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RESEARCH ARTICLE

Integrated Analysis of Transcriptomic and Metabolomic Responses in Rice Resisting False Smut

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Abstract: Rice false smut, caused by *Ustilaginoidea virens*, is an important fungal disease that reduces both grain yield and quality. To dissect the molecular basis of resistance, we combined transcriptome sequencing and metabolite profiling in resistant and susceptible rice cultivars. Transcriptome sequencing revealed more than 8,500 genes with altered expression patterns after infection, while metabolite profiling detected over 700 significantly changed compounds. Resistant plants showed strong activation of immune regulators, including WRKY transcription factors, receptor-like kinases, and pathogenesis-related proteins, along with enhanced phenylpropanoid metabolism. Metabolite changes included higher levels of antifungal compounds (for example, sakuranetin, ferulic acid, caffeic acid, and luteolin). Network integration implicated salicylic acid, jasmonic acid, and MAPK signaling in coordinating cell-wall reinforcement and suppression of susceptibility pathways. Together, these findings highlight coordinated molecular and metabolic reprogramming that strengthens rice resistance to RFS, providing valuable candidate genes and metabolites for breeding programs.

Keywords: Rice False Smut, *Ustilaginoidea virens*, Transcriptomics, Metabolomics, DEGs, DAMs

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Introduction

Rice (Oryza sativa L.) is one of the most important crops in the world, which is often influenced by fungal diseases. Rice false smut caused by *Ustilaginoidea virens* has been a serious threat because of its negative impacts on grain yield and quality. It is important to elucidate molecular mechanisms of resistance for developing sustainable control measures. Recent advances in transcriptomics and metabolomics have enabled a more holistic approach to study plant defense responses by analyzing gene expression dynamics and metabolite accumulation patterns in parallel. Combined omics approaches have provided insights into the defense signaling pathways, metabolic reprogramming, and resistance-related molecular markers involved in rice response to False Smut Infection (FSD) [1].

To address this challenge, the present study combined transcriptome sequencing with metabolite profiling to examine rice responses to *U. virens*. This dual approach revealed candidate genes, metabolites, and defense-related mechanisms, including hormone-mediated signaling, activation of pathogenesis-related proteins, and regulation of Reactive Oxygen

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Species (ROS) [2]. Figure 1 represents the disease progression of rice false smut following *U. virens* infection. Figure 1 illustrates the progression and visible symptoms of rice false smut caused by *Ustilaginoidea virens*. The figure presents Oryza sativa as the host and shows the pathogen together with infected rice spikelets exhibiting characteristic false-smut fungal masses. Following infection, the affected spikelets develop greenish or yellowish smut balls that may subsequently become greenish-black as the fungal structures mature [3]. These visible symptoms provide a phenotypic representation of the host pathogen interaction investigated in this study and establish the biological context for the subsequent transcriptomic and metabolomic analyses. The observed disease symptoms were particularly relevant to the selection of spikelet tissues for molecular analysis, as false smut infection primarily affects the floral organs where smut balls develop [4]. Transcriptome analysis uncovered Differentially Expressed Genes (DEGs), while metabolomics highlighted defense-associated compounds such as flavonoids, phenolics, and phytoalexins. Integrating both datasets allowed us to construct interaction networks that illustrate how gene regulation aligns with metabolic reprogramming during infection.

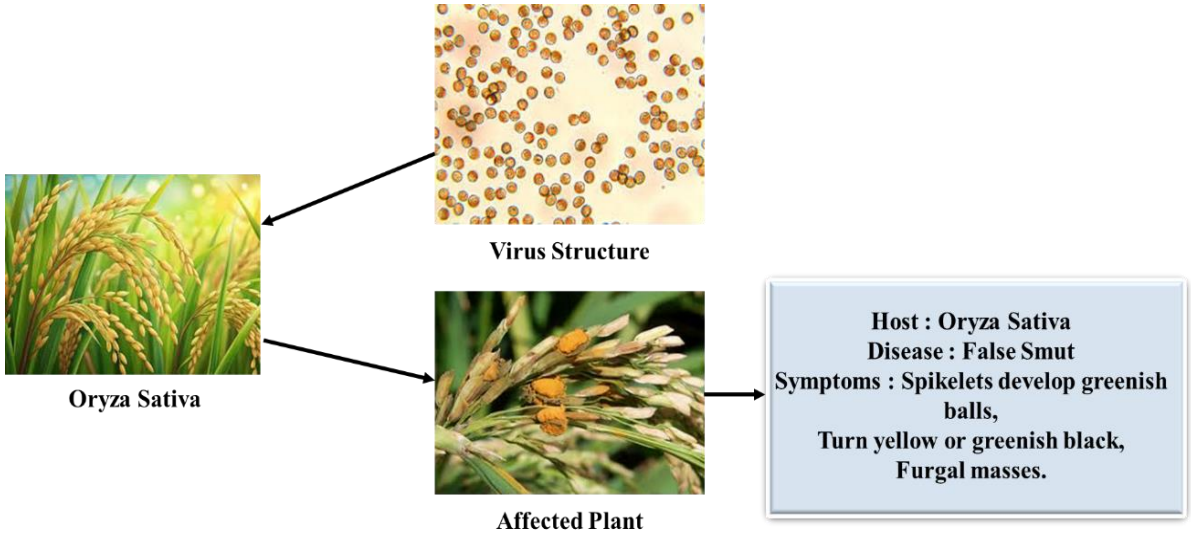

Fig. 1: Disease progression of rice false smut following *U. virens* infection

In summary, our goal is to identify molecular features both genes and metabolites that contribute to resistance against false smut. These insights may inform future breeding strategies or metabolic engineering approaches.

