3	48,715,514	45,896,346	43,282,184	94.3	51.83	42,512,372	92.63
2WC1	45,907,324	42,985,430	40,555,770	94.35	52.17	39,783,324	92.55
2WC2	44,627,062	42,649,116	40,661,318	95.34	52.36	39,856,425	93.45
2WC3	53,680,094	50,495,850	47,951,731	94.96	52.87	46,984,614	93.05
10W1	41,894,242	39,578,458	37,818,248	95.55	54.43	37,084,816	93.7
10W2	52,465,850	49,549,200	47,651,872	96.17	55.31	46,686,821	94.22
10W3	54,838,030	51,495,198	49,222,382	95.59	53.31	48,225,971	93.65
10WC1	55,802,720	52,568,468	50,652,964	96.36	55.32	49,676,156	94.5
10WC2	64,807,780	60,901,070	58,147,124	95.48	53.41	56,947,062	93.51
10WC3	47,206,392	44,396,494	42,575,308	95.9	53.58	41,682,439	93.89
20W1	42,948,436	40,419,176	38,668,957	95.67	52.95	37,822,055	93.57
20W2	43,302,670	40,328,210	38,657,280	95.86	52.22	37,224,244	92.3
20W3	51,816,448	48,500,640	45,752,457	94.33	53.02	44,810,027	92.39
20WC1	45,683,454	43,075,814	41,045,584	95.29	51.37	40,077,649	93.04
20WC2	47,385,500	44,986,236	42,552,937	94.59	51.51	41,549,760	92.36
20WC3	51,139,244	49,111,112	45,062,419	91.76	49.97	44,017,885	89.63
Z1	44,304,294	41,253,294	39,190,317	95.0	52.68	38,557,115	93.46
2Z2	47,929,282	44,902,204	42,796,610	95.31	52.28	42,059,983	93.67
2Z3	58,786,946	57,615,764	55,389,081	96.14	54.92	54,365,090	94.36
2ZC1	50,012,872	49,133,690	47,172,701	96.01	55.47	46,292,109	94.22
2ZC2	57,181,324	56,013,564	53,616,340	95.72	54.06	52,631,070	93.96
2ZC3	68,782,166	67,380,462	64,799,873	96.17	54.64	63,598,667	94.39
10Z1	46,786,756	43,446,924	41,703,324	95.99	54.01	41,037,860	94.46
10Z2	47,921,450	45,412,810	43,633,474	96.08	55.03	42,796,678	94.24
10Z3	51,225,840	48,248,680	46,354,884	96.07	55.35	45,551,061	94.41
10ZC1	44,740,220	42,174,566	40,525,853	96.09	55.33	39,760,336	94.28
10ZC2	46,039,562	43,302,132	41,503,585	95.85	54.96	40,707,189	94.01
10ZC3	53,489,768	51,111,822	49,155,464	96.17	54.83	48,205,045	94.31
20Z1	53,551,986	50,541,896	48,497,082	95.95	53.47	47,406,253	93.8

Sample	Raw reads	Clean Reads	Mapped Reads	Mapping Rate (%)	GC Percent (%)	Unique Mapping	Unique Mapping Rate (%)
20Z2	49,249,170	46,529,674	44,708,689	96.09	50.39	43,785,752	94.1
20Z3	53,736,288	50,348,402	48,072,368	95.48	52.72	47,048,640	93.45
20ZC1	43,531,072	40,498,626	38,913,693	96.09	53.32	38,027,570	93.9
20ZC2	43,519,758	40,737,856	39,160,542	96.13	53.51	38,268,896	93.94
20ZC3	51,943,718	49,171,608	47,314,826	96.22	52.82	46,277,535	94.11

Table S3-3: List of DEGs related to transcription factor

Time	Gene ID
2-w-down	Os09g0286600(AP2)/Os09g0287000(AP2)/Os01g0797600(AP2)/Os03g0341000(AP2)/Os02g0496100(HSF)/Os07g0617000(AP2)/Os10g0536100(SRF)/Os08g0386200(WRKY)/Os09g0280500(bZIP)/Os02g0203000(bZIP)/Os12g0152900(bZIP)/Os03g0322700(bZIP)/Os11g0152700(bZIP)/Os09g0572000(AP2)/Os01g0140700(AP2)/novel.900/Os08g0499300(WRKY)/Os08g0163400(HTH)
2-w-up	Os08g0474000(AP2)/Os09g0457900(AP2)/Os06g0127100(AP2)/Os05g0420300(AP2)/Os07g0685700(EIN3)/Os07g0410300/Os06g0649000(WRKY)/Os02g0806350/Os02g0764700(AP2)/Os04g0550200(AP2)/Os09g0522200(AP2)/Os04g0572200(AP2)/Os09g0522100(AP2)/Os02g0527300(HSF)/Os02g0181300(WRKY)/Os07g0644100(bZIP)/Os04g0546800(AP2)/Os05g0537100(WRKY)/Os03g0224700(HSF)/Os09g0417600(WRKY)/Os02g0654700(AP2)/Os01g0313300(AP2)/Os04g0669200(AP2)/Os02g0676800(AP2)/Os05g0343400(WRKY)/Os01g0821600(WRKY)/Os03g0188900/Os01g0750100(WRKY)/Os02g0766700(bZIP)/Os03g0758000(WRKY)/Os05g0129300(bZIP)/Os02g0781300(AP2)/Os05g0497200(AP2)/Os09g0456800(HSF)/Os02g0462800(WRKY)/Os01g0693400(AP2)/Os01g0656400(WRKY)/Os03g0161900/Os01g0968800(AP2)/Os06g0158100(WRKY)/novel.1333/Os02g0677300(AP2)/Os03g0191900(AP2)/novel.2859/Os09g0490200(EIN3)/novel.2770/Os05g0497300(AP2)/Os05g0474800(WRKY)/Os01g0749300(HSF)/Os06g0553100(HSF)/Os09g0526600(HSF)/Os05g0530400(HSF)/Os01g0186000(WRKY)/Os04g0287400(WRKY)/Os03g0335200(WRKY)/Os04g0572400(AP2)/Os05g0571200(WRKY)/Os05g0316800(AP2)/Os01g0908200/Os04g0482300
10-w-down	Os12g0116600(WRKY)/Os05g0583000(WRKY)/novel.2859/Os04g0541700(bZIP)/Os09g0379600/Os12g0116700(WRKY)/novel.2860/Os11g0117600(WRKY)/Os05g0569300(bZIP)/Os08g0481400/Os11g0168500(AP2)/Os02g0220400(GATA)/Os02g0175100(bZIP)/Os01g0542700(bZIP)/Os05g0129800(WRKY)/Os01g0626400(WRKY)/Os04g0398000(AP2)/Os10g0536100(AP2)/Os09g0280500(bZIP)/Os09g0306400(bZIP)/Os12g0507300(WRKY)/Os12g0116400(WRKY)/Os07g0227600(AP2)/Os03g0324200(EIN3)/Os12g0168100/Os03g0324300(EIN3)/Os01g0968800(AP2)/Os04g0549700(AP2)/Os01g0625300(HSF)/Os02g0203000(bZIP)/Os01g0920200/Os11g0615200
10-w-up	Os01g0826400(WRKY)/Os05g0343400(WRKY)/Os08g0386200(WRKY)/Os09g0334500(WRKY)/Os09g0457900(AP2)/Os02g0462800(WRKY)/Os06g0127100(AP2)/Os08g0474000(AP2)/Os09g0522100(AP2)/Os09g0522200(AP2)/Os05g0537100(WRKY)/Os02g0806350/Os02g0181300(WRKY)/Os05g0474800(WRKY)/Os01g0656400(WRKY)/Os01g0693400(B3)/Os05g0183100(WRKY)/Os05g0129300(bZIP)/Os01g0750100(WRKY)/Os08g0235800(WRKY)/Os02g0527300(HSF)/Os01g0821600(WRKY)/Os01g0313300(AP2)/Os07g0685700(EIN3)/Os04g0610400(AP2)/Os03g0191900(AP2)/Os01g0131600/Os06g0649000(WRKY)/Os07g0674800(AP2)/Os02g0654700(AP2)/Os04g0669200(AP2)/Os02g0677300(AP2)/novel.1333/Os06g0158100(WRKY)/Os04g0605100(WRKY)/Os04g0287400(WRKY)/Os02g0764700(AP2)/Os02g0676800(AP2)/Os01g0624700(WRKY)/novel.2770/Os03g0224700(HSF)/Os05g0497300(AP2)/Os04g0550200(AP2)/Os03g0231150/Os06g0571800(GATA)/Os05g0473300(AP2)/Os04g0572200(AP2)/Os09g0286600(AP2)/Os02g0645600(GATA)/Os05g0571200(WRKY)/Os04g0572400(AP2)/Os07g0644100(bZIP)/Os01g0584900(bZIP)/Os02g0157950/Os05g0316800(AP2)/Os04g0546800(AP2)/Os03g0335200(WRKY)/Os05g0530400(HSF)/Os04g0539500(GATA)/Os05g0420300(AP2)/Os01g0908200
20-w-down	Os02g0189100(Med11)/Os04g0661800(Med7)/Os12g0496900/Os03g0752800(SRF)/Os07g0605200(SRF)/Os12g0116600(WRKY)/novel.2859/Os11g0117600(WRKY)/Os11g0168500(AP2)/Os05g0121600(AP2)/Os09g0287000(AP2)/Os05g0129800(WRKY)/novel.2860/Os01g0658900(bZIP)/Os06g0720900(bZIP)/Os05g0316800(AP2)/Os08g0481400/Os01g0542700(bZIP)/Os02g0565600/Os01g0586800(WRKY)/Os02g0132500(bZIP)/Os01g0734000(WRKY)/Os01g0726400(SRF)/Os07g0204000(AP2)/Os12g0158800(E2F)/Os12g0116700(WRKY)/Os09g0286600(AP2)
20-w-up	Os02g0677300(AP2)/Os01g0826400(WRKY)/Os08g0386200(WRKY)/Os09g0522100(AP2)/Os04g0572400(AP2)/Os05g0474800(WRKY)/Os09g0522200(AP2)/Os08g0474000(AP2)/Os03g0188900/Os01g0821600(WRKY)/Os04g0669200(AP2)/Os02g0181300(WRKY)/Os09g0417600(WRKY)/Os02g0654700(AP2)/Os12g0618600/Os03g0335200(WRKY)/Os07g0227600(AP2)/Os02g0462800(WRKY)/Os02g0767800(AP2)/Os01g0885900(AP2)/Os12g0168800(GATA)/Os02g0157950/Os09g0457900(AP2)/Os02g0232000(HSF)/Os04g0456900(EIN3)/Os07g0680400(WRKY)/Os03g0191900(AP2)/Os04g0572200(AP2)/Os02g0806350/Os05g0343400(WRKY)/Os03g0411100/Os06g0127100()AP2/Os09g0528200/Os01g0752500(AP2)/Os01g0343300(GATA)/Os04g0649100/Os01g0624700(WRKY)/Os09g0490200(EIN3)/Os07g0608200/Os11g0160500(bZIP)/Os09g0334500(WRKY)/Os04g0637000(bZIP)/Os03g0647600/Os02g0638650(AP2)/Os07g0596900(WRKY)/Os09g0540800(bZIP)/Os06g0553100(HSF)

Time	Gene ID
20-w-up	Os08g0276200(WRKY)/Os07g0685700(EIN3)/Os06g0158100(WRKY)/Os04g0482300(ZF-TAZ)/Os08g0332800(Med14)/Os07g0648266(Med12)/Os09g0470900(SCAI)/Os08g0564800
2-z-down	Os02g0676800(AP2)/Os12g0168100/Os04g0541700(bZIP)/Os03g0758950(WRKY)/Os06g0712700(SRF)/Os03g0758000(WRKY)/Os08g0276200(WRKY)/Os12g0168800(GATA)/Os05g0572000(AP2)/Os03g0324200(EIN3)/Os09g0379600/novel.1333/Os02g0764700(AP2)/Os05g0492000(AP2)/Os07g0648266(Med12)/Os08g0332800(Med14)
2-z-up	Os01g0584900(WRKY)/Os05g0571200(WRKY)/Os03g0657400(WRKY)/Os05g0183100(WRKY)/Os07g0680400(WRKY)/Os05g0537100(WRKY)/Os06g0649000(WRKY)/Os11g0117500(WRKY)/Os03g0321700(WRKY)/Os11g0117400(WRKY)/Os05g0321900(WRKY)/Os03g0335200(WRKY)/Os05g0530400(HSF)/Os01g0186000(WRKY)/Os01g0826400(WRKY)/Os01g0289600(WRKY)/novel.2074/Os01g0542700(bZIP)/Os03g0306700/Os04g0544500(GATA)/Os09g0417600(WRKY)/Os07g0617000(AP2)/Os11g0117600(WRKY)/Os01g0730700(WRKY)/Os02g0782700(AP2)/Os12g0116700(WRKY)/Os09g0417800(WRKY)/Os09g0457900(AP2)/Os10g0377300/Os06g0158100(WRKY)/Os11g0490900(WRKY)/Os04g0610400(AP2)/Os05g0549800(B3)/Os05g0474800(WRKY)/Os12g0116600(WRKY)/novel.2072/Os01g0820400(WRKY)
10-z-down	Os05g0549800(B3)/Os11g0117500(WRKY)/Os01g0542700(bZIP)/Os12g0116700(WRKY)/Os01g0289600(WRKY)/Os08g0499300(WRKY)/Os06g0166400(AP2)/Os03g0306700/Os03g0657400(WRKY)/Os07g0617000(AP2)/Os09g0417800(WRKY)/Os09g0306400(bZIP)/Os01g0752500(AP2)/Os02g0833600(bZIP)/Os03g0182800(AP2)/Os03g0174900/Os03g0321700(WRKY)/Os11g0686250(WRKY)
10-z-up	Os09g0522100(AP2)/Os02g0806350/Os08g0474000(AP2)/Os04g0572400(AP2)/Os02g0527300(HSF)/Os06g0127100(AP2)/Os09g0522200(AP2)/Os04g0550200(AP2)/Os02g0676800(AP2)/Os02g0181300(WRKY)/Os05g0343400(WRKY)/Os02g0677300(AP2)/Os04g0572200(AP2)/Os01g0750100(WRKY)/Os02g0462800(WRKY)/Os01g0826400(WRKY)/Os04g0287400(WRKY)/Os07g0685700(EIN3)/Os01g0313300(AP2)/Os02g0766700(bZIP)/Os04g0669200(AP2)/Os09g0334500(WRKY)/novel.2770/Os03g0188900/Os01g0885900(AP2)/Os04g0605100(WRKY)/Os05g0572000(AP2)/Os01g0693400(B3)/Os02g0654700(AP2)/Os05g0537100(WRKY)/Os01g0821600(WRKY)/Os03g0224700(HSF)/Os01g0624700(WRKY)/Os12g0162500(bZIP)/Os05g0497200(AP2)/Os07g0644100(bZIP)/Os05g0129300(bZIP)/Os03g0191900(AP2)/Os02g0638650(AP2)/Os04g0546800(AP2)/Os03g0231150/Os02g0781300(AP2)/Os03g0741400(WRKY)/Os05g0420300(AP2)/Os01g0186000(WRKY)/Os04g0529100(AP2)/Os05g0530400(HSF)/Os01g0868000(AP2)/Os05g0474800(WRKY)/Os02g0157950/Os04g0548700/Os02g0764700(AP2)/Os03g0322700(bZIP)/Os01g0131600(DUF)/Os09g0490200(EIN3)/Os01g0968800(AP2)/Os04g0568700(HSF)/Os01g0893400(ZF-TAC)/Os01g0908200(ZF-TAC)/Os04g0482300(ZF-TAC)
20-z-down	Os02g0149900/Os04g0541700/Os01g0797600(AP2)/Os08g0408500(AP2)/Os05g0155400(GATA)/Os02g0132500(bZIP)/Os01g0542700(bZIP)/Os06g0571800/Os10g0561800(HD-ZIP)/Os12g0601800(bZIP)/Os09g0379600/Os02g0729700/Os01g0625300(HSF)/Os07g0648266(Med12)
20-z-up	Os04g0572400(AP2)/Os01g0885900(AP2)/Os08g0474000(AP2)/Os01g0313300(AP2)/Os04g0429050(AP2)/Os09g0522200(AP2)/Os02g0677300(AP2)/Os09g0522100(AP2)/Os04g0605100(WRKY)/Os03g0188900/Os11g0117500(WRKY)/Os01g0624700(WRKY)/Os02g0181300(WRKY)/Os02g0527300(HSF)/Os01g0826400(WRKY)/Os01g0821600(WRKY)/Os05g0420300(AP2)/Os09g0417600(WRKY)/Os06g0158100(WRKY)/Os09g0457900(AP2)/Os07g0608200/Os02g0806350/Os03g0411100/Os06g0649000(WRKY)/Os05g0343400(WRKY)/Os04g0287400(WRKY)/Os08g0480500(Med22)

Table S3-4: List of DEGs related to kinase

Time	Gene ID	Description
2-w-up	Os01g0923700	Histidine kinase
	Os01g0191700	pyrophosphate-fructose-6-phosphate1-phosphotransferase-like protein
	Os04g0691900	1-phosphatidylinositol-3-phosphate 5-kinase
	Os10g0136500	SRK5 protein
10-w-down	Os10g0571200	pyruvate kinase isozyme G
10-w-up	Os02g0738400	histidine kinase
	Os01g0191700	pyrophosphate-fructose-6-phosphate1-phosphotransferase-like protein
	Os03g0356582	phosphatidylinositol-4-phosphate 5-kinase
	Os04g0633800	Protein kinase
20-w-down	Os01g0784700	Serine/threonine protein kinase-related domain containing protein
	Os05g0501400	Receptor-like protein kinase 5
	Os09g0298100	diphosphate-dependent phosphofructokinase
20-w-up	Os05g0194900	Pyrophosphate-fructose-6-phosphate1-phosphotransferase-like protein
	Os01g0191700	Pyrophosphate-fructose-6-phosphate1-phosphotransferase-like protein
	Os03g0701800	Phosphatidylinositol-4-phosphate 5-kinase 1
2-z-up	Os10g0136500	SRK5 protein
	Os04g0633800	Protein kinase
	Os03g0672300	pyruvate kinase
	Os06g0151900	Phosphofructokinase family protein
10-z-down	Os09g0415800	Phosphofructokinase family protein
	Os03g0672300	pyruvate kinase
10-z-up	Os01g0191700	Pyrophosphate-fructose-6-phosphate 1-phosphotransferase-like protein
	Os02g0714200	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase alpha subunit
	Os03g0356582	Phosphatidylinositol-4-phosphate 5-kinase
	Os01g0784700	Serine/threonine protein kinase-related domain containing protein
20-z-down	Os09g0298100	diphosphate-dependent phosphofructokinase
20-z-up	Os05g0194900	Pyrophosphate-fructose-6-phosphate 1-phosphotransferase-like protein

Table S3-5: List of DEGs related to peroxidase

Time	Gene ID	Description
2-w-down	Os06g0306300	Haem peroxidase domain containing protein
	Os01g0326300	Haem peroxidase, plant/fungal/bacterial family protein
	Os06g0522300	Haem peroxidase family protein
	Os03g0235000	Peroxidase
	Os05g0135500	Haem peroxidase family protein
	Os06g0521500	Haem peroxidase family protein
	Os01g0963000	Peroxidase BP 1 precursor
	Os08g0113000	Peroxidase 47 precursor
	Os04g0688500	Peroxidase
	Os01g0326000	Peroxidase
	Os03g0234500	Haem peroxidase
	Os03g0285700	L-ascorbate peroxidase
	Os01g0263300	Peroxidase 72 precursor
	Os06g0521900	Plant peroxidase domain containing protein
	Os06g0522100	Plant peroxidase domain containing protein
2-w-up	Os02g0240300	Class III peroxidase GvPx2b
	Os04g0556300	Glutathione peroxidase
	Os01g0787000	Peroxidase
	Os04g0465100	Haem peroxidase, plant/fungal/bacterial family protein
	Os01g0270300	Cationic peroxidase isozyme 40K precursor
	Os07g0639400	Peroxidase 1
	Os08g0453700	ARABIDOPSIS THALIANA RESPIRATORY BURST OXIDASE PROTEIN F
	Os06g0274800	Peroxidase 11 precursor
10-w-down	Os06g0681600	Haem peroxidase family protein
	Os07g0677100	Peroxidase
	Os05g0135500	Haem peroxidase family protein
	Os11g0112200	Cationic peroxidase 1 precursor
	Os01g0360200	Respiratory burst oxidase homolog
	Os05g0499400	Haem peroxidase family protein
	Os01g0326000	Peroxidase
	Os12g0112000	Peroxidase precursor
	Os03g0358100	Glutathione peroxidase
	Os06g0274800	Peroxidase 11 precursor
	Os04g0688300	Haem peroxidase, plant/fungal/bacterial family protein
	Os03g0285700	L-ascorbate peroxidase
	Os07g0694300	Peroxidase
	Os01g0172600	electron carrier/ heme binding / peroxidase
10-w-up	Os06g0306300	Haem peroxidase domain containing protein
	Os09g0471100	Peroxidase 17 precursor
	Os07g0626700	Peroxidase
	Os07g0639400	Peroxidase 1
	Os04g0465100	Haem peroxidase, plant/fungal/bacterial family protein
	Os01g0294500	Class III peroxidase 9
20-w-down	Os02g0240300	Class III peroxidase
	Os12g0178200	Thylakoid-bound ascorbate peroxidase
	Os07g0677100	Peroxidase
	Os03g0762300	Peroxidase 51 precursor
	Os01g0263300	Peroxidase 72 precursor
	Os08g0522400	L-ascorbate peroxidase
20-w-up	Os05g0134400	Haem peroxidase, plant/fungal/bacterial family protein
	Os01g0270300	Cationic peroxidase isozyme 40K precursor
	Os01g0378100	Plant peroxidase domain containing protein
	Os03g0339300	peroxidase
	Os03g0121200	Peroxidase 1
	Os02g0236600	Peroxidase P7
	Os05g0528000	RbohAOsp
	Os08g0453700	ATRBOH F
	Os04g0465100	Haem peroxidase, plant/fungal/bacterial family protein

Time	Gene ID	Description
20-w-up	Os07g0115300	Peroxidase2 precursor
	Os01g0787000	Peroxidase
	Os12g0111800	Class III peroxidase 136
2-z-down	Os09g0471100	Peroxidase 17 precursor
	Os03g0339400	Haem peroxidase, plant/fungal/bacterial family protein
2-z-up	Os07g0677500	Peroxidase precursor
	Os12g0111800	Class III peroxidase 136
	Os07g0677200	Peroxidase
	Os01g0962700	Peroxidase 12 precursor
	Os06g0522300	Haem peroxidase family protein
	Os11g0112200	Cationic peroxidase 1 precursor
	Os01g0326000	Peroxidase
	Os04g0688100	Peroxidase
	Os09g0438000	RbohAOsp
	Os03g0234500	Haem peroxidase, plant/fungal/bacterial family protein
	Os04g0688600	Peroxidase
	Os05g0134400	Haem peroxidase, plant/fungal/bacterial family protein
	Os01g0963000	Peroxidase BP 1 precursor
10-z-down	Os07g0677200	Peroxidase
	Os07g0677500	Peroxidase precursor
	Os08g0453700	ATRBOH F
	Os07g0677100	Peroxidase
	Os04g0688500	Peroxidase
	Os10g0109600	Peroxidase
	Os06g0521500	Haem peroxidase family protein
	Os04g0434800	Thylakoid-bound ascorbate peroxidase
	Os04g0688600	Peroxidase
	Os08g0113000	Peroxidase 47 precursor
	Os06g0306300	Haem peroxidase domain containing protein)/
	Os01g0963000	Peroxidase BP 1 precursor
	Os01g0962700	Peroxidase 12 precursor
	Os03g0235000	Peroxidase
	Os11g0112200	Cationic peroxidase 1 precursor
	Os06g0522300	Haem peroxidase family protein
10-z-up	Os07g0677400	Peroxidase
	Os04g0465100	Haem peroxidase, plant/fungal/bacterial family protein
	Os01g0787000	Peroxidase
	Os01g0270300	Cationic peroxidase isozyme 40K precursor
	Os01g0294700	Haem peroxidase, plant/fungal/bacterial family protein
	Os04g0556300	Glutathione peroxidase
	Os01g0327100	Haem peroxidase family protein
20-z-down	Os05g0135500	Haem peroxidase family protein
20-z-up	Os02g0240100	Peroxidase 2
	Os05g0134400	Haem peroxidase, plant/fungal/bacterial family protein
	Os02g0236600	Peroxidase P7

Table S3-6: Primers of construction and detection of CRISPR and overexpression

Primer name	Primer sequence (5′-3′)	Remark
Os06g0267600-sg-F	GTGTGGACTGAACTAGAAGGTTCC	Primer of *Os06g0267600* sgRNA
Os06g0267600-sg-R	AAACGGAACCTTCTAGTTCAGTCC	
Os09g0538800-sg-F	GTGTGTCTCTCCGCCCTCATGATG	Primer of *Os09g0538800* sgRNA
Os09g0538800-sg-R	AAACCATCATGAGGGCGGAGAGAC	
Os06g0267600-OE-F	AGCCTGTTAAACGCCATGAATTTCTTCTCG GGGGTATGGG	Primer of *Os06g0267600* overexpression
Os06g0267600-OE-R	ACCGAGCTCACCCGGCTACAATTGAGGAG GCCCGTGGTAT	
Os09g0538800-OE-F	AGCCTGTTAAACGCCATGGGTGTGATTAGT ACGGTGCTTG	Primer of *Os09g0538800* overexpression
Os09g0538800-OE-R	ACCGAGCTCACCCGGCTATGAAGTTCTCCA CTGCATCTCT	
Os06g0267600-check-F	TGATGGTCTTTCAGATGGAG	Detection primers of *Os06g0267600* CRISPR mutant
Os06g0267600-check-R	CCAGTAATAGAGCCCCAGA	
Os09g0538800-check-F	AAGGTCTCACAGCACAAGG	Detection primers of *Os09g0538800* CRISPR mutant
Os09g0538800-check-R	GCATTTCTCCAGCAACTTT	
Os06g0267600-qPCR-F	CTGGGGCTCTATTACTGGTACG	Detection primers of *Os06g0267600* overexpression mutant
Os06g0267600-qPCR-R	GTCTACTTGCCGAATCCTTGC	
Os09g0538800-qPCR-F	CAAAGTTCGCCCACTTGTCG	Detection primers of *Os09g0538800* overexpression mutant
Os09g0538800-qPCR-R	GCCTTGTTGAGGTAAGGCCA	

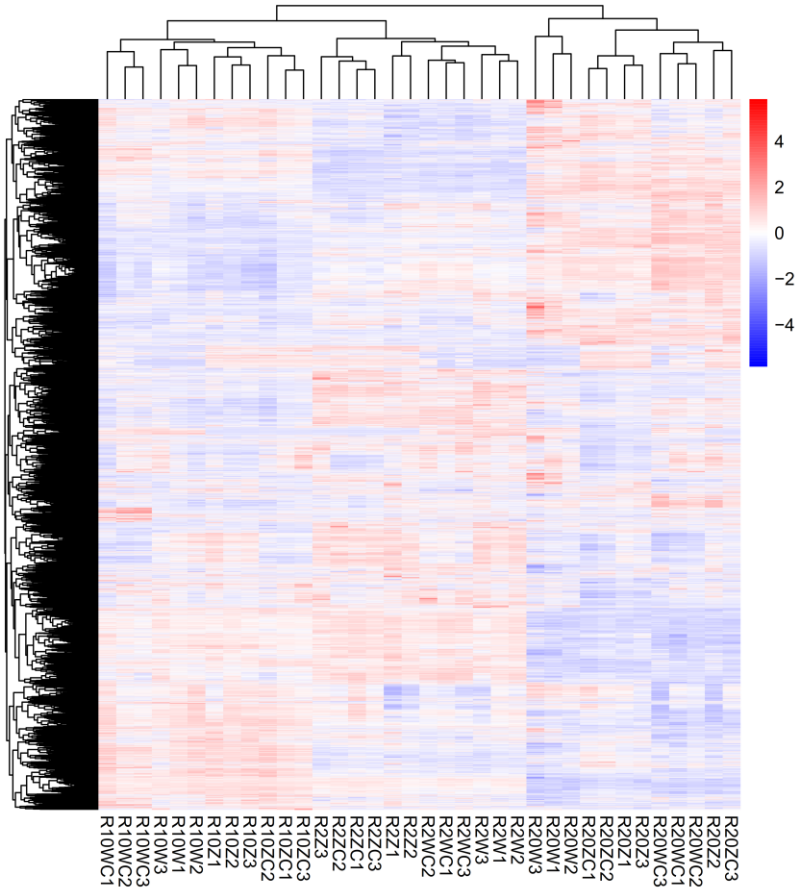
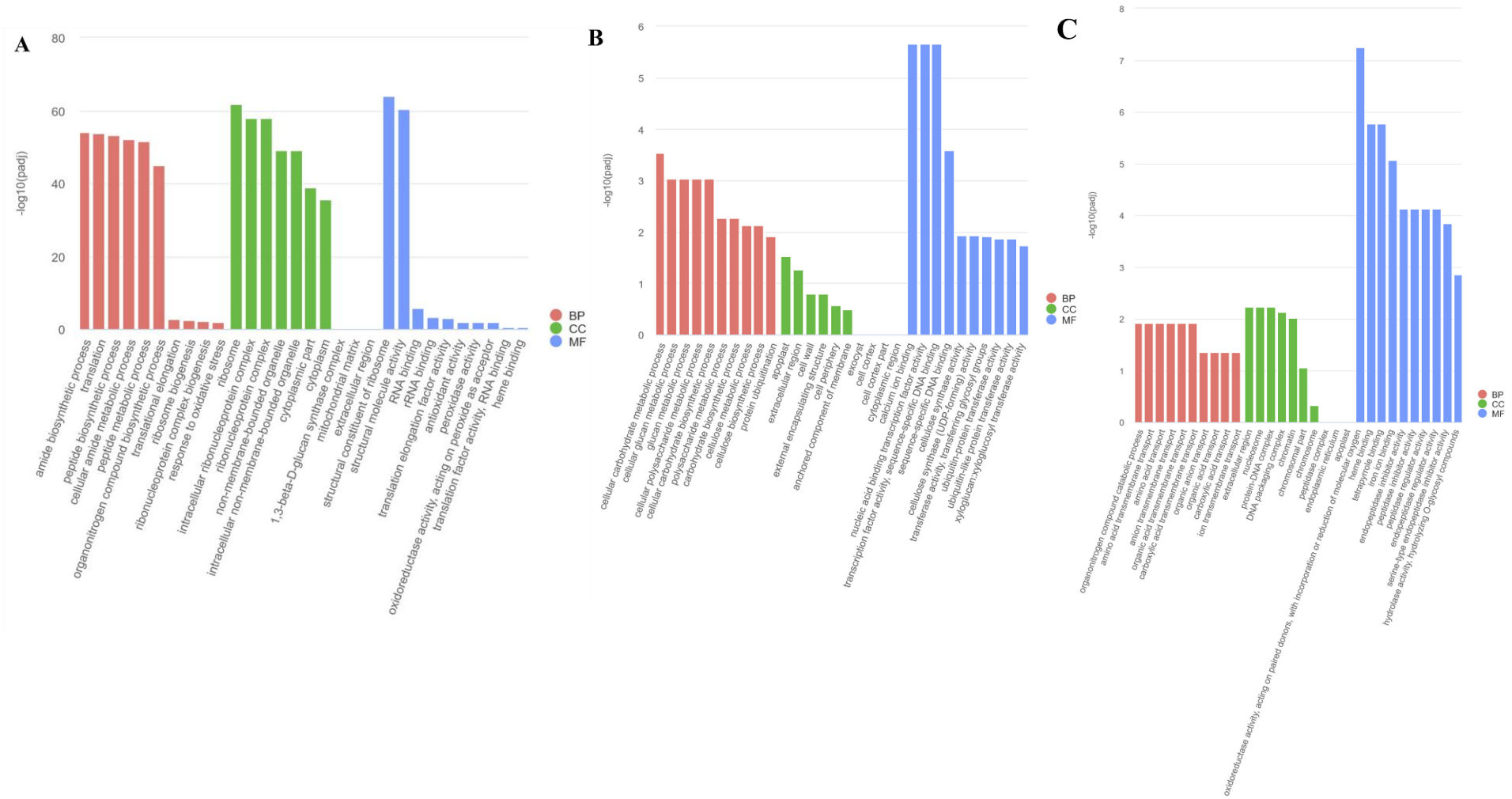


Figure S3-1: Hierarchical clustering analysis of the DEGs of all the samples. Heatmap shows that similar expression pattern of genes or samples are gathered together. For cross-sectional comparisons, red indicates high gene expression and blue indicates low gene expression.


A
-log10(padj)
amide biosynthetic process
translation
peptide biosynthetic process
cellular amide metabolic process
peptide metabolic process
organonitrogen compound biosynthetic process
translational elongation
ribosome biogenesis
ribonucleoprotein complex biogenesis
response to oxidative stress
ribosome
intracellular ribonucleoprotein complex
ribonucleoprotein complex
non-membrane-bounded organelle
intracellular non-membrane-bounded organelle
cytoplasmic part
cytoplasm
1,3-beta-D-glucan synthase complex
mitochondrial matrix
extracellular region
structural constituent of ribosome
structural molecule activity
RNA binding
rRNA binding
translation elongation factor activity
antioxidant activity
peroxidase activity
oxidoreductase activity, acting on peroxide as acceptor
translation factor activity, RNA binding
heme binding
BP
CC
MF
B
-log10(padj)
cellular carbohydrate metabolic process
cellular glucan metabolic process
glucan metabolic process
cellular polysaccharide metabolic process
cellular carbohydrate biosynthetic process
polysaccharide metabolic process
carbohydrate biosynthetic process
cellulose metabolic process
cellulose biosynthetic process
protein ubiquitination
apoplast
extracellular region
cell wall
external encapsulating structure
cell periphery
anchored component of membrane
exocyst
cell cortex
cell cortex part
cytoplasmic region
calcium ion binding
transcription factor activity, sequence-specific DNA binding
nucleic acid binding transcription factor activity
sequence-specific DNA binding
cellulose synthase (UDP-forming) activity
transferase activity, transferring glycosyl groups
ubiquitin-protein transferase activity
ubiquitin-like protein transferase activity
xyloglucan-xyloglucosyl transferase activity
BP
CC
MF
C
-log10(padj)
organonitrogen compound catabolic process
amino acid transmembrane transport
amino acid transport
anion transmembrane transport
organic acid transmembrane transport
carboxylic acid transmembrane transport
organic anion transport
carboxylic acid transport
ion transmembrane transport
extracellular region
nucleosome
protein-DNA complex
DNA packaging complex
chromatin
chromosomal part
chromosome
peptidase complex
endoplasmic reticulum
apoplast
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
heme binding
tetrapyrrole binding
iron ion binding
endopeptidase inhibitor activity
peptidase inhibitor activity
peptidase regulator activity
endopeptidase regulator activity
peptidase inhibitor activity
serine-type endopeptidase inhibitor activity
hydrolase activity, hydrolyzing O-glycosyl compounds
BP
CC
MF