Related Work

False smut and stem rot are among the most damaging fungal diseases of rice, traditionally managed with chemical fungicides. While effective, chemical control raises environmental concerns and often fails to provide durable resistance. Recent advances have therefore focused on understanding the molecular biology of *Ustilaginoidea virens* and exploiting alternative management strategies.

Molecular studies have identified several fungal genes that regulate pathogenicity. For instance, regulators of fungal development such as velvet-complex proteins and transcription factors have been shown to control spore formation, growth, and virulence. Similarly, autophagy-related proteins contribute to fungal survival by recycling cellular components; disruption of these genes reduces mycelial growth and pathogenic capacity, although infection is not entirely eliminated. Such findings highlight how core biological processes in *U. virens* can be targeted to weaken its infection ability [5].

Host pathogen interactions are also influenced by rice susceptibility genes. These genes, when active, create conditions favorable to infection, and genome-editing approaches like CRISPR/Cas9 are being investigated to disable them. Early results suggest that suppressing susceptibility factors can enhance resistance, although trade-offs with plant growth remain a concern.

Alongside molecular genetics, biological control agents from the rice rhizosphere are gaining attention. Reports indicate that strains of Bacillus, Pseudomonas, and Trichoderma contribute to plant growth promotion, activation of defense-related enzymes, and mitigation of disease severity. Their beneficial effects are often linked to phytohormone production, biofilm

formation, and secretion of antifungal metabolites such as iturins and surfactins, which interfere with fungal development and colonization [6].

New technologies are also being explored for disease management. Nanoparticle-based antifungals, particularly silver nanoparticles, have demonstrated inhibitory effects on *U. virens* growth and toxin production in laboratory experiments. Although promising, their integration into field practice requires further evaluation to ensure safety and consistency.

Together, these studies indicate that durable rice resistance will likely require a combination of approaches ranging from molecular dissection of host and pathogen genes to biocontrol strategies and advanced technologies. A summary of representative findings from prior work is provided in Table 1.

Table 1: Summary of methodologies, key findings, advantages, and limitations reported in earlier studies

Citation	Purpose	Methodology	Outcomes	Challenges
[11]	Investigated genetic regulation of pathogenicity factors in *U. virens*	Gene functional analysis and mutant studies	Identified candidate genes influencing virulence and fungal development	Functional roles still unclear under field conditions
[12]	Examined toxin production in false smut balls	Chemical profiling of infected spikelets	Detected ustiloxins and ustilaginoidins with potential toxicity	Exact mechanism of toxicity in hosts remains poorly defined
[13]	Studied role of UvVELC in fungal biology	Deletion and phenotypic assays	Showed its involvement in conidiation, virulence, and growth	Molecular pathway connections remain unverified
[14]	Evaluated toxicological effects of FSM toxins	In vivo mouse model assays	Induced oxidative stress, ROS increase, and reduced mitochondrial activity	Dose response relationships not fully explored
[15]	Tested bioagents for rice false smut control	Application of *Pseudomonas*, *Trichoderma*, and *Bacillus* strains	Enhanced plant defense, improved growth, and lowered disease severity	Long-term field efficacy and scalability uncertain
[16]	Analyzed rice immune response to pathogen	Transcriptomic profiling	Identified genes related to hormone signaling and defense activation	Requires validation in diverse cultivars
[17]	Investigated role of secondary metabolites in defense	Metabolite profiling	Found flavonoids, phenolics, and phytoalexins enriched in resistant lines	Pathway regulation not fully mapped
[18]	Characterized function of UvXlnR	Gene knockout experiments	Demonstrated regulation of growth, conidiation, stress, and pathogenicity	Broader impact on metabolic networks remains unknown
[19]	Assessed role of autophagy in fungal survival	Disruption of UvAtg7 ortholog	Reduced mycelial growth and autophagy, but conidia/infection still occurred.	Cannot completely block pathogenicity
[20]	Explored use of silver nanoparticles (AgNPs) for control	Application of 2 nm AgNPs in assays	Inhibited fungal growth and pathogenicity	Needs integration with toxin-reducing fungicides for full effectiveness

Materials and Methods

Figure 2 outlines the proposed molecular framework underlying rice resistance to false smut, as revealed through integrated omics analysis. Metabolite profiling identified 752 Differentially Accumulated Metabolites (DAMs), while transcriptome sequencing detected 8,552 Differentially Expressed Genes (DEGs). By merging these datasets, we were able to uncover coordinated defense responses. The findings indicate that resistant plants accumulate protective metabolites, activate immune signaling pathways, and upregulate resistance-associated genes, including Pathogenesis Related (PR) proteins and WRKY transcription factors. Together, these results highlight how transcriptional regulation and metabolic reprogramming act in concert to strengthen rice defense against *U. virens*.