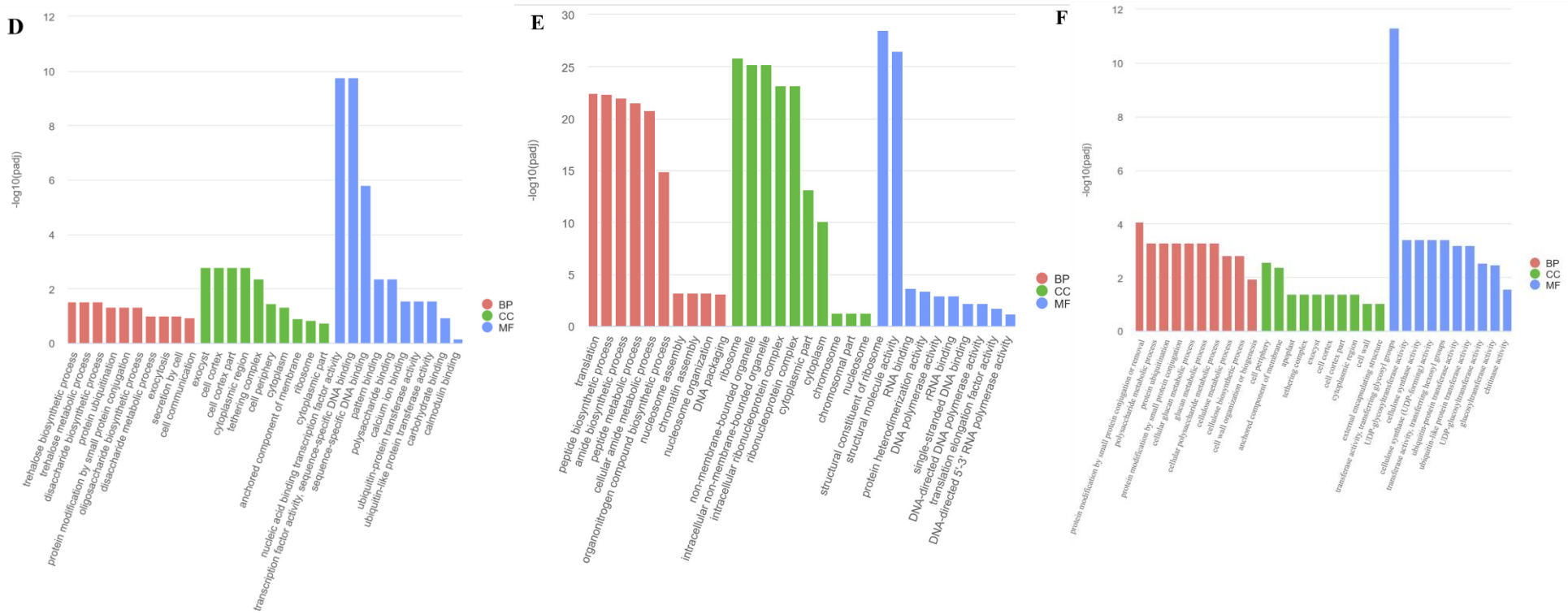
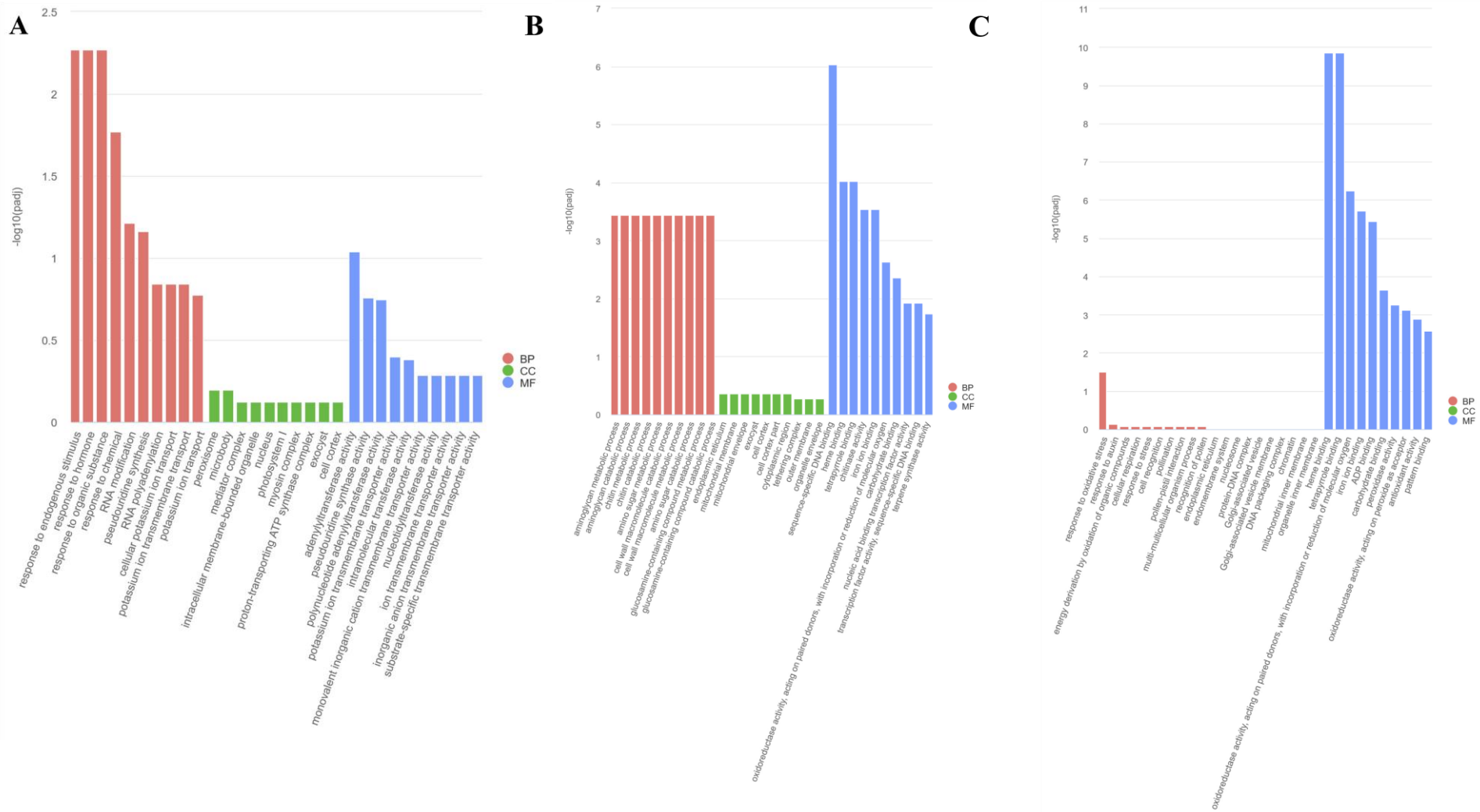


Figure S3-2: Classification of gene ontology (GO) analysis of DEGs for WYJ3. Top 30 GO terms enriched for differently expressed genes. The x-axis represents the enriched GO term, and the y-axis represents the number of DEGs in the term. Different colors are used to distinguish biological processes, cellular components, and molecular functions. A, Down-regulated DEGs of WYJ3 at 2 dpi. B, Up-regulated DEGs of WYJ3 at 2 dpi. C, Down-regulated DEGs of WYJ3 at 10 dpi. D, Up-regulated DEGs of WYJ3 at 10 dpi. E, Down-regulated DEGs of WYJ3 at 20 dpi. F, Up-regulated DEGs of WYJ3 at 20 dpi.


A
B
C
-log10(padj)
BP
CC
MF
response to endogenous stimulus
response to hormone
response to organic substance
response to chemical
RNA modification
pseudouridine synthesis
RNA polyadenylation
cellular potassium ion transport
potassium ion transmembrane transport
potassium ion transport
peroxisome
microbody
mediator complex
intracellular membrane-bounded organelle
nucleus
photosystem I
myosin complex
proton-transporting ATP synthase complex
exocyst
cell cortex
adenylyltransferase activity
pseudouridine synthase activity
polynucleotide adenylyltransferase activity
potassium ion transmembrane transporter activity
intramolecular transferase activity
monovalent inorganic cation transmembrane transporter activity
nucleotidyltransferase activity
ion transmembrane transporter activity
inorganic anion transmembrane transporter activity
substrate-specific transmembrane transporter activity
aminoglycan metabolic process
aminoglycan catabolic process
chitin catabolic process
amino sugar metabolic process
cell wall macromolecule catabolic process
amino sugar catabolic process
glucosamine-containing compound catabolic process
endoplasmic reticulum
mitochondrial membrane
mitochondrial envelope
exocyst
cell cortex
cell cortex part
cytoplasmic region
tethering complex
outer membrane
organelle envelope
sequence-specific DNA binding
heme binding
tetrapyrrole binding
chitinase activity
iron ion binding
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen
carbohydrate binding
nucleic acid binding transcription factor activity
transcription factor activity, sequence-specific DNA binding
terpene synthase activity
response to oxidative stress
response to auxin
energy derivation by oxidation of organic compounds
cellular respiration
response to stress
cell recognition
pollination
pollen-pistil interaction
multi-multicellular organism process
recognition of pollen
endoplasmic reticulum
endomembrane system
nucleosome
protein-DNA complex
Golgi-associated vesicle
Golgi-associated vesicle membrane
DNA packaging complex
chromatin
mitochondrial inner membrane
organelle inner membrane
membrane
heme binding
tetrapyrrole binding
iron ion binding
oxygen binding
ADP binding
carbohydrate binding
peroxidase activity
oxidoreductase activity, acting on peroxide as acceptor
antioxidant activity
pattern binding

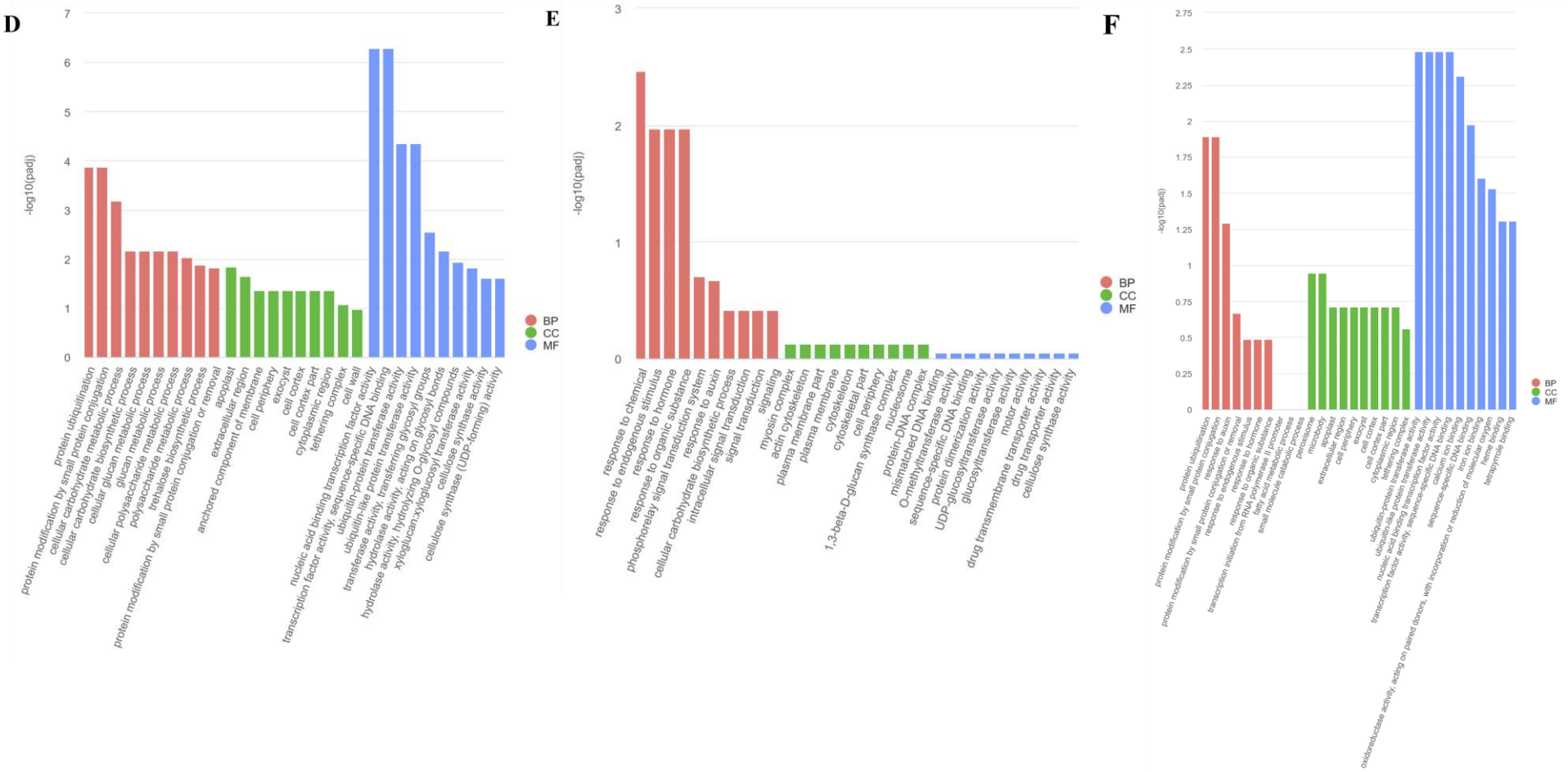


Figure S3-3: Classification of gene ontology (GO) analysis of DEGs for ZD88. Top 30 GO terms enriched for differently expressed genes. The x-axis represents the enriched GO term, and the y-axis represents the number of DEGs in the term. Different colors are used to distinguish biological processes, cellular components, and molecular functions. A, Down-regulated DEGs of ZD88 at 2 dpi. B, Up-regulated DEGs of ZD88 at 2 dpi. C, Down-regulated DEGs of ZD88 at 10 dpi. D, Up-regulated DEGs of ZD88 at 10 dpi. E, Down-regulated DEGs of ZD88 at 20 dpi. F, Up-regulated DEGs of ZD88 at 20 dpi.

Chapter 4

Enterobacter-infecting phages in nitrogen-deficient paddy soil impact nitrogen-fixation capacity and rice growth by shaping the soil microbiome

In this study, from the paddy soil where rice plants showed nitrogen deficiency, we isolated and identified a novel virulent phage (named here Apdecimavirus NJ2) that infects various Enterobacter species (nitrogen-fixing bacteria) and its impact on nitrogen fixation in the soil and in plants was characterized.

From Liu Y, Wang Y, Shi W, Wu N, Liu W, Frederic F, Wang X, 2024. *Enterobacter*-infecting phages in nitrogen-deficient paddy soil impact nitrogen-fixation capacity and rice growth by shaping the soil microbiome. Sci Total Environ. 956: 177382.

Abstract: Bacteriophages ("phage") play important roles in nutrient cycling and ecology in environments by regulating soil microbial community structure. Here, metagenomic sequencing showed that a low relative abundance of nitrogen-fixing bacteria but high abundance of Enterobacter-infecting phages in paddy soil where rice plants showed nitrogen deficiency. From soil in the same field, we also isolated and identified a novel virulent phage (named here as *Apdecimavirus* NJ2) that infects several species of *Enterobacter* and characterized its impact on nitrogen fixation in the soil and in plants. It has the morphology of the *Autographiviridae* family, with a dsDNA genome of 39,605 bp, 47 predicted open reading frames and 52.64 % GC content. Based on genomic characteristics, comparative genomics and phylogenetic analysis, *Apdecimavirus* NJ2 should be a novel species in the genus *Apdecimavirus*, subfamily *Studiervirinae*. After natural or sterilized field soil was potted and inoculated with the phage, soil nitrogen-fixation capacity and rice growth were impaired, the abundance of *Enterobacter* decreased, along with the bacterial community composition and biodiversity changed compared with that of the unadded control paddy soil. Our work provides strong evidence that phages can affect the soil nitrogen cycle by changing the bacterial community. Controlling phages in the soil could be a useful strategy for improving soil nitrogen fixation.

Keywords: Phage, *Enterobacter*, Phage-bacteria interaction, 16S rRNA amplicon sequencing, Nitrogen cycling

1. Introduction

Most crops require a high nitrogen content in the soil to meet needs for photosynthesis, growth, high yields and quality, and to produce seeds with a high protein content (Cechin et al., 2022). Numerous soil microbes, including mycorrhizal fungi (Wang et al., 2020), rhizobia (Koskey et al., 2017), other nitrogen-fixing bacteria (e.g. *Azotobacter*, *Klebsiella*, *Rhodospirillum*) (Aasfar et al., 2021; Jack et al., 1999; Masters and Madigan, 1983), and nitrifying (e.g. *Nitrosomonas*, *Nitrobacter*) (Mellbye et al., 2016; Uemoto and Saiki, 1996) and denitrifying bacteria (e.g. P*seudomonas*) (Best and Payne, 1965; Cameron et al., 1989) are involved in nitrogen fixation, nitrification and denitrification, critical processes in nitrogen cycling (Pashaei et al., 2022). Among the microbes involved in nitrogen cycling, bacteria in the genus *Enterobacter* (family *Enterobacteriaceae*) are involved in nitrogen fixation and have been isolated from the root nodules of certain crops, such as wheat and sorghum, and from the rhizospheres of rice (Chakraborty et al., 2019; Davin-Regli et al., 2019; James, 2000; Ji et al., 2020). Although some species of *Enterobacter* act as opportunistic pathogens of humans, other species of these common, widespread gram-negative bacilli are free-living or symbionts in terrestrial and aquatic environments including soil and sewers (Reitter et al., 2021; Singh et al., 2018). Some *Enterobacter* species promote plant growth by solubilizing phosphorus and potassium or producing indole-3-acetic acid (Kampfer, 2005; Roslan et al., 2020).

Many soil bacteria are also host to viruses such as bacteriophages (hereafter, phages), the most abundant of soil viruses (Suttle, 2005). Interactions between phages and their host bacteria play an important role in soil nutrient cycling by altering host abundance, the soil microbial community structure and function (Pratama et al., 2018). After phage infection, the host bacteria are lysed, thus significantly decreasing their abundance, but the abundance of other microbes increase as they compete for ecological niches and nutrients, thereby changing the microbial community structure (Escudero-Martinez et al., 2022; Yang et al., 2023; Zhang et al., 2022a). Bacterial nitrification and denitrification are more sensitive to environmental disturbance; thus, alterations in the community of these bacterial species are more likely to impact the diversity of other microbes and thus the nitrogen content in the soil (Xu et al., 2021). After treatment with the polyvalent virulent phage ΦNF-1, a species of a Nitrosomonas, a genus of nitrifying bacteria, in vitro bacterial growth and NH_4^+ consumption was inhibited (Quirós et al., 2023). Nitrogen-fixing bacteria are most likely specifically lysed by soil viruses through a lysogenic-based strategy in urea-treated paddy soil (Li et al., 2019). On the other hand, when phage-infected bacteria are lysed, their cytoplasm is released into the soil, thus increasing elements such as nitrogen, phosphorus, potassium and calcium that are critical for plant growth (Daly et al., 2019). In addition, phages can be indirectly involved in soil element cycling by the expression of virus-encoded auxiliary metabolic genes (AMGs) in the host bacteria after infection, thus altering host processes that are involved in biogeochemical cycling of elements, thereby endowing the host with new functions and broadening its ecological niche (Rosenwasser et al., 2016). Therefore, the existence of abundant phages infecting nitrogen cycling associated bacteria hold a critical role in various environments.

During a field investigation, we found rice plants showing typical symptoms of nitrogen deficiency in a field in Nanjing, Jiangsu Province, China. We collected soil samples in the field for metagenomic sequencing, which showed low relative abundance of nitrogen-fixing bacteria and high relative abundance of phages infecting *Enterobacter* spp. We further isolated a novel phage from the soil and verified that the phage can affect nitrogen fixation in the soil and the nitrogen content and growth of rice plants in several experiments in which the soils were amended with a novel phage. These results provide strong evidence that this phage affects plant growth by altering the soil bacterial community structure and reduces soil nitrogen-fixation capacity in the rice rhizosphere, which is an important factor causing soil nitrogen deficiency. This provides a new perspective for understanding the potential biological mechanism of soil barren, and also provides a new idea for soil remediation by adding bacteriophage resistant bacteria.

2. Materials and methods

2.1. Paddy soil, bacterial strains and culture conditions

Paddy soil samples were collected in Nanjing, Jiangsu Province (30°02′ N, 118°46′ E, 30 m a.s.l.). Three regions (5 m × 10 m) were randomly selected as three repeats, thirty cores of bulk soil samples (0-15 cm) were randomly collected from each repeated region. Isolates of *Enterobacter*, *Escherichia*, *Dickeya*, *Erwinia*, *Pectobacterium*, *Pseudomonas* and *Yersinia* (purchased from the Agricultural Culture Collection of China) used in the present study are listed in Table S4-1. These bacteria were cultured in Luria Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, and 1 % NaCl) by shaking for 16 h at 37°C.

2.2. Metagenomic sequencing

Genomic DNA was extracted from 0.5 g soil sample for each biological replicate using the E.Z.N.A. Soil DNA Kit (Omega Bio-TEK, Norcross, GA, USA) according to the manufacturer's instructions. Concentration and purity of extracted DNA were determined with a TBS-380 Mini-Fluorometer (Turner Biosystems, Sunnyvale, CA, USA) and NanoDrop2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. The extracted DNA was fragmented to an average size of about 400 bp using a Covaris M220 Focused Ultrasonicator (Covaris, Woburn, MA, USA). A paired-end library was constructed using NEXTFLEX Rapid DNA-Seq (Bioo Scientific, Austin, TX, USA). Paired-end sequencing was performed using the Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) at Majorbio BioPharm Technology Co., Ltd. (Shanghai, China), a HiSeq 3000/4000 PE Cluster Kit and a HiSeq 3000/4000 SBS Kit (Illumina) according to the instructions. All sequence data have been deposited in the NCBI Short Read Archive database (accession PRJNA732820). The paired-end Illumina reads were trimmed of adaptors, and low-quality reads (length < 50 bp or with a quality value < 20 or having N bases) were removed using fastp version 0.20.0 (https://github.com/OpenGene/fastp). Metagenomics data were assembled using MEGAHIT version 1.1.2 (https://github.com/voutcn/megahit). Contigs ≥300 bp were selected for further gene prediction and annotation. The representative sequences were blastp against the NCBI NR database for taxonomic annotations of bacteria and viruses with an e-value cut-off $1e^{-5}$. The most abundant bacteriophage was isolated and studied for functions.

2.3. Isolation, purification and TEM observations of the phage

Phages were isolated from soil samples collected from the rice field in Nanjing. Each soil sample was sieved, then 10 g of the sieved sample was added to 50 mL of LB broth and cultured overnight at 37 ℃. The culture was then centrifuged at 10,000 ×g for 20 min, and the supernatant was then filtered through a 0.22-μm filter to remove impurities and bacterial cells. This filtrate was used as the phage stock. One milliliter of the filtrate was added to 10 mL of a culture of *Enterobacter cloacae* at the logarithmic growth stage and grown overnight at 37 °C. For the double agar overlay plaque assay to purify the phage, a 10-fold dilution series of the phage stock in PBS buffer was prepared and 10 μL of a dilution (10^{-1}-10^{-8}) was mixed with 100 mL of *E. cloacae* at the logarithmic growth stage at room temperature, then plated the mixture of phage and E. cloacae was mixed with 5 mL of soft agar (0.35 % agar prepared in 1 % tryptone, 0.5 % NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.04 % [wt/vol] glucose) , then spread on a plate of solid LB agar plate and incubated overnight at 37 ℃. Phages were purified via five consecutive transfers of them from individual plaques to new bacterial cell lawns. The purified phage was stored in buffer (10 mM Tris [pH 7.6], 5 mM $MgSO_4 \cdot 7H_2O$, 0.01% gelatin) at 4 °C. The concentrated phage suspension (about 20 μL) was dropped on copper grids. After 15 min, a drop of 2 % (w/v) phosphotungstic acid was placed on each grid and incubated for 5 min, then removed the phosphotungstic acid and dried at room temperature. Phage particles on the grids were visualized with a H-7500 transmission electron microscope (TEM, Hitachi, Tokyo, Japan) at 80 kV as previously described (Summer et al., 2011).

2.4. Determination of phage host range

Seventeen strains representing species of *Enterobacter*, *Escherichia*, *Dickeya*, *Erwinia*, *Pectobacterium*, *Pseudomonas* and *Yersinia* (Table S4-1) were used to assess the host range of the phage in a phage lysis assay as described by Li and Tang (2011), indicating that the phage had lysed the bacterial cell.

2.5. Isolation of genomic DNA, genome sequencing and analysis

Phage DNA was extracted using a Phage Genomic DNA Extraction Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturers' protocol. The quality and integrity of genomic DNA were assessed using 1 % agarose gel electrophoresis and densitometry and comparison to appropriate size standards. DNA yield and purity were measured as described earlier. Only high-quality DNA (OD260/280 = 1.8~2.0, >1 μg) was used. The phage DNA was sent to WinnerBio Technology Co., Ltd (Shanghai, China) for third-generation sequencing of the genome using the Illumina NovaSeq 6000 platform (Illumina). Briefly, at least 1 μg genomic DNA was used to construct the sequencing library. DNA sample was sheared into 400–500-bp fragments using a Covaris M220 Focused-Ultrasonicator (Covaris) following the manufacturer's protocol. Illumina sequencing libraries were prepared from the sheared fragments (Ribarska et al., 2022). The prepared libraries were then used for paired-end Illumina sequencing (2 × 150 bp) on an NovaSeq 6000 (Illumina). The genome was assembled using SOAPdenovo2 (version 2.0.4, https://sourceforge.net/projects/soapdenovo2/) (Luo et al., 2012). Each assembled nucleotide sequence was used in a search against the National Center for Biotechnology Information (NCBI) non-redundant database (https://www.ncbi.nlm.nih.gov/), the UniProt database version 2023_04 (https://www.uniprot.org/), the protein families (Pfam) database version 36.0 (http://pfam.xfam.org/), the Clusters of Orthologous Group (COG) database version 2020 (https://www.ncbi.nlm.nih.gov/research/cog), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database version 92.0 (https://www.genome.jp/kegg/)

for annotation. Tri-generation data were assembled using Canu software V21.1 (https://github.com/marbl/canu/releases/tag/) (Koren et al., 2017). Coding sequences for the assembled sequences were predicted using Glimmer 3 (http://ccbjhu.edu/software/glimmer/index.shtml) (Delcher et al., 2007). The sequence has been deposited in the NCBI BankIt database (GenBank accession: OR822025).

2.6. Impact of the novel phage on nitrogen-fixation capacity of the soil and on rice growth

To investigate the effect of the novel phage (*Apdecimavirus* NJ2) on the composition and diversity of the bacterial community, the nitrogen-fixing capacity of soil bacteria and rice growth, we set up experiments with a reciprocal transplant design using sterilized and natural field soils. Natural soil from same field was sterilized at 121 ℃ for 30 min, cooled to room temperature, sterilized again, then cooled and stored at 4 ℃ until use. *E. cloacae*, *E. cancerogenus* and *E. ludwigii* were cultured in LB Borth overnight at 37 ℃. Then they were mixed together at a 1:1:1 ratio by volume, and collected by centrifugation at 6,600 ×g for 10 min. The bacteria were then resuspended in ddH_2O to achieve an OD600 of 0.02. Rice seeds (cv. Nipponbare) were soaked in 75 % alcohol for 3 min, then washed with ddH_2O three times, then placed in a 25 ℃ incubator for 2–3 days to germinate. Seeds with sprouts that were 1–2 cm long were then selected and planted, five in each of three replicate pots containing either natural soil or sterilized soil that had been treated as follows:

The natural soils were either watered with 50 mL of ddH_2O (blank control 1) or inoculated with 50 mL of a phage suspension (2×10^5 plaque forming units [PFUs]) (natural soil with phage) per pot. The control pots with sterilized soil were either watered with 50 mL of ddH_2O (blank control 2) or inoculated with 50 mL of the suspension of the 1:1:1 mixture of nitrogen-fixing *Enterobacteria* (described above) (nitrogen-fixing bacteria control) per pot. Sterilized soil in another three pots was inoculated with 50 mL of the mixed *Enterobacteria* suspension per pot. After 1 week, the pots in blank control 2 and nitrogen-fixing bacteria were each watered with 50 mL ddH_2O, and each pot with sterilized soil with nitrogen-fixing bacteria was added with 50 mL of the phage suspension (2×10^5 PFUs).

All rice plants were grown in a greenhouse at 26 ℃ with 14 h of fluorescent light and 10 h of darkness, watering with 20 mL ddH_2O every 3 days per pot. After 7 weeks, rhizosphere soil samples were collected (Berlanas et al., 2019), plant height, dry mass were measured. Plant nitrogen concentrations were determined using the modified Kjeldahl digestion method (Nelson and Sommers, 1973). Nitrogen-fixation capacity of soil was assessed using the acetylene reduction assay measuring ethylene formation from acetylene (Lopez-Lozano et al., 2016).

2.7. DNA extraction from rhizosphere soil and 16S rRNA sequencing

Total DNA was extracted from the rice rhizosphere soils and its concentration and purity assessed as described above. The V3-V4 region of the 16S rRNA gene of soil bacteria was amplified by PCR in a thermocycler PCR system (GeneAmp 9700, ABI, Foster, CA, USA) using primer pair 338F (5'-ACTCCTACGGGAGGCAGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') designed by Biomarker Technologies. Co. LTD (Beijing, China). The PCR mixture contained 4 μL 5× TransStart FastPfu buffer, 2 μL 2.5 mM dNTPs, 0.8 μL 5 μM forward primer, 0.8 μL 5 μM reverse primer, 0.4 μL TransStart FastPfu DNA Polymerase, 10 ng template DNA, with ddH_2O added to reach 20 μL. PCR cycling conditions were initial denaturation at 95 °C for 3 min; 35 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s; and single extension at 72 °C for 10 min, and end at 4 °C. The amplified products were purified with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), and a 16S rRNA library was constructed and used for high-throughput sequencing with the Illumina Novaseq 6000 platform by Biomarker Technologies. The raw sequencing reads were demultiplexed, quality-filtered (Benjamin et al., 2017). High-quality sequences from all samples were clustered into operational taxonomic units (OTUs) at 97 % sequence similarity using the default QIIME2 pipeline UCLAST (https://qiime2.org/) (Bolyen et al., 2019). The taxonomy of each gene sequence was analyzed using Silva reference gene data base (http://www.arb-silva.de/) with a confidence threshold of 70 % (Kõljalg et al., 2013).

2.8. Statistical analyses

The alpha diversity of the bacterial community (including Shannon and Simpson index) was calculated using Mothur v1.30 software (https://mothur.org/wiki/download_mothur/) for the bacterial communities of the rhizosphere samples based on their OTUs levels (Schloss et al., 2009), Principal coordinate analysis (PCoA) analysis was performed in R using Bray-Curtis distances, which was used to compare the beta diversity of the bacterial community. Alpha diversity indices and relative abundance of major genus which were non-normally distributed were tested for significant differences date using nonparametric statistics Kruskal-Wallis test conducted in SPSS version 16.0 (Wickham, 2016).

The R package VennDiagram (Chen and Boutros, 2011) was used construct a Venn diagram to visualize the number and identity of shared and unique genera between the blank control samples and the samples with the phage. Species distribution analysis was performed by matplotlib version 1.5.1 (Hunter, 2007) in Python to show the distribution of bacterial relative abundance in the rhizosphere samples.

3. Results

3.1. Community composition of bacteria and viruses in nitrogen-deficient paddy soil

In the analysis of the community composition of soil bacteria and viruses through metagenomic sequencing in the nitrogen- deficient soil in Nanjing, 1878 genera of bacteria and 218 virus species were detected. Of the bacterial genera, 20 were dominant (abundance greater than 1 % of total). The four most abundant genera were unclassified-p-*Chloroflexi*, *Nocardioides*, *Gemmatimonas* and *Solirubrobacter*, with relative abundance of 5.1 %, 3.4 %, 2.5 % and 2.4 %, respectively (Fig. 4-1a and b). The abundance of *Enterobacter*, the potential host of *Enterobacter* phages, was extremely low (0.005 %). Of the 218 virus species, 19 were dominant with relative abundance greater than 1 %. *Enterobacter* phages were the most abundant with a relative abundance of 15.89 % (Fig. 4-1c and d).

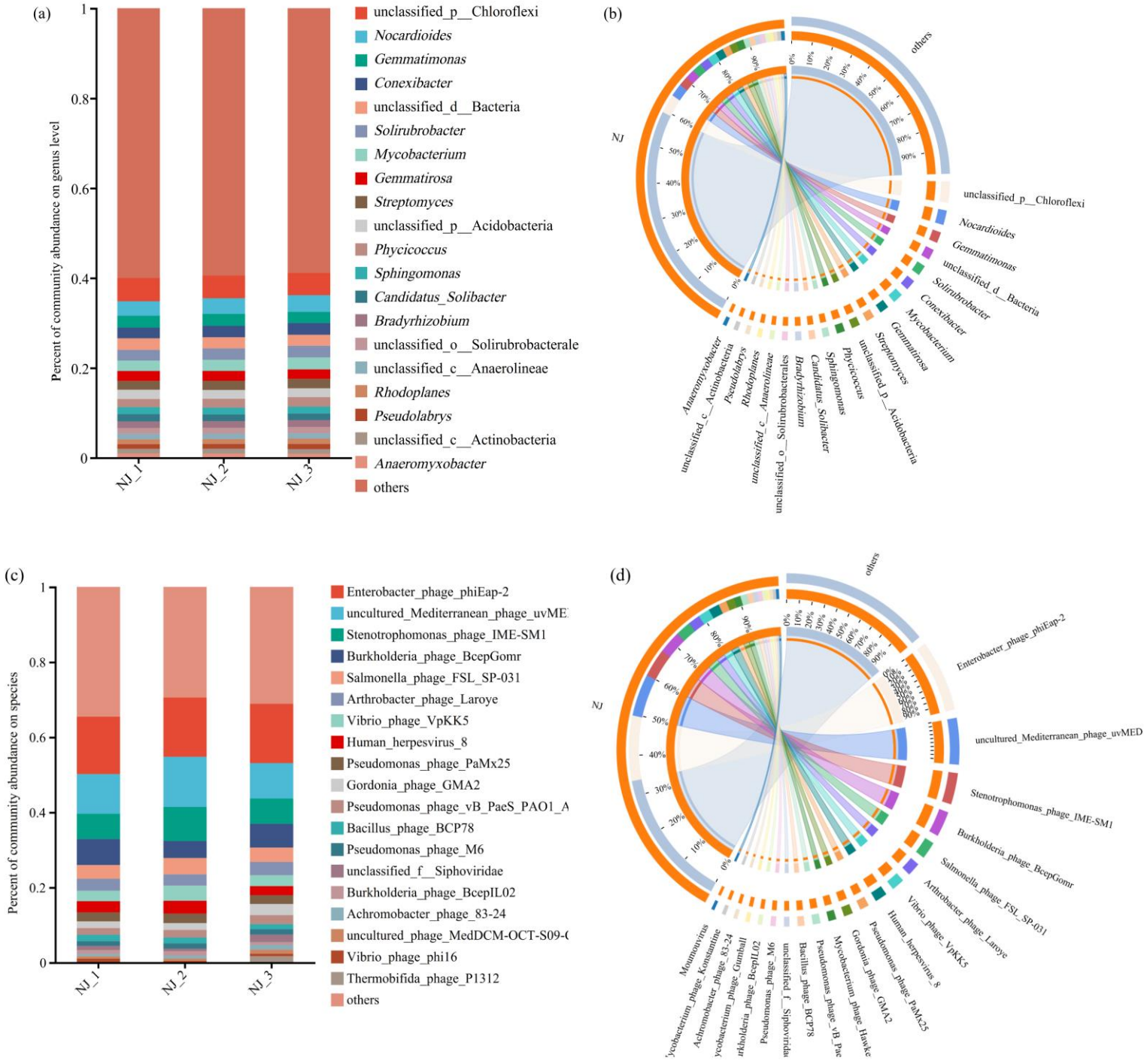


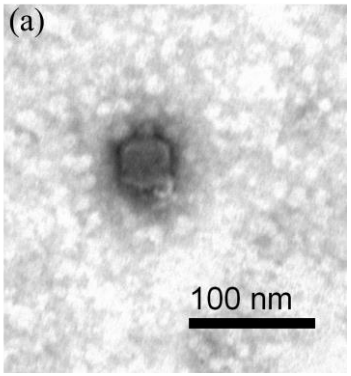
Figure 4-1: Composition of soil bacterial and viral communities in nitrogen-deficient paddy soil. (a) Bacterial community at genus level. (b) Circos analysis of distribution and proportion of the dominant bacterial genera. (c) Composition of viral community at species level. (d) Circos analysis of distribution and proportion of the dominant viral species. NJ-1, NJ-2 and NJ-3 represent three soil samples.

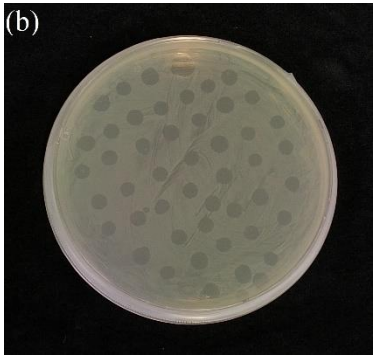
3.2. Phage isolation, visualization and host specificity

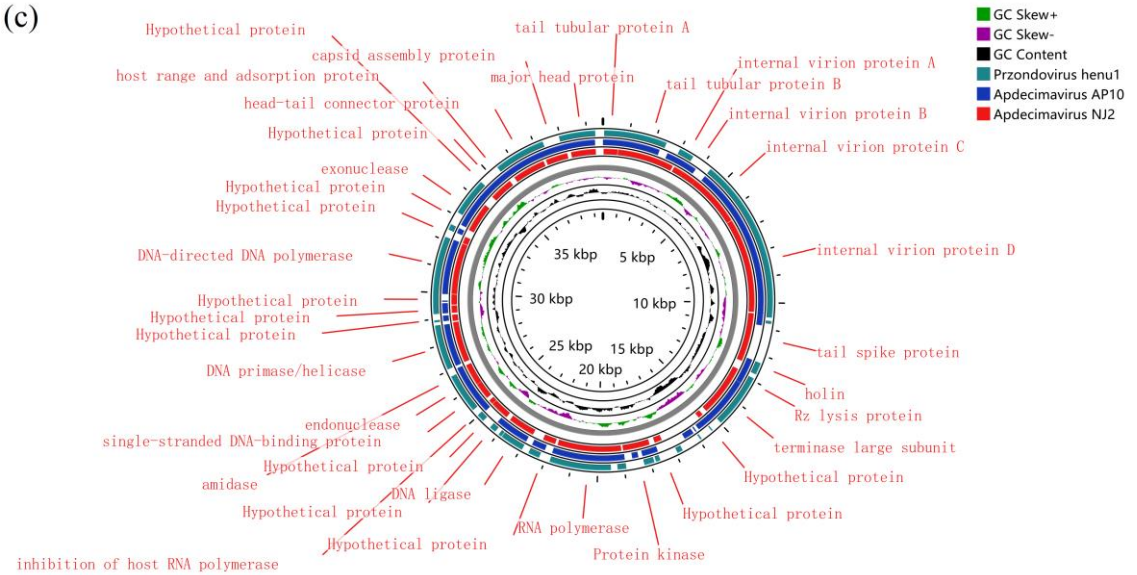
A novel phage was isolated from the collected soil samples. TEM micrographs revealed that the phage has an icosahedral head with a diameter of approximately 58 nm and a short tail approximately 15 nm long (Fig. 4-2a). Based on these characteristics and the 9th Report of the International Committee on Taxonomy of Viruses (ICTV), this phage was morphologically classified as a member of the *Autographiviridae* family. As shown in the phage lysis test, the phage infected *Enterobacter cloacae* (Fig. 4-2b). In the double agar overlay plaque assay of the 17 species of bacteria, the phage only infected *Enterobacter* species (*E. cloacae*, *E. cancerogenus*, *E. ludwigii*, *E. aerogenes* and *E. hormaechei*), meaning a narrow host range for it (Table S4-1).