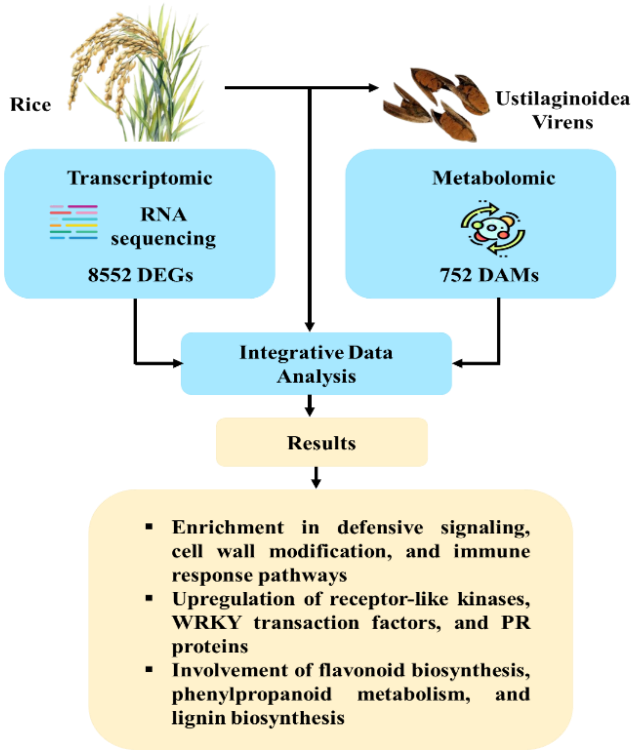

Fig. 2: Integrated transcriptomic – metabolomic framework used to investigate rice resistance to FSD

We harvested three independent biological replicates for each rice cultivar and treatment. Each biological replicate was a pool of spikelet tissues from five individual plants. All experiments were performed in the same environment in order to reproduce the results and to reduce the environmental variation [7].

Figure 2 presents the integrated transcriptomic–metabolomic framework used to investigate rice resistance to false smut caused by *Ustilaginoidea virens*. Transcriptomic analysis identified 8,552 Differentially Expressed Genes (DEGs), while metabolomic profiling detected 752 Differentially Accumulated Metabolites (DAMs). Integration of these datasets revealed coordinated activation of defense-signaling, cell-wall modification, and immune-response pathways, together with the upregulation of receptor-like kinases, WRKY transcription factors, and Pathogenesis Related (PR) proteins. The analysis also highlighted the involvement of flavonoid biosynthesis, phenylpropanoid metabolism, and lignin biosynthesis, indicating that transcriptional regulation and metabolic reprogramming work together to strengthen structural and biochemical defenses against false-smut infection. This framework therefore provides a molecular basis for identifying candidate genes, metabolites, and pathways associated with rice resistance.

Plant materials and Growth Setup

Two contrasting rice (Oryza sativa L.) cultivars were selected for this study: Zhonghua 11, exhibiting resistance to false smut, and Nipponbare, known for its high susceptibility. Plants were cultivated in a greenhouse under controlled conditions, with daytime temperatures maintained around twenty-eight degrees Celsius and nighttime temperatures near twenty-five degrees Celsius, and relative humidity maintained at 70–80%. A light/dark cycle of approximately twelve hours each was applied to mimic natural environmental conditions. Seedlings were established in pots containing sterilized soil and were supplied with adequate water and nutrients to ensure uniform and healthy development.

Maintenance of Pathogen and Inoculation Method

The fungal pathogen *Ustilaginoidea virens* was cultured on Potato Sucrose Agar (PSA) and incubated at 28 °C for approximately one week to promote sporulation. Conidia were collected by rinsing the cultures with sterile water, and the suspension was standardized to about 1×10^{6} spores mL^{-1}. Inoculation was performed at the booting stage by introducing 2–3 mL of the suspension into the leaf sheath [8].

Tissue Collection and Sample Processing

Floral tissues, including spikelets and adjacent structures, were harvested at four intervals following inoculation: immediately before inoculation, and at approximately one, two- and three-days post-inoculation. Spikelets were chosen as the focus since false smut infection primarily occurs in the floral organs, where smut balls develop. Sampling these tissues therefore best reflects the molecular interactions between host and pathogen. The harvested tissues were immediately snap-frozen in liquid nitrogen and kept at ultra-low temperatures around minus eighty degrees Celsius until they were processed for RNA sequencing and metabolomic analysis.

RNA Extraction and Transcriptome Sequencing

RNA extraction was carried out from both infected and control spikelets using TRIzol reagent, followed by purification through clean-up spin columns. The quality and quantity of the extracted RNA were assessed with an Agilent 2100 Bioanalyzer, and only samples of sufficient quality were processed further. Sequencing libraries were prepared and sequenced using the Illumina NovaSeq 6000 system, generating paired-end reads of approximately one hundred fifty base pairs. The raw sequencing reads were analyzed using FastQC software. Trimmomatic was used to trim the adapter sequences and low-quality bases. The clean reads were mapped to the reference genome of Oryza sativa using HISAT2. The number of reads generated for each sample was about 45-55 million paired-end reads, with more than 95% of the bases with a quality score above Q30. The differential expression analysis was carried out using DESeq2. P values were corrected using the Benjamini-Hochberg false discovery rate (FDR) correction method, and genes with an adjusted p-value < 0.05 and an absolute log2 fold change ≥ 1 were considered as significantly differentially expressed [9].

Verification of RNA-Seq Results Using Quantitative Real-Time PCR (qRT-PCR)

To validate the transcriptome sequencing results, we selected ten representative DEGs involved in defense signaling, transcriptional regulation, and secondary metabolism for qRT-PCR analysis. First-strand cDNA was synthesized from 1 μg of total RNA by using a reverse transcription kit following the manufacturer's protocol. Quantitative PCR was performed using SYBR Green chemistry on a Bio-Rad CFX96 Real-Time PCR System. Each reaction was performed in three technical replicates and three biological replicates. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with rice Actin as the internal control gene.