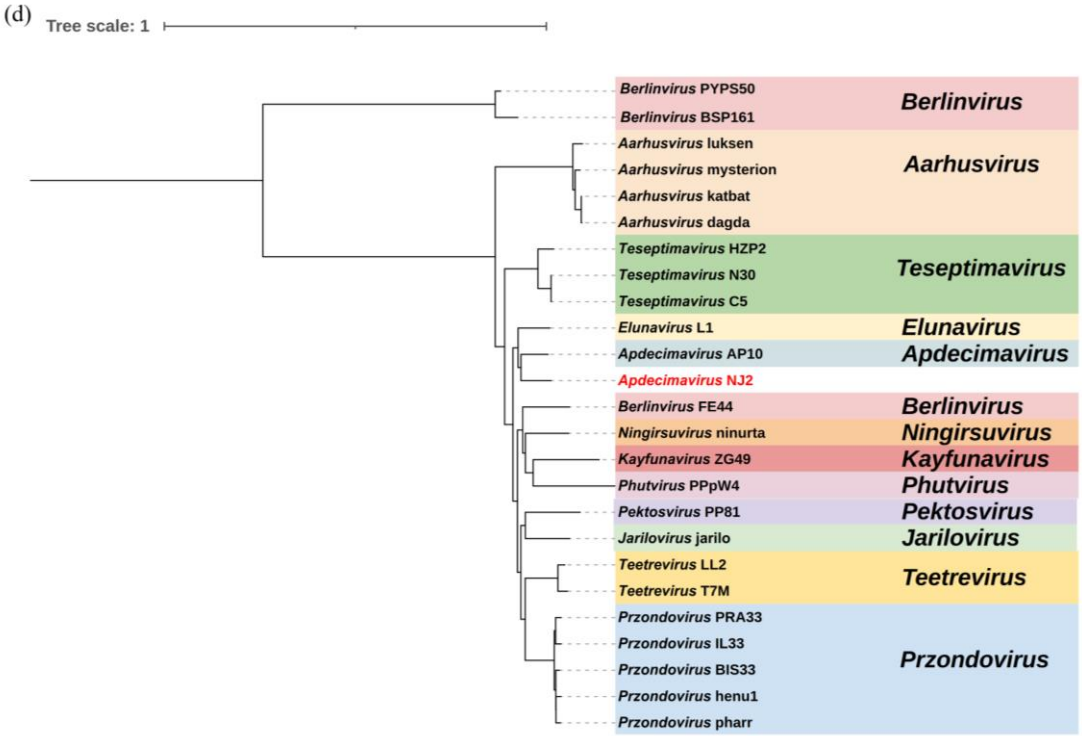
3.3. Genome sequencing, annotation and analysis

Whole-genome sequencing of the novel phage followed by de novo assembly revealed a 39,605-bp linear genome with a GC content of 52.64 %. The NCBI ORF finder (www.ncbi.nlm.nih.gov/orffinder) predicted 47 putative ORFs in the genome, and a putative function was assigned to 27 predicted proteins (Table S4-2). The 27 proteins represented four main categories: (1) phage structure (tail tubular protein A, tail tubular protein B, internal virion protein A, internal virion protein B, internal virion protein C, internal virion protein D, major head protein, capsid assembly protein and head-to-tail connector protein), (2) package (terminase large subunit), (3) release (Rz-like lysis protein, amidase and holin) and (4) replication recombination (phage exonuclease, DNA-directed DNA polymerase, DNA helicase, endonuclease, single-stranded DNA-binding protein, DNA ligase, RNA polymerase and protein kinase). In the Nucleotide BLAST (blastn) of the NCBI database (https://www.ncbi.nlm.nih.gov/), the genome sequence of the phage shared 73.76~78.91 % nucleotide sequence identity (query cover 34~72 %) with 100 phages, all of which are members of the subfamily *Studiervirinae*, family *Studiervirinae*. The genome sequence of the novel phage had the highest query coverage (72 %) with that of *Apdecimavirus* AP10, which then served as a closely related phage in subsequent analyses. The genome map of the novel phage and the blastn comparison of the phage nucleotide sequence with that of *Apdecimavirus* AP10 (KT852574) are shown in Fig. 4-2c. For analyzing the genetic evolutionary relationship of the novel phage, nucleotide sequences of MHP (major head protein), RNAP (RNA polymerase) and terminase large subunit (terL) of related phages were downloaded from the NCBI database. The phylogenetic tree constructed using MHP sequences showed that the novel phage and *Apdecimavirus* AP10 shared the highest homology and were located on the same branch (Fig. 4-2d). The phylogenetic trees based on RNAP and terL had a similar topology to the tree based on MHP (Fig. 4-2e and f). All above results suggested that the isolated phage is a novel species in the genus *Apdecimavirus*, family *Autographiviridae*, and named it *Apdecimavirus* NJ2.


(a)
100 nm


(b)


(c)
GC Skew+
GC Skew-
GC Content
Przondovirus henu1
Apdecimavirus AP10
Apdecimavirus NJ2
35 kbp
5 kbp
30 kbp
10 kbp
25 kbp
15 kbp
20 kbp
Hypothetical protein
capsid assembly protein
host range and adsorption protein
head-tail connector protein
Hypothetical protein
exonuclease
Hypothetical protein
Hypothetical protein
DNA-directed DNA polymerase
Hypothetical protein
Hypothetical protein
Hypothetical protein
DNA primase/helicase
endonuclease
single-stranded DNA-binding protein
Hypothetical protein
amidase
DNA ligase
Hypothetical protein
Hypothetical protein
inhibition of host RNA polymerase
RNA polymerase
Protein kinase
Hypothetical protein
terminase large subunit
Hypothetical protein
Rz lysis protein
holin
tail spike protein
internal virion protein D
internal virion protein C
internal virion protein B
tail tubular protein B
internal virion protein A
major head protein
tail tubular protein A


(d)
Tree scale: 1
Berlinvirus PYPS50
Berlinvirus BSP161
Berlinvirus
Aarhusvirus luksen
Aarhusvirus mysterion
Aarhusvirus katbat
Aarhusvirus dagda
Aarhusvirus
Teseptimavirus HZP2
Teseptimavirus N30
Teseptimavirus C5
Teseptimavirus
Elunavirus L1
Elunavirus
Apdecimavirus AP10
Apdecimavirus
Apdecimavirus NJ2
Berlinvirus FE44
Berlinvirus
Ningirsuvirus ninurta
Ningirsuvirus
Kayfunavirus ZG49
Kayfunavirus
Phutvirus PPpW4
Phutvirus
Pektosvirus PP81
Pektosvirus
Jarilovirus jarilo
Jarilovirus
Teetrevirus LL2
Teetrevirus T7M
Teetrevirus
Przondovirus PRA33
Przondovirus IL33
Przondovirus BIS33
Przondovirus henu1
Przondovirus pharr
Przondovirus

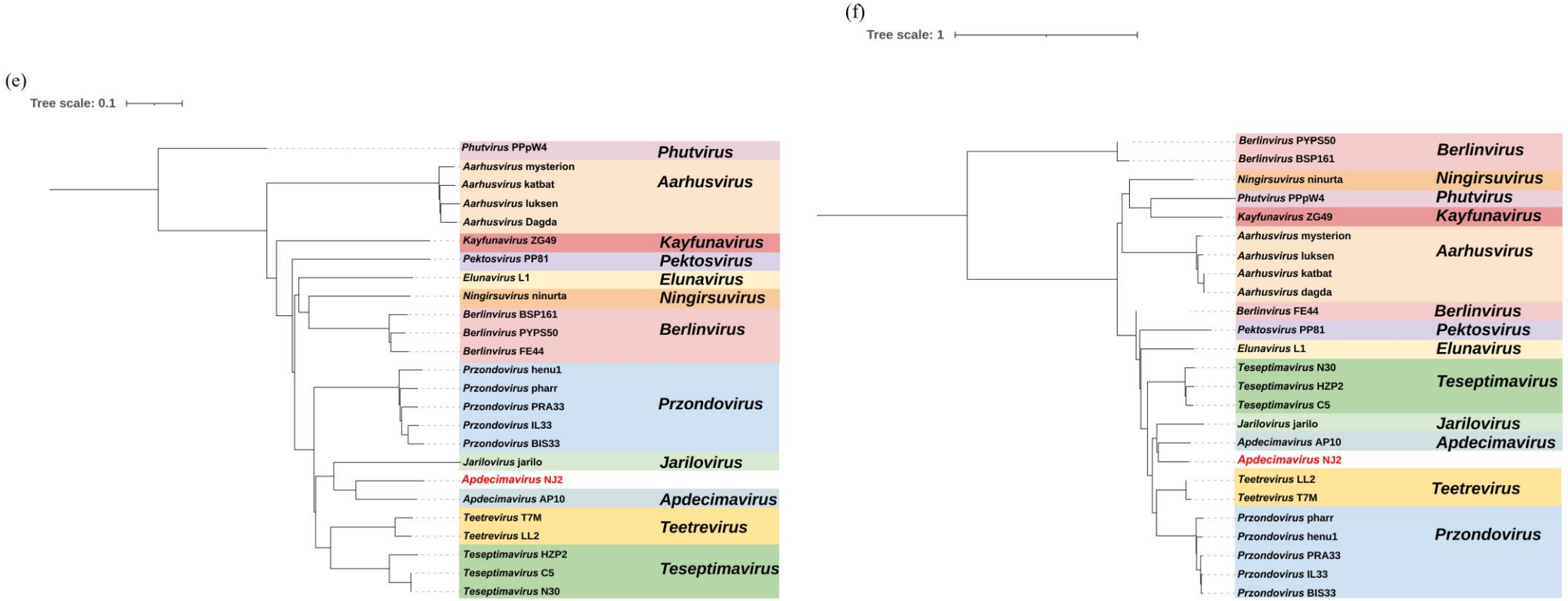


Figure 4-2: Particle morphology, plaque lysis assay, and genomic structure of *Apdecimavirus* NJ2 and phylogenetic trees based on three genes in *Apdecimavirus* NJ2 and 24 other virus species in subfamily *Studiervirinae* using neighbor-joining method. (a) TEM of *Apdecimavirus* NJ2 particle. (b) Phage lysis assay for *Enterobacter cloacae*. (c) Complete gene map, numbering from outside inward: first circle represents open reading frames; the second and third circles are Blastn results for percentage similarity for *Apdecimavirus* NJ2 with *Apdecimavirus* AP10 and with *Przondovirus* henu1 DNA; the fourth circle indicates G+C skew of G-C/G+C; the innermost represents (G+C) mol

3.4. Apdecimavirus NJ2 reduced the nitrogen-fixation capacity of soil by lysing host bacteria

When *Apdecimavirus* NJ2 was added to untreated field soil, the nitrogen-fixation capacity of the soil was 15.36 % lower than in the blank control soil (Fig. 4-3f), and the height (Fig. 4-3a and c), dry mass (Fig. 4-3d), and nitrogen content (Fig. 4-3e) of rice plants was 25.21 %, 64.81 %, and 25.02 % lower than those of plants in the nature soil blank control respectively. When *Apdecimavirus* NJ2 was added to sterilized soil that had been inoculated with the mixture of nitrogen-fixing *Enterobacter* strains (*E. cloacae*, *E. cancerogenus* and *E. ludwigii*), the nitrogen-fixation capacity was 11.35% lower than in the sterilized soil added with nitrogen-fixing bacteria (Fig. 4-3f), and the height (Fig. 4-3b and c), dry mass (Fig. 4-3d) and nitrogen content (Fig. 4-3e) of rice plants were 10.68 %, 26.39 % and 8.79 % lower than those of plants in the sterilized soil amended with nitrogen-fixing bacteria respectively.

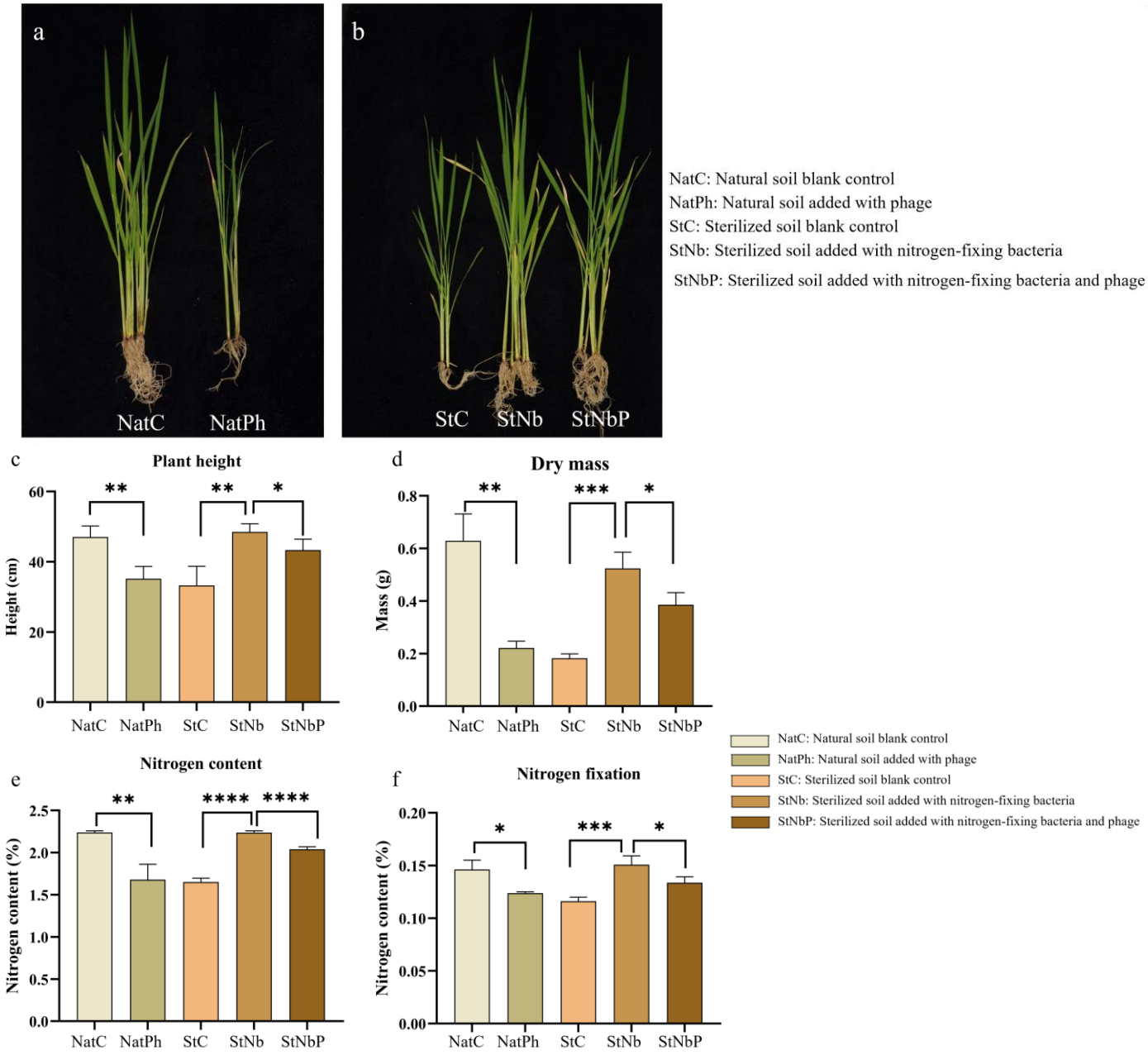


Figure 4-3: Addition of *Apdecimavirus* NJ2 to soil reduced nitrogen fixation capacity of three *Enterobacter* species and inhibited rice growth. (a) Representative phenotypes of rice grown in natural field soil from Nanjing without (NatC) or with *Apdecimavirus* NJ2 (NatPh). (b) Representative phenotypes of rice grown in sterilized field soil (StC) or in sterilized soil with either three nitrogen-fixing *Enterobacter* species (StNb) or with nitrogen-fixing *Enterobacter* species and *Apdecimavirus* NJ2 (StNbP). Effects of the treatments on (c) plant height, (d) dry mass, (e) nitrogen content in rice, (f) nitrogen fixation capacity of *Enterobacter* species in soil. Asterisks above the bars indicate a significant difference between treatment means ($P < 0.05$) in a *t*-test.

3.5. Treatment with Apdecimavirus NJ2 significantly changed the composition and diversity of rice rhizosphere bacteria

After quality control of the 16S rRNA sequence for all the samples, 1,439,290 high-quality sequences were obtained, 1,435,401 were valid sequences, accounting for 99.73 % of the total high-quality sequences. An average of 79,745 valid sequences were obtained for each sample, indicating that the sequencing data were sufficient to cover most of the rice rhizosphere bacteria. The rarefaction curve showed a rapid linear increase in the number of OTUs when the sequencing volume was small. However, when the sequencing volume was larger, the rate of increase in the OTUs gradually decreased, then leveled off, indicating that the amount of sequencing data obtained was sufficient to reflect the species diversity in the samples and thus ensured the reliability of the subsequent analyses (Fig. 4-S1).

In the alpha-diversity analysis of the richness and diversity of the rice rhizosphere bacteria in the different treatments, the Chao1 index (735.03) for the natural soil amended with the novel phage was significantly lower (t test, $P = 0.0461$) than for the natural soil control without the phage (740) (Fig. 4-4a). Similarly, the Shannon index for the natural soil amended with the phage (8.64) was significantly lower (t test: $P = 0.0273$) than for the natural soil control (8.85) (Fig. 4-4b). The Chao1 index for the sterilized soil treated with nitrogen-fixing bacteria (640.12) or with nitrogen-fixing bacteria and phage (596.86) was higher (t test: $P = 0.0160$, 0.5135, respectively) than for the sterilized soil control (588.19), and the index for the sterilized soil amended with nitrogen-fixing bacteria and phage was significantly lower (t test: $P = 0.0137$) than for the sterilized soil amended with only nitrogen-fixing bacteria (Fig. 4-4d). Similarly, the Shannon index for the sterilized soil treated with nitrogen-fixing bacteria (8.09) or with nitrogen-fixing bacteria and phage (7.95) was higher (t test: $P = 0.0229$, 0.5177, respectively) than for the sterilized soil control (7.89), and the index for the sterilized soil amended with nitrogen-fixing bacteria and phage was significantly lower (t test: $P = 0.0460$) than for the sterilized soil amended with nitrogen-fixing bacteria (Fig. 4-4e). The diversity of soil bacteria in the sterilized soil should be very low, so the difference between the sterilized soil and the sterilized soil amended with nitrogen-fixing bacteria and phage was not significant.

The PCoA of the Bray-Curtis distances to assess the beta-diversity of rice rhizosphere bacteria composition revealed significant separation ($P < 0.05$) in the composition of rice rhizosphere bacteria between natural soil amended with the phage and natural soil control at the OTU level (PerMANOVA test: $R^2 = 0.304$, $P = 0.001$; Fig. 4-4c). Thus, the addition of the phage *Apdecimavirus* NJ2 significantly changed the bacterial community composition of rice rhizosphere in the natural and the sterilized soil.