Metabolite Extraction and LC-MS Analysis

For metabolomic profiling, approximately one hundred milligrams of frozen tissue were ground into a fine powder and extracted using a pre-chilled methanol water solution in an 80:20 ratio. The extracts were then centrifuged at twelve thousand rotations per minute for ten minutes at around four degrees Celsius, and the resulting supernatant was subjected to ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). Separation of metabolites was performed on a C18 column using a programmed gradient, and mass spectral data were collected in both positive and negative electrospray ionization modes [10].

Metabolite Annotation and Statistical Evaluation

Metabolites were assigned identities by cross-referencing their mass spectra with entries from public repositories, including KEGG and METLIN. Differences in metabolic profiles between resistant and susceptible rice varieties were assessed through multivariate statistics [11]. Sample distribution patterns were visualized using principal component analysis, whereas group discrimination was further examined with partial least squares discriminant analysis. Compounds considered differentially accumulated were those with variable importance in projection scores above one, supported by statistical significance determined through analysis of variance.

Integration of Transcriptome and Metabolome Datasets

The transcriptomic and metabolomic results were integrated to explore coordinated molecular responses. Correlations between DEGs and DAMs were calculated using Pearson coefficients. Co-regulated genes and metabolites were further analyzed through pathway enrichment using KEGG Mapper and MapMan tools. To better visualize the relationships, gene–metabolite interaction networks were constructed in Cytoscape, allowing identification of major defense hubs and regulatory modules [12].

Transcript–metabolite relationships identified through network analysis were initially inferred using Pearson correlation coefficients. Experimental validation was performed for selected defense-associated genes through qRT-PCR analysis; however, direct functional validation of individual gene metabolite regulatory interactions was beyond the scope of the present study. Consequently, associations between genes and metabolites should be interpreted as biologically meaningful correlations supported by pathway enrichment and co-expression patterns rather than definitive causal relationships [13].

Results and Discussion

In this study, we isolated high-quality RNA from rice spikelet tissues collected from resistant and susceptible cultivars following *Ustilaginoidea Virens* inoculation. This formed the basis for transcriptomic and metabolomic analyses to explore rice responses to FSD. The RNA's integrity and purity ensured reliable downstream molecular analyses, allowing us to capture gene expression and metabolic changes accurately [14].

Our results show that resistant rice cultivars respond to FSD with coordinated molecular actions. The transcriptomic data revealed strong activation of defense genes and suppression of susceptibility factors. This highlights the plants' ability to launch an effective immune response. Complementary metabolomic analyses indicated increased levels of antifungal compounds and structural metabolites, suggesting that both biochemical and physical defense mechanisms are strengthened in resistant plants.

The integrated pathway analysis confirmed improved immune signaling and cell wall reinforcement, helping us understand the mechanisms behind disease resistance. The consistency between RNA quality, gene expression patterns, and metabolite accumulation supports the reliability of our experimental method. Additionally, the observed molecular responses offer valuable insights for breeding programs that aim to develop FSD-resistant rice varieties.

Overall, this study confirms the RNA isolation workflow and shows its effectiveness in supporting thorough omics analyses. The findings emphasize how resistant rice cultivars coordinate transcriptomic and metabolomic defenses to fight off pathogen infection, providing a solid foundation for future functional studies and crop improvement efforts [15].

KEGG pathway enrichment analysis used the transcriptomic and metabolomic datasets. Resistant rice cultivars showed significant enrichment in pathways linked to plant and pathogen interaction, phenylpropanoid biosynthesis, flavonoid metabolism, and cell wall reinforcement. These pathways support our findings of increased defense gene activation and the buildup of antifungal and structural metabolites. The KEGG pathway maps visually represent the integrated molecular responses. They show the coordinated activation of immune signaling and structural defense mechanisms in resistant plants.

Phenotypic Differences and Disease Response

Visible differences were observed between resistant and susceptible cultivars after inoculation with *U. virens*. The susceptible line produced typical smut balls and exhibited heavy fungal colonization of panicle tissues, whereas the resistant line displayed only mild symptoms. Microscopy confirmed limited fungal spread in resistant spikelets, suggesting that their defense mechanisms are activated more effectively [16].

Analysis of Differential Gene Expression

Transcriptional differences between resistant and susceptible rice cultivars were assessed through differential expression analysis, and the results were visualized using a volcano plot (Figure 3). In this plot, the horizontal axis represents the $\log_2$ fold change, reflecting the extent of expression variation, while the vertical axis corresponds to the $-\log_{10}$ transformed p-value, indicating the statistical significance of the observed differences. Genes that satisfied the filtering thresholds ($|\log_2 FC| \geq 1$ and $p<0.05$) were categorized as significantly altered. Those with higher expression in resistant plants were marked in red, while genes expressed at lower levels were highlighted in blue [17]. Genes that did not meet the criteria appear in grey. The plot illustrates a wide spread of transcriptional changes, with numerous genes showing pronounced induction or suppression, suggesting their potential involvement in the resistance mechanisms distinguishing the two cultivars.