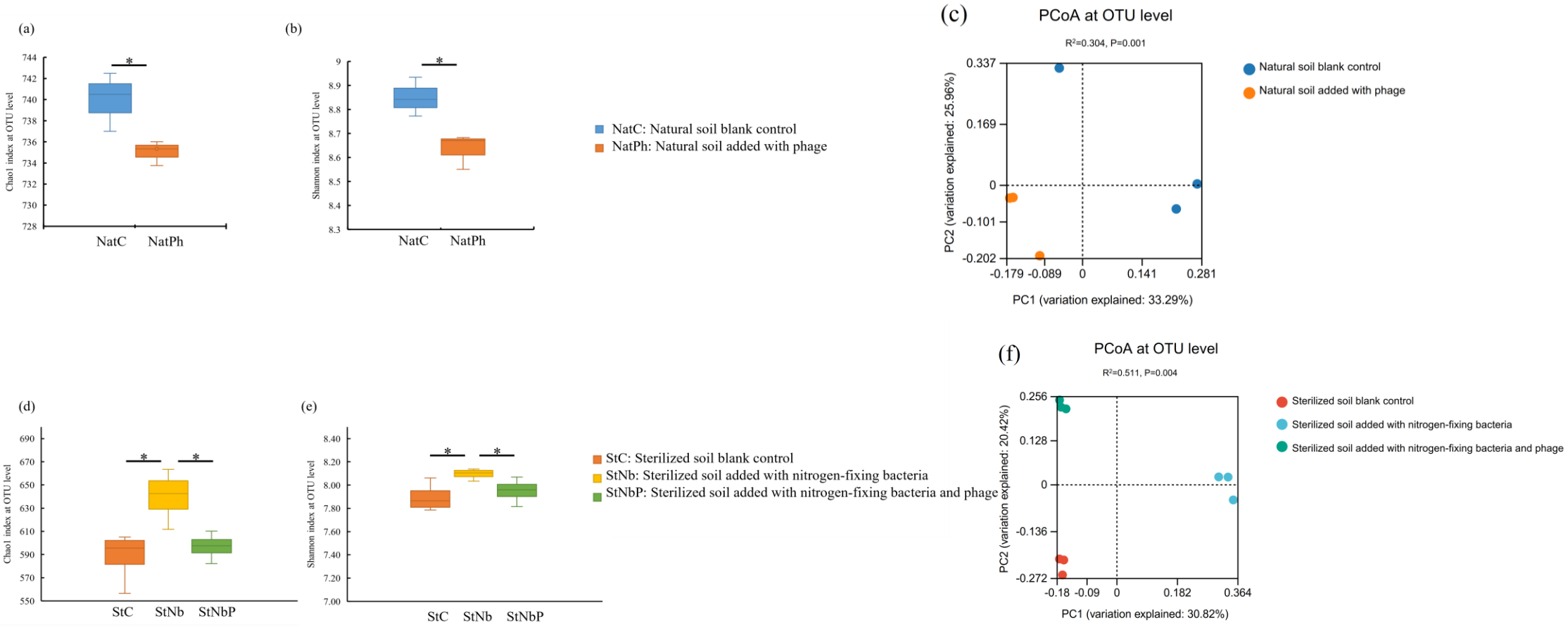


Figure 4-4: Effects of *Apdecimavirus* NJ2 on bacterial diversity in natural and sterilized rhizosphere soil from rice. (a, b) Alpha diversity in natural field soil based on (a) Chao1 index and on (b) Shannon index. (c) PCoA of bacterial community in sterilized field soil. (d, e) Alpha diversity in (d) natural field soil based on Chao1 index and in (e) sterilized field soil based on Shannon index. (f) PCoA of bacterial community in sterilized field soil. Bars on a, b, d and e mean indicate standard error; an asterisk indicates a significant difference between treatments ($P < 0.05$) in a *t*-test.

3.6. The main changing bacterial taxa in rice rhizosphere after Apdecimavirus NJ2 addition

At the genus level, for natural soil, 399 genera were identified in the soil treated with the phage and 379 genera in the blank control; 354 genera were common to both treatment groups; 45 were unique to the soil with the phage (Table S4-3), and 25 were unique to the blank control (Fig. 4-5a). Among the 45 unique genera in the soil treated with *Apdecimavirus* NJ2, two genera have been hypothesized to be nitrogen-fixers and one to be a denitrifier (Aasfar et al., 2021; Gao et al., 2022; Gumaelius et al., 2001). In the sterilized soil, 360 genera were identified after nitrogen-fixing bacteria and the phage were added and 373 after only nitrogen-fixing bacteria were added; 311 genera were common to both treatment groups, 49 were unique to the soil with nitrogen-fixing bacteria and the phage (Table S4-4), and 62 were unique to soil with only nitrogen-fixing bacteria (Fig. 4-5b). Among 49 genera unique to sterilized soil with nitrogen-fixing bacteria and the phage, one genus is presumed to have a nitrogen fixation function and four genera to have a denitrification function.

For the top 30 most-abundant genera of rice rhizosphere bacteria, in natural soil after the addition of phage, the relative abundance of *Hydrogenophaga*, *Pseudomonas* and *Azospirillum* was 13.66 %, 48.19 % and 7.60 %, respectively, higher than in the blank control (Fig. 4-5c). For the sterilized soil after the addition of nitrogen-fixing bacteria and phage, the abundance of *Arenimonas*, *Hydrogenophaga* and *Azospirillum* was 23.55 %, 7.18 % and 76.53 %, respectively, lower than in the sterilized with nitrogen-fixing bacteria treated sterilized soil and the abundance of *Pseudomonas* was 176.19 % higher (Fig. 4-5d).

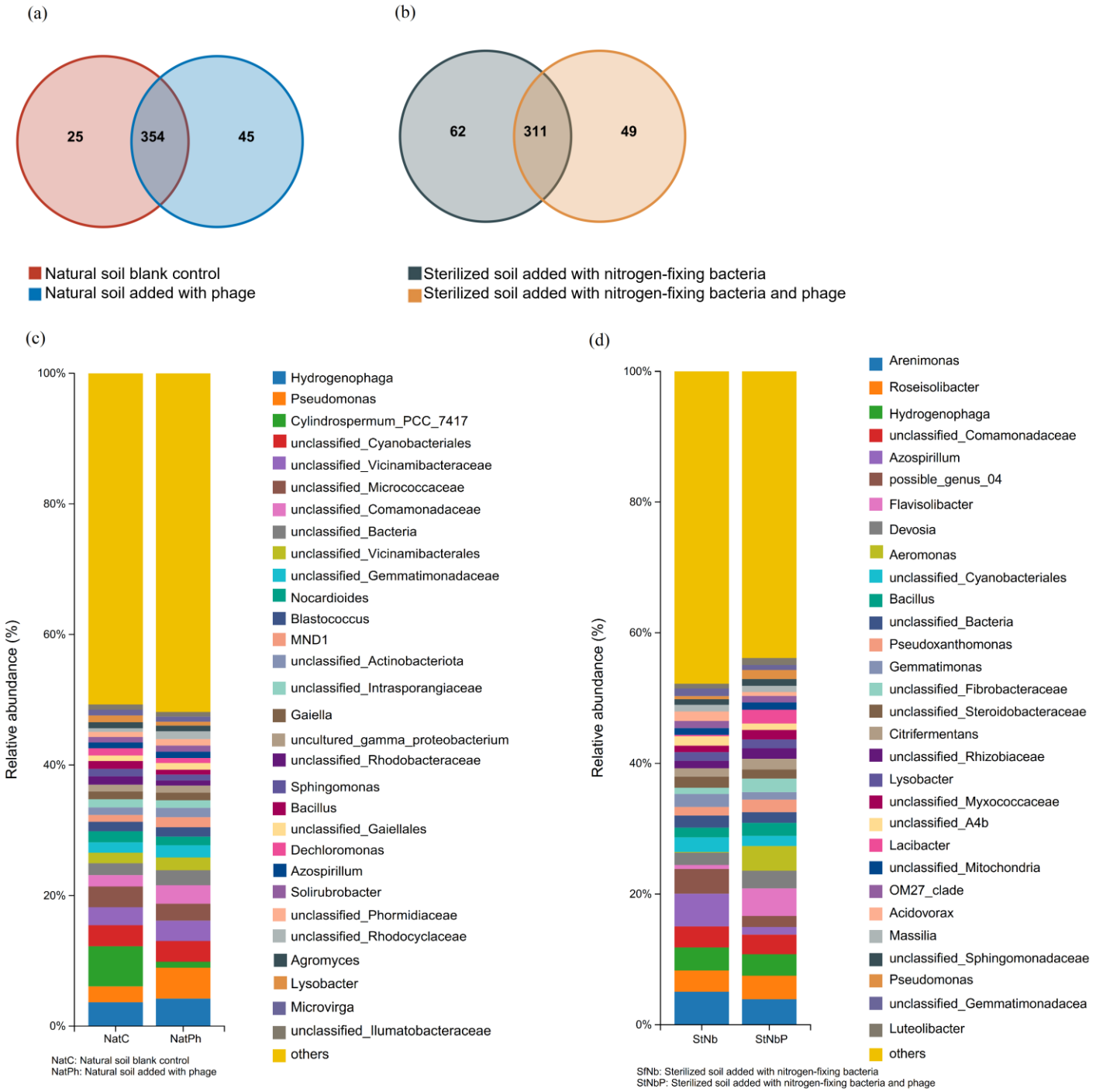


Figure 4-5: Effects of *Apdecimavirus* NJ2 on bacterial community in rice rhizosphere. (a, b) Venn diagrams of the number of bacterial genera shared between treatments and unique to treatments. a, Natural soil blank control vs natural soil with phage *Apdecimavirus* NJ2; b, sterilized soil with nitrogen-fixing *Enterobacter* species vs sterilized soil with nitrogen-fixing *Enterobacter* species and phage *Apdecimavirus* NJ2. (c, d) Top 30 most-abundant genera of rice rhizosphere bacteria in (c) natural soil blank control and with phage *Apdecimavirus* NJ2, (d) sterilized soil with nitrogen-fixing *Enterobacter* species and with nitrogen-fixing *Enterobacter* species and phage *Apdecimavirus* NJ2.

4. Discussion

Soil phage is an important component of soil ecosystems, and its abundance is closely related to the biogeochemical content and cycling (Kuzyakov et al., 2019; Wang et al., 2022a). Biogeochemical elements can influence the growth and diversity of bacteriophage host microbial communities, which in turn indirectly regulates the abundance and community structure of bacteriophages (Liang et al., 2023). Conversely, phages participate in host metabolism through their encoded AMGs, and regulate the abundance of bacteria associated with nutrient cycling which affect the biogeochemical cycling efficiency and content in soil (Tran et al., 2021). In our study, low abundances of nitrogen-fixing bacteria and high abundances of *Enterobacter* phages were found where rice plants had symptoms of nitrogen deficiency in a field of Nanjing, China. But, in our previous study, a high abundance of nitrogen-fixing bacteria and low abundances of *Enterobacter* phages were found in was found in the soil of a field in Jiamusi, Heilongjiang Province which has fertile soil, high levels of nitrogen, and rice grows well (Wang et al., 2022a). Therefore, we hypothesized that the presence of *Enterobacter* phages lead to a decrease in the relative abundance of nitrogen-fixing bacteria and thus results in nitrogen deficiency and poor rice growth, and phage inoculation experiments confirmed this hypothesis. In nitrogen-poor environments, nitrogen-fixing bacteria are crucial for maintaining soil nitrogen levels (Yun et al., 2023). Our findings suggest that high bacteriophage abundance may exacerbate nitrogen deficiency by lysing nitrogen-fixing bacteria, potentially limiting plant productivity in nutrient-poor soils. This role of bacteriophages may explain why some barren soils struggle to naturally regain fertility. Understanding phages' impact is significant for agricultural and ecological restoration. Effective strategies could include using bacteriophage-resistant nitrogen-fixing strains or adjusting soil conditions (e.g., pH, moisture) to reduce bacteriophage activity, thus improving nitrogen supply and supporting plant recovery.

Phages can influence the biogeochemical content in ways that directly or indirectly the structure and functions of bacterial communities (Rodriguez-Valera et al., 2009; Koskella and Brockhurst, 2014). In direct way, by causing the lysis of host bacteria, phages can directly control the abundance of host populations in soils, thus altering their biological functions (Braga et al., 2018; Koskella and Brockhurst, 2014; Rodriguez-Valera et al., 2009). For example, cyanophages are probably responsible for lysing a small but significant portion of the *Synechococcus* population on a daily basis and play an important role in the cycling of nutrients and energy in the ocean (Suttle and Chan, 1994). In wetlands bacteriophages infect and lyse sulfate-reducing bacteria and methanogenic bacteria, decreasing their abundance, potentially repressing sulfate reduction and methane production (Paula et al., 2018). In the present study, after adding the phage *Apdecimavirus* NJ2 to soils, and the abundance of the host *Enterobacter* bacteria was 35.62 % lower than in the control soil, the nitrogen fixation capacity was 15.36 % lower, and plant height, dry mass, and nitrogen content was 25.21 %, 64.81 %, and 25.02 % respectively, lower (Fig. 4-3). These results provide strong evidence that virulent phages can lyse host bacteria, reducing their abundance and impairing their biological functions.

In indirect way, phages effectively lyse host bacteria, which makes space and resources available to other microorganisms, eventually altering the composition of the microbial community in the environments (Ankrah et al., 2014; Wang et al., 2019). Notably, in the present study, the composition and abundance of bacterial species that are not hosts of the phage *Apdecimavirus* NJ2 after the phage was added to nonsterilized paddy soil (Fig. 4-5). For example, the abundance of members of *Azospirillum* (Naqqash et al., 2022), *Kosakonia* (Gao et al., 2022), *Azotobacter* (Aasfar et al., 2021), which are involved in nitrogen fixation and of *Pseudomonas* (Daims et al., 2006), which is involved in nitrification and plant-growth promotion increased. We assume that, after the phage addition, the abundance of nitrogen-fixing host species of *Enterobacte*r decreased, which opened ecological niches for other nitrogen-cycling bacteria, leading to their increased abundance. The addition of the phage *Apdecimavirus* NJ2 decreased the nitrogen content in the soil and in rice plants and adversely affected plant growth, illustrating that phages can be responsible for substantially reduce nitrogen levels in the soil to the extent that nitrogen additions by more abundant bacterial species cannot meet the immediate plant demand for nitrogen.

The selection of phage-resistant bacteria is closely linked to the characteristics of the phage, such as genome size, infection mechanisms, and host range (Labrie et al., 2010). Small-genome phages typically encode fewer functions and have simpler infection mechanisms. Bacteria can acquire resistance through a single mutation or a few resistance mechanisms, making the selection of resistant strains relatively straightforward. In contrast, large-genome phages may carry AMGs, multiple receptor-binding proteins, or immune evasion strategies (Dennehy et al., 2021), requiring bacteria to develop complex resistance mechanisms, which complicates the selection process (Sanson et al., 2013). Narrow-host-range phages infect only specific bacterial species, allowing for a more focused and straightforward selection of resistant strains. However, broad-host-range phages can infect various bacteria, complicating the selection of phage-resistant bacteria due to the need to account for different resistance mechanisms across multiple bacterial species (Hyman et al., 2010). The novel phage NJ2 was isolated from the rice field in Nanjing and characterized here belongs to genus *Apdecimavirus*

by morphology (Adriaenssens et al., 2020) and molecular analysis (Bujak et al., 2022; Lavigne et al., 2008). It has a narrow host range, capable of infecting only *Enterobacter* species among the 17 tested bacteria. This novel phage NJ2 has a dsDNA genome of 39,605 bp (Fig. 4-2c), which is small and simple, containing only the essential genes for phage structure, replication, assembly, and host lysis (Table S4-2). It lacks auxiliary genes related to complex infection mechanisms, such as cell wall-degrading enzymes, virulence factors, immune evasion genes, etc, suggesting a simple infection mechanism. These findings indicate that the phage NJ2 is a small, simple phage with a narrow host range, which suggests that it may be relatively easy to select phage-resistant nitrogen-fixing bacteria in future studies.

This study has identified a new bacteriophage, NJ2, which has been shown to reduce soil nitrogen fixation and plant growth by lysing nitrogen-fixing bacteria, making it a major factor contributing to soil nitrogen deficiency and infertility. To control this phage and improve soil fertility, we will undertake the following work: (1) Identify Key Genes for NJ2 Infection: We will identify the critical genes involved in NJ2 infection to elucidate its mechanism of recognizing and binding to specific receptors on nitrogen-fixing bacteria. This will provide theoretical support for developing phage-resistant nitrogen-fixing strains. (2) Determine Environmental Factors Affecting NJ2: We will identify environmental factors such as soil pH, temperature, humidity, and organic matter content that influence NJ2's distribution, activity, and infection. This will inform soil management strategies. (3) Screen for Phage-Resistant Nitrogen-Fixing Bacteria: We will select nitrogen-fixing strains resistant to NJ2 to provide microbial resources for improving nitrogen-deficient soils. (4) Implement Soil Fertility Improvement Strategies: Finally, we will use comprehensive measures such as adding phage-resistant nitrogen-fixing strains, applying organic fertilizers, and adjusting pH and moisture levels to suppress NJ2 activity, protect nitrogen-fixing bacterial communities, enhance soil fertility, and promote better plant growth conditions.

5. Conclusion

This work highlights the importance of phage communities in paddy soil for nitrogen cycle, because nitrogen-deficient rice plants were associated with low abundance of nitrogen-fixing bacteria in a field in Nanjing where the dominant viruses were *Enterobacter* bacteriophages. From the same field, we identified a novel bacteriophage belonging to genus *Apdecimavirus*, family *Autographiviridae*. By adding this phage to the soil with its host *Enterobacteria* species, the biodiversity and community structure of the soil bacteria changed, and the nitrogen-fixing capacity of the soil microbial community and thus the nitrogen availability for rice plants were reduced. Our results provide new insights on the contributions of phages in altering soil bacterial communities, ecological functions and plant growth. Controlling phages in the soil could be a useful strategy for improving soil nitrogen fixation in the future.

Acknowledgement

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Supplementary Materials

Table S4-1: Host range analysis in lysis assay of phage *Apdecimavirus* NJ2

Bacterial species	Plaque
Enterobacter cloacae	+
Enterobacter cancerogenus	+
Enterobacter ludwigii	+
Enterobacter aerogenes	+
Enterobacter hormaechei	+
Escherichia coli	-
Escherichia fergusonii	-
Dickeya zeaea	-
Dickeya chrysanthemi	-
Erwinia teleogrylli	-
Erwinia pyrifoliae	-
Pectobacterium cacticida	-
Pectobacterium carotovorum	-
Pseudomonas aeruginosa	-
Pseudomonas syringae	-
Yersinia enterocolitica	-
Yersinia rohdei	-

+, plaques observed, indicating lysis; -, no lysis.