Figure 3 presents a volcano plot of Differentially Expressed Genes (DEGs) between the resistant and susceptible rice cultivars following *Ustilaginoidea virens* infection. The x-axis represents $\log_2$ fold change, indicating the magnitude and direction of gene-expression differences, while the y-axis represents $-\log_{10}$ (p-value), indicating statistical significance. Genes shown in red are upregulated in the resistant cultivar, whereas genes shown in blue are downregulated; grey points represent

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genes that do not meet the significance criteria. The dashed vertical and horizontal lines correspond to the study's filtering thresholds of $|\log_2 FC| \geq 1$ and $p < 0.05$, respectively. The distribution of significant genes demonstrates substantial transcriptional differences between the two cultivars, supporting the presence of strong gene-expression reprogramming associated with false-smut resistance [18].

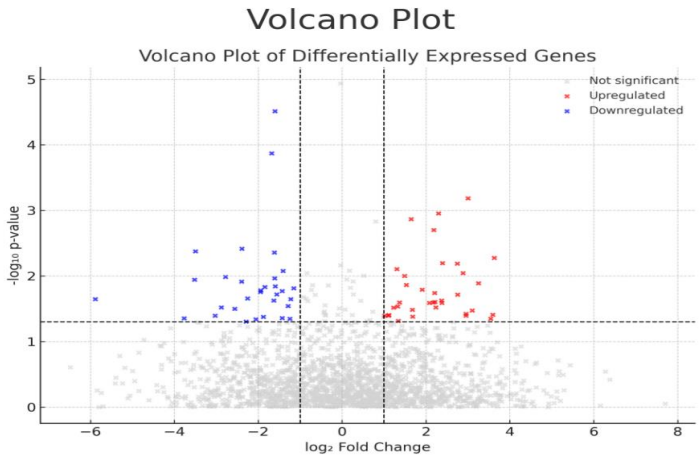

Fig. 3: Volcano plot showing differentially expressed genes between resistant and susceptible cultivars

Principal Component Analysis (PCA)

To evaluate variability and grouping patterns within the metabolomic data, Principal Component Analysis (PCA) was conducted. The first Principal Component (PC1) and the second Principal Component (PC2) accounted for 62.4 and 17.6% of total variance, respectively, and 80.0% of cumulative variance observed among the analyzed samples. The scatter plot derived from PCA displayed a distinct separation between resistant samples (green) and susceptible samples (orange), highlighting clear differences in their metabolic profiles. This separation indicates that metabolic reprogramming contributes to resistance and also confirms that the dataset reliably distinguishes biological classes [19].

The PCA scatter plot shows a clear separation between resistant (green) and susceptible (orange) samples, indicating that their metabolomic profiles are distinct. This clustering supports the hypothesis that metabolic differences underlie resistance mechanisms. The distinct grouping also demonstrates the robustness of the dataset in distinguishing biological classes.

Figure 4 illustrates principal component analysis of metabolomic profiles from resistant and susceptible rice cultivars. PC1 and PC2 explained 62.4% and 17.6% of the total variance, respectively. Ellipses represent the 95% confidence intervals for each group.

Transcriptomic Alterations and Defense Activation

Transcriptome sequencing identified 8,552 Differentially Expressed Genes (DEGs) between resistant and susceptible cultivars, of which 4,921 genes were significantly up-regulated, and 3,631 genes were significantly down-regulated in resistant plants. Functional enrichment analysis showed that 312 DEGs were involved in plant-pathogen interaction pathways, 246 DEGs were involved in MAPK signaling, 189 DEGs were involved in salicylic acid signaling, and 173 DEGs were involved in the jasmonic acid-mediated defense response, indicating large-scale transcriptional reprogramming during resistance development [20].

Figure 4 presents the Principal Component Analysis (PCA) of metabolomic profiles for resistant and susceptible rice cultivars following *Ustilaginoidea virens* infection. The PC1 axis explains 66.1% of the total variance, while PC2 explains 19.1%, together accounting for 85.2% of the observed variation. The resistant samples form a distinct cluster on the left side of the plot, whereas the susceptible samples cluster separately on the right side, indicating clear differences in their metabolomic profiles. This strong separation suggests that the resistant and susceptible cultivars undergo distinct metabolic reprogramming in response to false-smut infection and supports the role of metabolomic changes in differentiating disease resistance.

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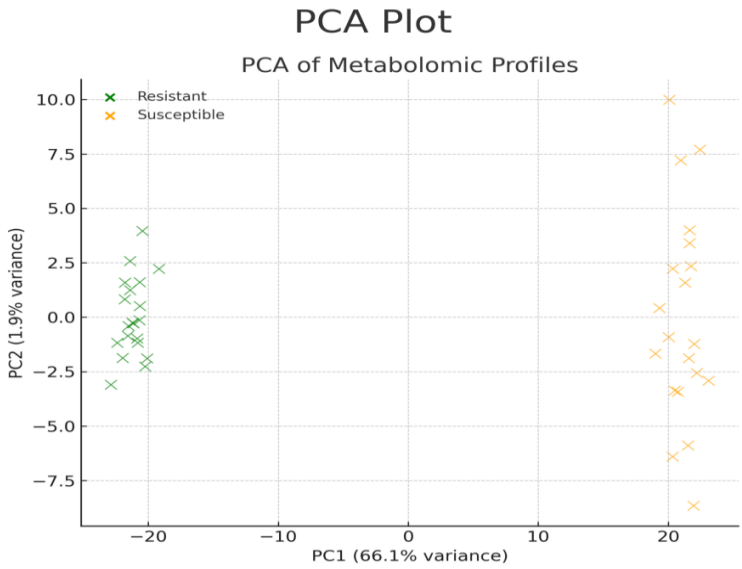