Table S4-2: Predicted ORFs for phage *Apdecimavirus* NJ2

Strat	End	Length (bp)	Encoded protein
27	617	591	tail tubular protein A
634	3024	2391	tail tubular protein B
3097	3504	408	internal virion protein A
3507	4121	615	internal virion protein B
4126	6387	2262	internal virion protein C
6405	10382	3978	internal virion protein D
10442	12292	1851	tail spike protein
12292	12495	204	holin
12864	13307	444	Rz-like lysis protein
13309	15063	1755	terminase large subunit
17826	18962	1137	protein kinase
19032	21722	2691	RNA polymerase
22834	23970	1137	DNA ligase
24965	25153	189	inhibition of host RNA polymerase
25294	25992	699	single-stranded DNA-binding protein
26001	26456	456	endonuclease
26457	26912	456	amidase
27061	28788	1728	DNA primase/helicase
29972	32125	2154	DNA-directed DNA polymerase
33081	33995	915	exonuclease
35098	35430	333	host range and adsorption protein
35667	37064	1398	head-tail connector protein
37170	38102	933	capsid assembly protein
38263	39306	1044	major head protein

Table S4-3: Details of 45 genera specific to natural soil after the addition of phage *Apdemavirus* NJ2

Phylum	Class	Order	Family	Genus
Acidobacteriota	*Vicinamibacteria*	*Vicinamibacterales*	*Vicinamibacteraceae*	*uncultured_Acidobacteriaceae_bacterium*
Bacteroidota	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*Cnuella*
Bacteroidota	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*Parasegetibacter*
Bacteroidota	*Bacteroidia*	*Cytophagales*	*Bernardetiaceae*	*Bernardetia*
Bacteroidota	*Bacteroidia*	*Cytophagales*	*Cyclobacteriaceae*	*unclassified_Cyclobacteriaceae*
Bacteroidota	*Bacteroidia*	*Cytophagales*	*Hymenobacteraceae*	*Pontibacter*
Bacteroidota	*Bacteroidia*	*Cytophagales*	*Microscillaceae*	*OLB12*
Bacteroidota	*Bacteroidia*	*Flavobacteriales*	*Flavobacteriaceae*	*Confluentibacter*
Bacteroidota	*Bacteroidia*	*Flavobacteriales*	*Weeksellaceae*	*Chryseobacterium*
Bdellovibrionota	Bdellovibrionia	Bacteriovoracales	Bacteriovoracaceae	Bacteriovorax
Chloroflexi	*Chloroflexia*	*Chloroflexales*	*Chloroflexaceae*	*Chloronema*
Chloroflexi	*Chloroflexia*	*Chloroflexales*	*Herpetosiphonaceae*	*Herpetosiphon*
Cyanobacteria	*Cyanobacteriia*	*Cyanobacteriales*	*Cyanobacteriales_Incertae_Sedis*	*unclassified_Cyanobacteriales_Incertae_Sedis*
Cyanobacteria	*Cyanobacteriia*	*Cyanobacteriales*	*Phormidiaceae*	*Phormidium_IAM_M_71*
Cyanobacteria	*Cyanobacteriia*	*Cyanobacteriales*	*Phormidiaceae*	*Planktothrix_NIVA_CYA_15*
Cyanobacteria	*Cyanobacteriia*	*Leptolyngbyales*	*Leptolyngbyaceae*	*Leptolyngbya_ANT.L52.2*
Cyanobacteria	*Cyanobacteriia*	*Leptolyngbyales*	*Leptolyngbyaceae*	*Leptolyngbya_PC6306*
Cyanobacteria	*Cyanobacteriia*	*Limnotrichales*	*Limnotrichaceae*	*Limnothrix*

Cyanobacteria	*Cyanobacteriia*	*Oxyphotobacteria_Incertae_Sedis*	*Unknown_Family*	*Leptolyngbya_EcFYyyy_00*
Cyanobacteria	*Cyanobacteriia*	*unclassified_Cyanobacteria*	*unclassified_Cyanobacteria*	*unclassified_Cyanobacteriia*
Desulfobacterota	*unclassified_Desulfobacterota*	*unclassified_Desulfobacterota*	*unclassified_Desulfobacterota*	*unclassified_Desulfobacterota*
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Jeotgalibacillus*
Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
Gemmatimonadota	*Longimicrobia*	*Longimicrobiales*	*Longimicrobiaceae*	*unclassified_Longimicrobiaceae*
Hydrogenedentes	*Hydrogenedentia*	*Hydrogenedentiales*	*Hydrogenedensaceae*	*unclassified_Hydrogenedensaceae*
Patescibacteria	*Parcubacteria*	*Candidatus_Kaiserbacteria*	*bacterium_SH4_10*	*unclassified_bacterium_SH4_10*
Patescibacteria	*Saccharimonadia*	*Saccharimonadales*	*LWQ8*	*unclassified_LWQ8*
Patescibacteria	*Saccharimonadia*	*Saccharimonadales*	*unclassified_Saccharimonadales*	*unclassified_Saccharimonadales*
Planctomycetota	*Phycisphaerae*	*Phycisphaerales*	*Phycisphaeraceae*	*SM1A02*
Proteobacteria	*Alphaproteobacteria*	*Alphaproteobacteria_Incertae_Sedis*	*Unknown_Family*	*Acuticoccus*
Proteobacteria	*Alphaproteobacteria*	*Defluviicoccales*	*unclassified_Defluviicoccales*	*unclassified_Defluviicoccales*
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Falsirhodobacter*
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*SM2D12*	*unclassified_SM2D12*
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Porphyrobacter*
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingopyxis*
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Sphingosinicella*
Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*
Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Kosakonia*
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Alcanivoracaceae*	*Ketobacter*

Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Azotobacter*
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*unclassified_Pseudomonadales*	*unclassified_Pseudomonadales*
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Rhodanobacteraceae*	*Chiayiivirga*
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Rhodanobacteraceae*	*Pseudofulvimonas*
Verrucomicrobiota	*Verrucomicrobiae*	*Verrucomicrobiales*	*Verrucomicrobiaceae*	*Verrucomicrobium*

Table S4-4: Details of 49 genera specific to sterilized soil after the addition of nitrogen-fixing Enterobacteria and phage *Apdecimavirus* NJ2

Phylum	Class	Order	Family	Genus
Acidobacteriota	*Acidobacteriae*	*Elev_16S_1166*	*Acidobacterium_sp._WY65*	*unclassified_Acidobacterium_sp._WY65*
Acidobacteriota	*Acidobacteriae*	*PAUC26f*	*unclassified_PAUC26f*	*unclassified_PAUC26f*
Actinobacteriota	*Actinobacteria*	*Corynebacteriales*	*Nocardiaceae*	*Rhodococcus*
Actinobacteriota	*Actinobacteria*	*Micrococcales*	*Micrococcaceae*	*Paenarthrobacter*
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Paludibacteraceae*	*unclassified_Paludibacteraceae*
Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prolixibacteraceae*	*WCHB1_32*
Bacteroidota	*Bacteroidia*	*Chitinophagales*	*Chitinophagaceae*	*UTBCD1*
Bacteroidota	*Bacteroidia*	*Cytophagales*	*Cyclobacteriaceae*	*unclassified_Cyclobacteriaceae*
Bacteroidota	*Bacteroidia*	*Cytophagales*	*Cytophagaceae*	*Rhodocytophaga*
Bacteroidota	Bacteroidia	*Flavobacteriales*	*Flavobacteriaceae*	*Confluentibacter*
Bacteroidota	*Bacteroidia*	*Flavobacteriales*	*Flavobacteriaceae*	*Muricauda*
Bacteroidota	*Bacteroidia*	*Flavobacteriales*	*Weeksellaceae*	*Chryseobacterium*
Bacteroidota	*Bacteroidia*	*Sphingobacteriales*	*Sphingobacteriaceae*	*Solitalea*
Chloroflexi	*Anaerolineae*	*SBR1031*	*A4b*	*uncultured_Chloroflexi_bacterium*
Chloroflexi	*Anaerolineae*	*SBR1031*	*A4b*	*uncultured_gamma_proteobacterium*
Chloroflexi	*Anaerolineae*	*SBR1031*	*uncultured_Gemmatimonadetes_bacterium*	*uncultured_Gemmatimonadetes_bacterium*
Cyanobacteria	*Cyanobacteriia*	*Cyanobacteriales*	*Nostocaceae*	*unclassified_Nostocaceae*
Cyanobacteria	*Cyanobacteriia*	*Limnotrichales*	*Limnotrichaceae*	*Limnothrix*

Desulfobacterota	*Desulfuromonadia*	*Geobacterales*	*Geobacteraceae*	*unclassified_Geobacteraceae*
Desulfobacterota	*Desulfuromonadia*	*PB19*	*unclassified_PB19*	*unclassified_PB19*
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Jeotgalibacillus*
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
Firmicutes	*Bacilli*	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
Firmicutes	*Clostridia*	*Gracilibacteraceae*	*Gracilibacter*	*unclassified_Gracilibacter*
Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
Firmicutes	*Clostridia*	*Peptostreptococcales_Tisierellales*	*Peptostreptococcaceae*	*Sporacetigenium*
Firmicutes	*Clostridia*	*Peptostreptococcales_Tisierellales*	*Thermotaleaceae*	*Anaerosolibacter*
Firmicutes	*Desulfotomaculia*	*Desulfotomaculales*	*Desulfotomaculaceae*	*Desulfotomaculum*
Hydrogenedentes	*Hydrogenedentia*	*Hydrogenedentiales*	*Hydrogenedensaceae*	*uncultured_Fusobacteria_bacterium*
Myxococcota	*Polyangia*	*Polyangiales*	*BIrii41*	*uncultured_proteobacterium*
Myxococcota	*Polyangia*	*Polyangiales*	*Polyangiaceae*	*Sorangium*
Patescibacteria	*Parcubacteria*	*Candidatus_Kaiserbacteria*	*Parcubacteria_bacterium_OLB19*	*unclassified_Parcubacteria_bacterium_OLB19*
Patescibacteria	*Saccharimonadia*	*Saccharimonadales*	*Saccharimonadaceae*	*TM7a*
Planctomycetota	*Phycisphaerae*	*Phycisphaerales*	*Phycisphaeraceae*	*unclassified_Phycisphaeraceae*
Proteobacteria	*Alphaproteobacteria*	*Acetobacterales*	*Acetobacteraceae*	*Roseococcus*
Proteobacteria	*Alphaproteobacteria*	*Elsterales*	*unclassified_Elsterales*	*unclassified_Elsterales*
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Tropicimonas*
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Magnetospiraceae*	*unclassified_Magnetospiraceae*
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Magnetospirillaceae*	*unclassified_Magnetospirillaceae*

Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*unclassified_Rhodospirillaceae*
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Terasakiellaceae*	*unclassified_Terasakiellaceae*
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Erythrobacter*
Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Chromobacteriaceae*	*Vogesella*
Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*
Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Rivibacter*
Proteobacteria	*Gammaproteobacteria*	*PLTA13*	*uncultured_gamma_proteobacterium*	*uncultured_gamma_proteobacterium*
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Halomonadaceae*	*Halomonas*
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Porticoccaceae*	*C1_B045*
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	*Thermomonas*

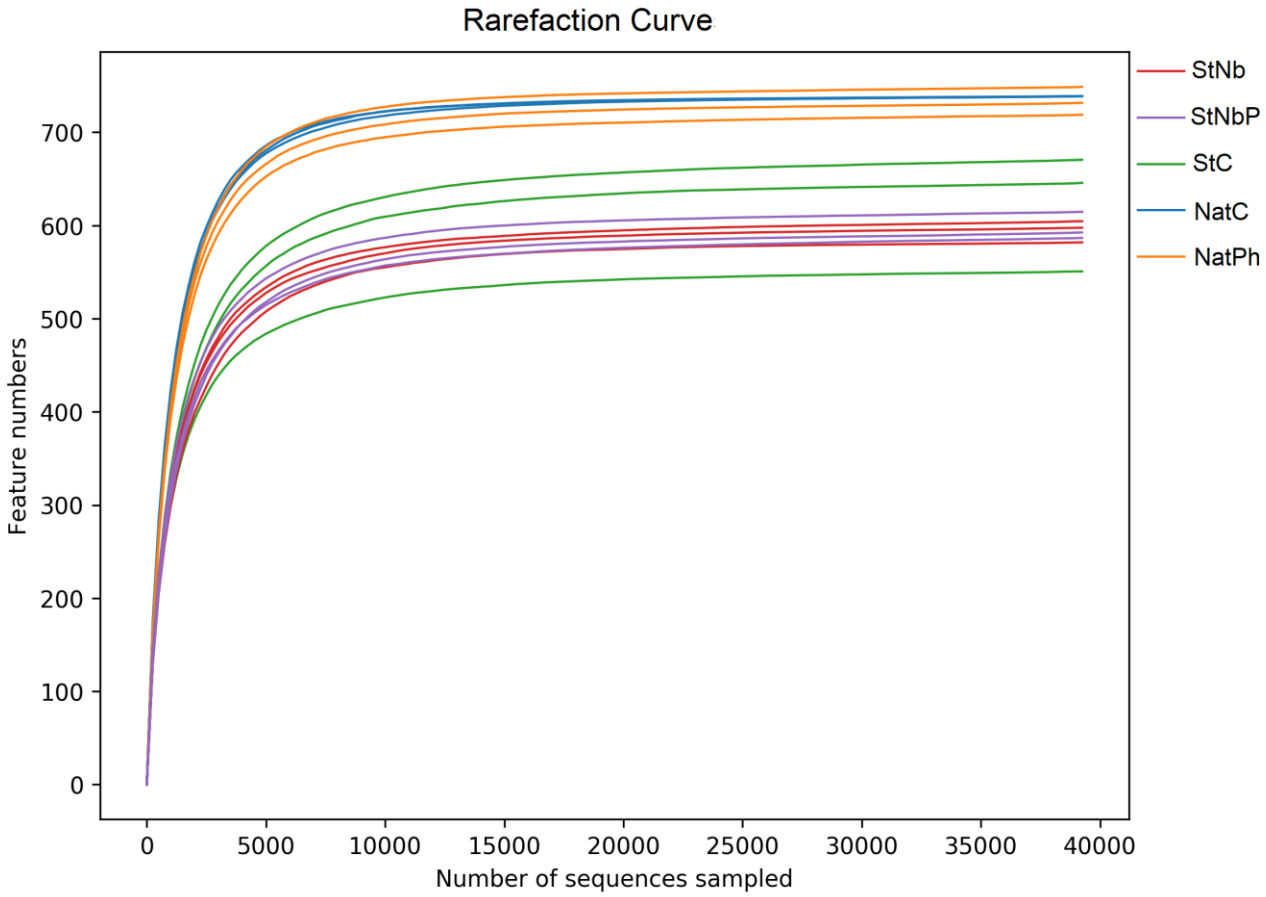

Figure S4-1: Rarefaction curve for bacterial sequences in samples from natural and sterilized soil. The curves tended to be flat, indicating that the amount of sequencing data was reasonable. NatC, natural soil blank control; NatPh, natural soil added with phage; StC, sterilized soil blank control; StNb, sterilized soil added with nitrogen-fixing bacteria; StNbP, sterilized soil added with nitrogen-fixing bacteria and phage.

Chapter 5

Conclusions and perspectives

1. General conclusion

For the study on the impact of insect-transmitted virus on rice growth, RNA-Seq was used to analyze the transcriptomic differences between the resistant and susceptible rice varieties at different times after RSV infection. Through GO annotation, the DEGs related to transcription factors, peroxidases, and kinases of 2 varieties at 3 time points were identified. Comparing these 2 varieties, the number of DEGs associated with these 3 proteins in the resistant variety was lower than in the susceptible variety, with significant regulation trends. Then through KEGG annotation, DEGs involved in some pathways that have a contribution to disease resistance including plant hormone signal transduction and plant-pathogen interaction were found. The results showed that resistance responses regulated by ABA and BR were the same for the 2 varieties, but the one mediated by SA and JA/ET were different. The DEGs in resistant and susceptible varieties at the 3 time points were identified in both PTI and ETI, but most of the DEGs of the susceptible variety were involved in PTI, whereas the number of DEGs involved in ETI in the resistant variety was higher. These results revealed the different responses of resistant and susceptible varieties in the transcription level to RSV infection. Our study provides valuable and novel information to obtain putative resistance-related genes by bioinformatics analysis. In the transcriptome data, we screened two candidate genes, constructed knockout and overexpression vectors through CRISPR/Cas9 gene editing and overexpression technology, and transformed the vectors into rice varieties Zhendao 88 and Wuyujing 3 through *Agrobacterium*-mediated transformation. T0 knockout and overexpression mutant rice plants were successfully obtained, laying the foundation for further verification of the function of candidate genes.

And for the study on the impact of soil virus that lyses nitrogen-fixing bacteria on rice growth, this work highlights the importance of phage communities in paddy soil for nitrogen cycle, because nitrogen-deficient rice plants were associated with low abundance of nitrogen-fixing bacteria in a field in Nanjing where the dominant viruses were *Enterobacter* phages. From the same field, we identified a novel phage, *Apdecimavirus* NJ2, belongs to genus *Apdecimavirus*, family *Autographiviridae*. By adding this phage to the soil with its host *Enterobacter*ia species, the biodiversity and community structure of the soil bacteria changed, and the nitrogen-fixing capacity of the soil microbial community and thus the nitrogen availability for rice plants were reduced. Our results provide new insights on the contributions of phages in altering soil bacterial communities, ecological functions and plant growth. Controlling phages in the soil could be a useful strategy for improving soil nitrogen fixation in the future.

2. Perspectives

2.1 Rice agro-system management

The effects of RSV and the novel phage on rice growth provide specific directions for rice argo-system management. Before sowing, selecting disease-resistant varieties is one of the key measures to prevent rice stripe disease. According to local climatic conditions and soil types, suitable varieties should be planted. Seed disinfection can effectively kill viruses carried on the surface of seeds. During the rice seedling stage, covering with insect-proof nets is an effective physical control measure. Insect nets can prevent feeding by the viruliferous insect vector. The growth of seedlings should be regularly checked, especially for symptoms of rice stripe disease such as curling and striping on the leaves. Once diseased plants are found, they should be promptly removed. Reasonable planting density can ensure good ventilation and light in the field, reduce humidity, and create an environment unfavorable to the occurrence of virus disease. During the tillering stage, monitoring and control of insect vectors should be strengthened, and weeds should be promptly removed to reduce the hiding places of insect vectors (Liu, 2018). If nitrogen deficiency is found in the rice field, urea can be applied to provide nitrogen nutrition for rice. Increased application of organic fertilizers can improve soil structure, improve the soil's ability to retain fertilizer and water. And the humus in organic fertilizers can adsorb and preserve nitrogen to prevent nitrogen loss, and as soil microbes decompose it, nitrogen can be slowly released for long-term absorption and utilization by rice (An et al., 2015).

2.2 Biocontrol on plant viruses

Biocontrol on viruses is an environmentally friendly method that uses biological resources to control the spread and infection of viruses. Here are some effective biocontrol strategies: (1) Biopesticides such as nucleotides, glycine, fatty acids, fungi proteins, and oligosaccharides, not only supplement leaf nutrients and induce plants to enhance resistance but also form a protective film on the leaf surface, reducing the likelihood of virus transmission (Xu et al., 2022b). (2) Some antibiotics, such as Ningnanmycin and Peptide Mycin, can inhibit the proliferation of viral diseases to some extent (An et al., 2019). (3) Botanical pesticides such as matrine and osthole, can effectively control viruliferous insect vectors, reduce the spread of viral diseases, and directly

inhibit the proliferation of viral diseases, alleviating their harm (Xu et al., 2020). (4) Some beneficial bacteria can induce ISR in plants. They can colonize the rhizosphere of plants and activate the plant's own immune system, making the plants resistant to virus infections (Pršić and Ongena, 2020). (5) Utilizing natural enemies can manage pest populations and reduce the spread of viruses, for example, using *Harmonia axyridis* Pallas to control aphids (Li et al., 2023a). (6) The use of disease-resistant varieties and genetic engineering methods can effectively prevent and control plant virus diseases, reduce the use of chemical pesticides, protect the environment, and also increase crop yield and quality (Cao et al., 2019). By using these methods, it is possible to effectively control the spread and infection of viruses while not using or reducing chemical pesticides, thus protecting crops and the environment.

2.2.1 Breeding of disease-resistant varieties

The cultivation of disease-resistant varieties is an important way to improve crop yield and quality, relying on in-depth research into plant disease resistance (Sha et al., 2023). The latter is divided into non-host resistance and host resistance, with non-host resistance exhibiting broad-spectrum and durable characteristics, while host resistance is usually controlled by specific disease resistance genes (Hafez et al., 2020; White et al., 2021). The discovery and utilization of disease resistance genes are key steps in breeding disease-resistant varieties, including qualitative resistance genes and quantitative resistance genes. Qualitative resistance is typically controlled by major effect genes, while quantitative resistance involves the cumulative effects of multiple genes (Roux et al., 2014). Breeding methods for disease-resistant varieties include traditional hybrid breeding, molecular marker-assisted selection, genomic selection, and gene editing technologies such as CRISPR/Cas9 (Hasan et al., 2021; Moser et al., 2009; Xie et al., 2022). Through molecular marker-assisted selection, disease resistance genes can be introduced into crop varieties to enhance their resistance to specific diseases (Das et al., 2017). Genomic selection uses whole-genome data to predict the breeding value of individuals, thereby selecting individuals with desirable traits (Meher et al., 2022). Additionally, research has found that the expression of certain genes is related to plant disease resistance, such as the *OsPsaL* gene in rice, which plays a role in photosynthesis and is associated with disease resistance and yield increase through CRISPR/Cas9 (Zhang et al., 2024). These findings help to understand the mechanisms of plant disease resistance and provide potential gene resources for breeding new disease-resistant varieties. Future directions for breeding disease-resistant varieties include expanding the collection and utilization of germplasm resources, constructing the plant pan-genome, identifying plant immune receptors, elucidating the interaction mechanisms between *Avr* genes and *R* genes, and balancing immunity and crop yield through the regulation of plant hormone signaling networks and epigenetics (Hao et al;., 2022; Rudolf et al., 2024; Yang et al., 2020). These studies will help to improve crop disease resistance and yield, ensuring food security.

2.2.2 Application of gene editing in breeding disease-resistant varieties

Gene editing technology, especially the CRISPR/Cas9 system, has shown great potential and application value in the breeding of disease-resistant varieties. This technology allows researchers to make precise modifications to specific genes in the plant genome, including insertions, deletions or replacements, thereby achieving targeted improvement of plant traits (Liu et al., 2019). In disease-resistant breeding, gene editing technology can directly act on the susceptible genes of plants, conferring new disease resistance to plants by mutating these genes. For example, a study used CRISPR/Cas9 technology to induce targeted mutations in the susceptibility gene Mlo of wheat, resulting in wheat varieties with broad-spectrum and durable resistance to powdery mildew. Additionally, through genome editing, precise manipulation of the susceptible gene MLO-related genetic alleles in major wheat varieties has quickly led to the development of new germplasm with broad-spectrum resistance to powdery mildew while maintaining high yield and quality, providing a new pathway for the practical application of susceptibility genes in disease-resistant breeding (Li et al., 2022a). Besides wheat, other crops such as soybeans and rice have also been bred for disease resistance using gene editing technology. For instance, knocking out the GmWRI1a gene in soybeans using CRISPR/Cas9 technology can increase oil content, editing the promoter of the xa23 gene in rice enhances resistance to bacterial blight (Wang et al., 2024; Yang et al., 2024). Furthermore, the application of gene editing technology is not limited to editing single genes, it can also introduce new allelic genotypes into crops through precise large fragment insertion techniques, such as the application of *RBL1*$^{\Delta 12}$ in rice, which enhances the broad-spectrum disease resistance and stable yield of the crops. It is noteworthy that the application of gene editing technology also involves a deep understanding of the interactions between pathogens and hosts, as well as research on the molecular mechanisms and signaling pathways of disease-resistant genes (Sha et al., 2023). Based on the transcriptomic data, we screened two candidate susceptibility genes and created knockout and overexpression mutants. Susceptibility genes are also important genetic resources, through in-depth study and rational utilization, we can better explore the plant's own disease resistance potential and enrich the database of disease resistance gene resources (Li et al., 2022a). Compared with traditional breeding methods, the manipulation of susceptibility genes through technologies such as gene editing can achieve precise and targeted improvement of plant disease resistance. Known

susceptibility genes can be directly knocked out, avoiding a large amount of hybridization and screening work in traditional breeding, and greatly improving breeding efficiency and accuracy. And gene editing technology can accurately edit susceptibility genes without affecting other desirable agronomic traits of plants (Manzoor et al., 2024). These studies help reveal the balance mechanisms between crop yield and disease resistance, providing new strategies and technical routes for breeding high-yielding, disease-resistant crop varieties. In summary, gene editing technology has significant advantages and broad application prospects in the breeding of disease-resistant varieties, promising to bring revolutionary changes to agricultural production.

2.2.3 Application of genetically modified organisms in the world

The application of genetically modified organisms (GMOs) varies around the world, according to the International Service for the Acquisition of Agri-biotech Applications (ISAAA), more than 19 million hectares of genetically modified (GM) crops were planted in 29 countries in 2019, including 19 developing countries. China allows the production and sale of genetically modified (GM) food products, but there are strict regulatory and approval procedures. China implements strict safety evaluation and management of GMOs, and only GM products that have been scientifically evaluated and proven to be safe and in compliance with the relevant regulatory requirements are authorized to enter the market. Meanwhile, clear labeling is also required for GM foods to protect consumers' right to know and right to choose. The United States is the world's largest grower of GM crops, with soybeans, corn, cotton, oilseed rape and other major GM crops. The U.S. does not have comprehensive legislation specifically for GMOs at the national level, and for GM foods, the U.S. adopts a three-power, decentralized management system, and on the labeling of GM foods, the U.S. has long adopted the principle of voluntary labeling of GM foods at the federal level. Until December 2018, the U.S. stipulated that foods involving GM can be labeled through text, icons, QR codes and other forms on packaging, and from January 1, 2022, it will be mandatory. The EU is the world's largest importer of GM crops, mainly for feed and processing. However, the EU has very limited cultivation of GM crops and more extensive and strict restrictions on GMOs. In Europe, restrictions on GMOs are more extensive. France, Switzerland, Austria, Germany, Bulgaria, Hungary, and Poland are among the countries with total or partial bans on GMOs. In Italy, 16 of the country's 20 regions are declared GMO-free in agriculture. EU related products have been widely used in many fields, such as enzymes, food additives and novel food, and have become an important part of the food industry. To summarize, the U.S. is more open to the application of GM technology, while the European Union has stricter restrictions on the cultivation and use of GM crops. China, on the other hand, has adopted a middle ground by allowing GM products to enter the market after rigorous evaluation and labeling (Li et al., 2023b; Turnbull et al., 2021; Zhang et al., 2022b). The application of GMOs still faces a great deal of testing.

2.3. Applications of transcriptomics in plant disease resistance

Through transcriptomics technology, genes and signaling networks associated with plant disease resistance can be identified. The plant innate immune system consists of two layers of immune responses, PTI and ETI, and that ETI is a major target for crop disease resistance breeding. Through the application of genomic and transcriptomic technologies, a series of different types of broad-spectrum disease resistance and regulatory genes have been identified and cloned, elucidating the molecular mechanism of broad-spectrum disease resistance in plants (Li et al., 2020). In a study, transcriptomic analysis revealed that the dynamics of the transcription factor *TaWRKY24* was significantly and positively correlated with the synthesis of indole acetaldehyde and melatonin during wheat infection with Fusarium crown rot, as well as the dynamics of the key enzymes in this synthesis pathway. This transcription factor was able to activate the expression of the rate-limiting key enzyme genes of the melatonin synthesis pathway and maintain its enzymatic activity under pathogen stress conditions, thereby enhancing the disease resistance of wheat (Xu et al., 2024). In the study of black rot disease in cauliflower seedling, through RNA-Seq technology, researchers conducted transcriptome analysis of resistant variety EC-247 and susceptible variety Y1-2, and identified 3,195 DEGs, which were mainly enriched in phenylpropane metabolism, secondary metabolite synthesis, response to adversity and MAPK signaling pathway (Yao et al., 2020). In the study of pepper rust resistance, through transcriptome and metabolome analysis, significant differences in DEGs and metabolites between resistant and susceptible varieties were found, and genes and metabolites related to phenylpropanoid metabolism were highly enriched in the resistant varieties, suggesting that phenylpropanoid metabolism may mediate the mechanism of rust resistance in peppers (Han et al., 2023). In addition, a novel mechanism for the activation of plant disease resistance proteins was found to involve TIR structural domain proteins, which are activated by substrate-induced phase separation to activate the immune response in plants (Song et al., 2024).

Transcriptomics has also been used to study plant responses to abiotic stresses such as heavy metal stress. Among the highly expressed genes in the roots of metal hyperaccumulating plants, some belong to the ZRT/IRT-like protein (ZIP) transporter gene family, which absorb and transport heavy metals in the roots of hyperaccumulating plants (Li, 2023). Genome-wide association analysis (GWAS) revealed that the *Arabidopsis* transcription factor REPLUMLESS (RPL) plays a role in promoting both disease resistance and growth. RPL

can directly bind to the promoter region of the GH3 gene and inhibits the expression of this gene. Further inhibition of the transport and expression of the pathogen effector proteins and reduction of the pathogen propagation leaded to inhibit the occurrence of disease. RPL can also affect the accumulation of flavonoids by binding to the promoter of the CHI gene, relieving the inhibitory effect of flavonoids on growth hormone transport, and ultimately promoting growth (Xu et al., 2022a). In summary, the application of transcriptomics in plant disease resistance research includes identifying disease resistance genes, analyzing plant hormone regulatory mechanisms, studying plant responses to abiotic stresses, and coordinating plant growth and immunity, which provides an important theoretical basis and technical support for plant disease resistance breeding and molecular mechanism research.

2.4. Application of nitrogen-fixing bacteria

Nitrogen-fixing bacteria are a class of microbes that can convert atmospheric nitrogen into plant-available ammonia, and they have important applications in agriculture and environmental management. The development and application of nitrogen-fixing bacterial agents is an effective strategy to replace chemical nitrogen fertilizers and promote high-quality agricultural development. Through the use of nitrogen-fixing bacteria, dependence on chemical nitrogen fertilizers can be reduced, energy can be saved and crop yields can be increased (Ai et al., 2024). Compared with single microbial agents, synthetic flora can reduce the metabolic burden of individual strains, improve the overall execution efficiency of ecological functions, and enhance the ability to withstand environmental fluctuations. Currently, multifunctional synthetic flora, including biological nitrogen fixation, have been constructed and applied to a variety of cereal crops, including bioprophylaxis and biocontrol (Ai et al., 2024). The imposition of nitrogen-fixing bacteria can enhance nitrogen fixation in plants and effectively improve their disease and stress resistance. For example, the application of nitrogen-fixing cyanobacteria not only fixes nitrogen but also reduces the rate of nitrogen loss from the soil (Hu et al., 2021). The application of nitrogen-fixing bacteria is of great significance to sustainable ecological development, and they can reduce the use of chemical fertilizers and environmental pollution. Nitrogen-fixing bacteria also have certain applications in environmental management. In the process of water treatment, nitrogen-fixing bacteria can help remove nitrogen from the water and reduce the risk of eutrophication of water bodies. Nitrogen-fixing bacteria can improve the soil structure, increase the organic matter content in the soil, and improve the fertility of the soil (Hu et al., 2021). Nitrogen-fixing bacteria have great prospects for future application, and they will play an important role in increasing crop yields, enhancing crop resistance, reducing environmental pollution and realizing sustainable development of agriculture. Synthetic biology, which has emerged in the 21st century, will provide a revolutionary solution for the agricultural application of biological nitrogen fixation. Through synthetic biology technology, it is possible to create artificial high-efficiency biological nitrogen fixation systems, which include enhancing the efficiency of rhizosphere association nitrogen fixation, constructing nitrogen-fixing cereal crops, and realizing the autonomous nitrogen fixation of eukaryotes (Yan et al., 2021). Artificial high-efficiency biological nitrogen fixation technology has the advantages of being green, energy-saving and emission reduction, and every ton of nitrogen fertilizer used can save a lot of energy and reduce greenhouse gas emissions, which is important for achieving the goal of "carbon neutrality" (Yan et al., 2021).

2.5. Biocontrol and application of phages

This study isolated the phage *Apdecimavirus* NJ2 from soil samples in Nanjing, which has lytic activity against three nitrogen-fixing *Enterobacter*iaceae. Adding *Apdecimavirus* NJ2 to untreated field soil and sterilized soil containing nitrogen-fixing bacteria resulted in a decrease in soil nitrogen-fixing capacity and nitrogen content, significantly affecting rice growth. This result provides direct evidence that phages inhibit biogeochemical cycles through lysis. To control this phage and improve soil fertility, we will undertake the following works in the future: (1) Identify key genes for phage infection nand target knockout operations on the genes of the phage. For example, using the gene editing technology CRISPR-Cas9, different genes can be gradually mutated from the phage, and then tested for their ability to recognize and bind to the host bacteria (Yuan and Ma, 2020). In addition, the sequences of receptor proteins of different phages can be compared to identify highly conserved regions or amino acid residues. These conserved sequences may be closely related to the recognition and binding functions of phages, providing clues for identifying binding sites. Meanwhile, the role of key sites in the conserved sequences can be verified in combination with mutant experiments (Boeckaerts et al., 2020). (2) Determine environmental factors affecting phage infection: Under the condition that other factors remain consistent, only a specific environmental factor changes, such as temperature, humidity or pH, and then the changes in the activity, propagation rate, and infectivity of the phage are observed (Li et al., 2022b). This will inform soil management strategies. (3) Screen for phage-resistant nitrogen-fixing bacteria: nitrogen-fixing strains resistant to the phage will be selected to provide microbial resources for improving nitrogen-deficient soils. Finally, comprehensive measures such as adding phage-resistant nitrogen-fixing strains, applying organic fertilizers, and adjusting temperature, pH and moisture levels to suppress phage activity, protect nitrogen-fixing

bacterial communities, enhance soil fertility, and promote better plant growth conditions.

Despite the adverse effects of this phage on rice growth, phages have promising applications in the future. Phages have important roles in regulating soil microbial communities, promoting plant health, improving soil quality, and preventing and controlling soil-borne diseases (Wang et al., 2019). Phages are able to precisely lyse specific soil-borne pathogens and reduce the number of these pathogens, thus reducing the occurrence of diseases. For example, phage therapy has been applied in the control of plant diseases such as green wilt, which reduces the spread of soil-borne diseases and promotes the growth of crops by lysing host pathogens (Wang et al., 2019). By lysing their host bacteria, phages can change the structure and diversity of soil bacterial communities, which in turn affects nutrient turnover and ecological functions in the soil (Gao et al., 2022). Phage therapy helps to improve soil quality and promote the application of this method in soil pollution management and face source pollution control by regulating the cycling of life elements and the detoxification and transformation of pollutants (Ye et al., 2020). Phages can reduce bacteria that are harmful to plant roots, thus indirectly promoting healthy plant growth (Yang et al., 2023). Phages may help reduce heavy metal pollution in soil by regulating the metabolic process of bacteria on heavy metals through horizontal gene transfer with microbial hosts (Huang et al., 2021). By killing bacteria, phages reduce the mineralization of soil organic carbon and affect the soil carbon cycle (Wei et al., 2021). Phages can stimulate plant rhizosphere defense mechanisms and enhance plant resistance to soil-borne pathogens (Wang et al., 2024). Phages can be used as bioindicators of soil condition and pollution levels (Ye et al., 2020). The narrow lysis spectrum of this phage limits its application, and one of the future research directions is to determine how to screen phage with wide lysis range and how to apply a cocktail pattern of phage mixtures. Meanwhile, the safety issue of phage use is also the focus of research, and people are beginning to focus on the direct use of lysins derived from phage (Wu et al., 2019; Zhou et al., 2021). The study of soil phages helps us to better understand the mechanism of viruses in soil ecosystems and provides new research perspectives for soil ecology and environmental microbiology. With further research, these potential applications of soil phages will bring new opportunities in the fields of sustainable agriculture, environmental protection and biotechnology.

2.6. Applications of 16S rRNA Sequencing

16S rRNA sequencing technology has been widely used in agricultural soil management. By analyzing 16S rRNA gene sequences in soil samples, it is possible to assess the impact of different agricultural management practices on soil microbial diversity, such as fertilizer application, irrigation and tillage practices (Yang et al., 2019). 16S rRNA sequencing can reveal the structural composition of soil microbial communities including bacteria, archaea, and fungi, which can be helpful to understand the impact of different agricultural activities on soil microbial communities. The structure and function of soil microbial community are closely related to soil fertility, and 16S rRNA sequencing can help assess soil microbial diversity and abundance of beneficial and pathogenic bacteria, thus providing a basis for soil improvement and management (Liu et al., 2018b). By analyzing the microbial community in plant rhizosphere soils, beneficial microbes related to plant health can be identified, providing guidance for disease control (Luo et al., 2022). 16S rRNA sequencing can help to predict and analyze the metabolic functions of soil microbes in agro-ecosystems, such as decomposition of organic matter and nutrient cycling, and then optimize the agricultural management measures to improve soil productivity (Maretto et al., 2022). By comparing the structure of soil microbial communities under different environmental conditions, the effects of environmental factors such as soil pH, temperature, humidity on microbial communities can be revealed (Jiang et al., 2023). 16S rRNA sequencing technology, combined with neutral and null modeling analysis, can reveal the deterministic and stochastic processes of microbial community assembly, which can provide important information for understanding the mechanism of microbial community construction (Dini-Andreote et al., 2015). 16S rRNA sequencing can be used to assess the effects of soil pollution, such as heavy metal contamination, on microbial community structure and provide scientific basis for soil pollution remediation (Hur and Park, 2019). With the rapid development of sequencing technology, 16S rRNA sequencing has been widely used in various microbial diversity analyses. Although there are still some problems in sequence specificity, accuracy of sequencing technology and data processing, with the emergence of multi-group primer design, more precise sequencing instruments and continuously optimized data processing software, highly accurate and high-quality 16S rRNA analyses will be realized (Wang et al., 2024c). 16S rRNA sequencing technology plays a crucial monitoring role in pandemics, which can help humans to predict and warn of pandemic diseases. In the early stage, when specific pathogenic bacteria increase significantly in the population, corresponding prevention and control measures can be taken, such as vaccination and early treatment, to effectively prevent the outbreak and spread of the disease (Wang et al., 2024c). 16S rRNA sequencing will also become a new trend to be used in conjunction with metagenomic sequencing, metabolomics and other omics technologies. Among them, 16S rRNA sequencing is mainly used to identify and classify bacteria and understand the structure of microbial populations; metagenomic sequencing can obtain information on gene composition and function, and related metabolic pathways; metabolomics can obtain the

relative relationship between metabolites and physiological and pathological changes, and the cross-fertilization of multiple methods can provide more accurate information for early warning, prevention and control of diseases (Wang et al., 2024c). Overall, the future application prospects of 16S rRNA sequencing technology are very broad, and it will play an important role in improving the accuracy of microbial analysis, epidemic disease surveillance, other omics research, interdisciplinary integration, and environmental and agricultural fields.

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Appendix-Publications

1. **Liu Y**, Wang Y, Shi W, Wu N, Liu W, Francis F, Wang X, 2024. *Enterobacter*-infecting phages in nitrogen-deficient paddy soil impact nitrogen-fixation capacity and rice growth by shaping the soil microbiome. Science of the Total Environment. 956: 177382.
2. **Liu Y**, Liu W W, Li L, Francis F, Wang X F. Transcriptome analysis reveals different response of resistant and susceptible rice varieties to rice stripe virus infection. Journal of Integrative Agriculture, 2023, 22(6): 2–14.
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