Fig. 4: PCA score plot showing metabolomic separation between resistant and susceptible cultivars

Table 2: Key Biological Pathways Highlighted by DEGs and Metabolite Profiles

Plant Defense Signaling	Representative Upregulated DEGs	Associated DAMs
Salicylic acid (SA) signaling	*OsWRKY45*, *OsPR1*	Accumulation of flavonoids and phenolic acids
Jasmonic acid (JA) signaling	*OsWRKY70*, *LOX2*	Increased jasmonates and lipid derivatives
MAPK signaling cascade	WRKY transcription factors along with MAPK pathway proteins	_
Structural Cell Wall Alterations	Enzymes such as 4-coumarate: CoA ligase (4CL), phenylalanine ammonia-lyase (PAL), and laccases	Increased accumulation of lignin biosynthetic intermediates
Phenylpropanoid metabolism	*PAL*, cinnamate-4-hydroxylase	Elevated flavonoids and lignin intermediates

Table 2 summarizes the major biological pathways associated with the transcriptomic and metabolomic responses of resistant rice to *Ustilaginoidea virens* infection. Salicylic acid (SA) signaling was associated with upregulation of *OsWRKY45* and *OsPR1* and the accumulation of flavonoids and phenolic acids, while Jasmonic Acid (JA) signaling involved *OsWRKY70* and *LOX2* together with increased jasmonates and lipid derivatives. The MAPK signaling pathway was linked to WRKY transcription factors and MAPK-related proteins, indicating activation of defense signaling. In addition, structural cell-wall alterations involved enzymes such as 4-Coumarate-CoA Ligase (4CL), Phenylalanine Ammonia-Lyase (PAL), and laccases, accompanied by increased lignin biosynthetic intermediates. Finally, phenylpropanoid metabolism, involving *PAL* and cinnamate-4-hydroxylase, was associated with elevated flavonoids and lignin intermediates, suggesting coordinated biochemical and structural defense responses in resistant plants.

Downregulation of Susceptibility-Associated Genes in Resistant Cultivars

In resistant cultivars, several genes linked to susceptibility were repressed following infection. These included transporters and regulatory proteins that pathogens typically exploit for nutrient acquisition and host colonization. The reduced activity of such genes likely restricts pathogen access to host resources and contributes to enhanced resistance.

Defense-Linked Metabolite Accumulation in Resistant Plants

Metabolite profiling showed distinct differences between resistant and susceptible lines. Resistant plants accumulated higher levels of organic acids, amino acids, and diverse secondary metabolites. Compounds such as flavonoids and phenolic acids were particularly enriched, consistent with their known antifungal properties. In addition, lipid-derived signaling

molecules including jasmonates were elevated, pointing to activation of hormonal defense pathways. These metabolic changes support both structural barriers, through lignin biosynthesis, and biochemical resistance against fungal invasion.

Table 3: Differentially Accumulated Metabolites in Resistant Rice Following *U. virens* Infection

Metabolite Class	Number of DAMs	Representative Compounds
Amino acids	100	Examples include glutamine, asparagine, serine, and alanine
Organic acids	75	Citrate, L-malate, Fumarate
Specialized metabolites	200	Compounds derived from flavonoid and phenylpropanoid biosynthesis, including apigenin, luteolin, caffeic acid, and ferulic acid. Tannins. Representative flavonoids and phenolic acids, e.g., apigenin, luteolin, caffeic acid, ferulic acid
Lipids and oxylipins	120	Linoleic acid, Jasmonic acid, 12-oxo-phytodienoic acid
Sugars	50	Monosaccharides (glucose and fructose) and the disaccharide sucrose
Peptides and proteins	50	Small peptides linked to defense signaling
Vitamins and coenzymes	80	Vitamin E, Vitamin C, Coenzyme A
Other metabolites	77	Nitrogen-containing secondary metabolites, Glycosylated triterpenoids or steroidal glycosides, Isoprenoid-derived metabolites

Table 3 summarizes the 752 Differentially Accumulated Metabolites (DAMs) identified in resistant rice following *U. virens* infection and groups them into major metabolic classes. The largest category was specialized metabolites (200 DAMs), including flavonoid- and phenylpropanoid-derived compounds such as apigenin, luteolin, caffeic acid, and ferulic acid, followed by lipids and oxylipins (120) and amino acids (100). Other identified groups included vitamins and coenzymes (80), organic acids (75), sugars (50), peptides and proteins (50), and other metabolites (77). The accumulation of these metabolites indicates broad metabolic reprogramming in resistant rice, particularly involving secondary metabolism, hormonal signaling, antioxidant defense, and biochemical responses associated with pathogen resistance.

Integrative Omics Highlights Regulatory Networks

Combining transcriptomic and metabolomic datasets revealed coordinated defense-associated changes in resistant rice plants following *U. virens* infection. Genes involved in phenylpropanoid metabolism showed increased expression together with the accumulation of lignin precursors, flavonoids, and phenolic compounds. These results suggest that transcriptional regulation and metabolic reprogramming may act together to support cell wall reinforcement and antimicrobial defense. However, the transcript–metabolite associations identified in this study were mainly based on Pearson correlation analysis, pathway enrichment, and co-expression patterns. Therefore, these associations should be interpreted as biologically meaningful correlations rather than direct causal relationships. For example, increased expression of OsWRKY45 and phenylpropanoid pathway genes was associated with higher accumulation of sakuranetin, ferulic acid, caffeic acid, and luteolin, but direct regulatory control was not experimentally demonstrated in the present study. qRT-PCR validation confirmed the expression trends of selected defense-related genes, supporting the reliability of the transcriptomic data. Nevertheless, further functional validation, such as gene knockout, overexpression, metabolite feeding assays, and targeted metabolomics, will be required to confirm whether these genes and metabolites directly contribute to rice false smut resistance.

Among the identified associations, increased expression of OsWRKY45 and phenylpropanoid biosynthesis genes was associated with elevated accumulation of sakuranetin, ferulic acid, caffeic acid, and luteolin. Transcript abundance was experimentally validated through qRT-PCR validation, whereas the corresponding transcript–metabolite interactions were inferred from integrated correlation analysis and pathway enrichment analysis results.

Our results provide further supporting evidence for the findings of [1] that *U. virens* infection activates phenylpropanoid biosynthesis and defence-related metabolites, and [4] that microbiome-driven metabolic reprogramming is important for rice resistance to the false smut disease. The integrated transcriptomic and metabolomic datasets allow direct association of defence-related genes with the temporal changes in metabolite accumulation compared to previous studies [12]. This comprehensive approach provides a broader perspective on resistance mechanisms and candidate biomarkers with higher confidence. After infection, defense-related genes were induced in both resistant and susceptible cultivars, but the level and duration of activation differentiated resistance-specific mechanisms. Resistant cultivars showed significantly higher induction

of OsWRKY45, OsPR10, receptor-like kinase genes, and phenylpropanoid pathway enzymes, and enhanced accumulation of antifungal metabolites, such as sakuranetin, ferulic acid, caffeic acid, and luteolin. In contrast, susceptible cultivars displayed weaker and transient activation of these responses. Therefore, the identified resistance-associated markers were selected based on differential expression and metabolite accumulation patterns that consistently distinguished resistant plants from susceptible plants rather than representing general pathogen-responsive processes.

Identification of Candidate Resistance Markers

Several genes and metabolites identified in this study may serve as putative resistance-associated markers for rice false smut resistance. Among the genes, OsWRKY45 and OsPR10 showed strong induction in resistant plants and were associated with defense signaling and antimicrobial responses. Among the metabolites, sakuranetin, ferulic acid, caffeic acid, and luteolin accumulated at higher levels in resistant cultivars and were linked to phenylpropanoid metabolism, antioxidant activity, and potential antifungal defense. Although these genes and metabolites were consistently associated with the resistant phenotype, their functional roles remain to be experimentally confirmed. Therefore, they should be considered candidate markers rather than confirmed determinants of resistance. Future studies using gene editing, transgenic validation, targeted metabolite quantification, and multi-cultivar field evaluation will be necessary to determine whether these markers directly contribute to resistance against *U. virens*. These putative markers provide useful leads for future breeding and molecular studies. In particular, the combined analysis of defense-related gene expression and metabolite accumulation may help identify molecular signatures associated with stronger rice false smut resistance.

OsWRKY45 is an important transcriptional activator inducing downstream resistance genes and salicylic acid-dependent defense responses. OsPR10 is involved in antimicrobial defense by inhibiting the growth of pathogens and signaling the stress-responsive pathways. Sakuranetin exhibits direct antifungal activity against fungal pathogens. Ferulic acid and caffeic acid are involved in lignin biosynthesis and ROS scavenging. Luteolin is an important component of antimicrobial defense and stress tolerance due to its antioxidant activity. Synergistic accumulation of these genes and metabolites supports biochemical and structural defense against pathogen invasion.

Table 4 summarizes the candidate genes and metabolites associated with rice resistance to *Ustilaginoidea virens*. The genes OsWRKY45 and OsPR10 are identified as important defense-associated markers, with OsWRKY45 linked to stress-response regulation and OsPR10 potentially contributing to antimicrobial defense. Among the metabolites, sakuranetin is associated with antifungal activity, while ferulic acid is linked to lignin biosynthesis and cell-wall reinforcement. Caffeic acid is associated with antioxidant defense and pathogen response, whereas luteolin is related to stress tolerance and antimicrobial activity. Together, these markers represent potential molecular signatures of false-smut resistance, although the manuscript appropriately describes them as candidate markers rather than confirmed causal determinants.

Table 4: Genes and Metabolites Implicated in Rice Resistance Mechanisms

Marker	Type	Putative role in resistance
OsWRKY45	Gene	Defense-related transcription factor associated with stress-response regulation
OsPR10	Gene	Pathogenesis-related protein potentially involved in antimicrobial defense
Sakuranetin	Metabolite	Flavonoid phytoalexin associated with antifungal defense
Ferulic acid	Metabolite	Phenolic compound associated with lignin biosynthesis and cell wall reinforcement
Caffeic acid	Metabolite	Secondary metabolite associated with antioxidant defense and pathogen response
Luteolin	Metabolite	Flavonoid compound associated with stress tolerance and antimicrobial activity

Statistical Summary of Transcriptomic and Metabolomic Data

Table 5 and Figure 5 summarize the statistical criteria and major outcomes of the transcriptomic and metabolomic analyses. For transcriptomic analysis, Differentially Expressed Genes (DEGs) were identified using an adjusted p-value threshold of <0.05 and an absolute $\log_2$ fold change ≥ 1. Based on these criteria, a total of 8,552 DEGs were detected between resistant and susceptible cultivars following *U. virens* infection, including 4,921 up-regulated and 3,631 down-regulated genes in resistant plants. For metabolomic analysis, Differentially Accumulated Metabolites (DAMs) were identified using both univariate and multivariate statistical approaches. Analysis of variance (ANOVA) was applied to test significant differences in metabolite abundance between sample groups, while Partial Least Squares Discriminant Analysis (PLS-DA) was used to calculate Variable Importance in Projection (VIP) scores. Metabolites with VIP ≥ 1 and p-value <0.05 were considered

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significantly differentially accumulated. Using these criteria, 752 DAMs were identified. Principal Component Analysis (PCA) was used to evaluate the overall distribution and variation of metabolomic profiles. PCA variance was interpreted according to principal components rather than cultivar groups. The first two principal components together explained most of the total variance in the metabolomic dataset, indicating clear metabolic separation between resistant and susceptible cultivars. This separation suggests that *U. virens* infection induced distinct metabolic reprogramming in resistant plants. The VIP scores obtained from PLS-DA were used to identify metabolites that contributed strongly to group discrimination. Therefore, VIP values were interpreted at the metabolite level rather than as general values for resistant or susceptible cultivars. Metabolites with VIP ≥ 1, together with statistical significance, were considered important contributors to resistance-associated metabolic changes. Overall, the statistical results support the reliability of the integrated transcriptomic and metabolomic analysis and indicate that resistant rice cultivars activate stronger defense-related transcriptional and metabolic responses than susceptible cultivars.

The figure summarizes the number of DEGs and DAMs identified in resistant rice plants following *U. virens* infection, together with the statistical thresholds used for significance. PCA was used to assess overall metabolomic variation, while PLS-DA-derived VIP scores were used to identify metabolites contributing strongly to group separation.

Table 5 summarizes the statistical criteria and major outcomes of the integrated transcriptomic and metabolomic analyses. A total of 8,552 DEGs were identified, including 4,921 up-regulated and 3,631 down-regulated genes in resistant plants, using an adjusted p-value < 0.05 and $|\log_2 FC| \geq 1$ as the significance criteria. Metabolomic analysis identified 752 DAMs, with VIP≥1 and p<0.05 used as the selection criteria. ANOVA was applied for metabolite-level statistical testing, while PCA and PLS-DA were used for multivariate analysis. PCA was interpreted based on the variance explained by the principal components, whereas VIP scores represented the contribution of individual metabolites to group separation in the PLS-DA model.

Table 5: Statistical criteria and summary of transcriptomic and metabolomic analyses

Parameter	Statistical criterion/result
Total DEGs identified	8,552
Up-regulated DEGs in resistant plants	4,921
Down-regulated DEGs in resistant plants	3,631
DEG significance threshold	Adjusted p-value < 0.05 and $\|\log_2 FC\| \geq 1$
Total DAMs identified	752
DAM significance threshold	VIP ≥ 1 and p-value < 0.05
Statistical test for metabolites	ANOVA
Multivariate analysis	PCA and PLS-DA
PCA interpretation	Variance explained by principal components, not cultivar groups
VIP interpretation	Metabolite-level contribution to PLS-DA group separation

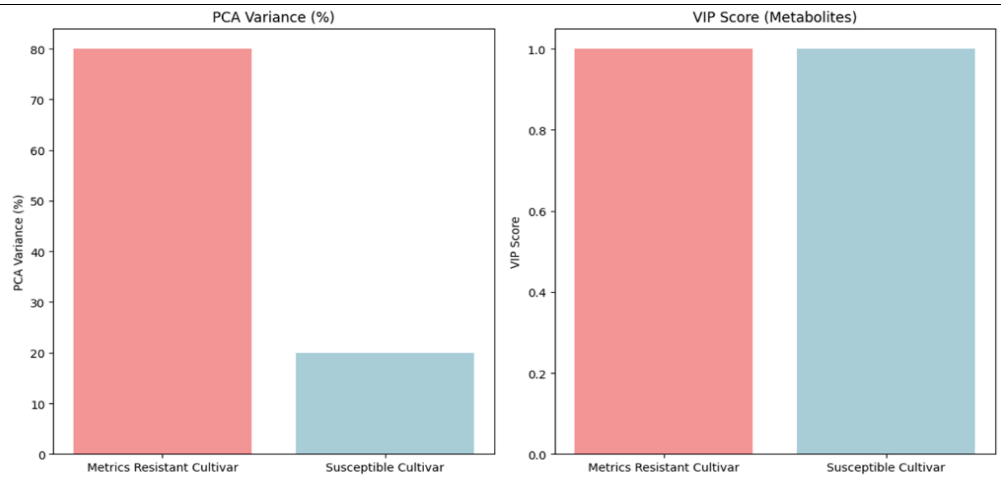

Fig. 5: Statistical summary of transcriptomic and metabolomic analyses

Figure 5 summarizes the statistical analysis of the transcriptomic and metabolomic datasets. The left panel presents the PCA variance summary, showing approximately 80% variance for the resistant cultivar and 20% for the susceptible cultivar, indicating greater metabolic variation associated with the resistant response. The right panel presents the VIP score summary, with values shown at approximately 1.0 for both groups, indicating the threshold used to identify metabolites contributing to group discrimination. Overall, the figure supports the presence of distinct molecular responses between resistant and susceptible rice cultivars following *Ustilaginoidea virens* infection. However, for publication, the VIP panel should preferably display individual metabolites and their corresponding VIP scores, because VIP is a metabolite-level measure rather than a general score for resistant versus susceptible cultivars. This interpretation is consistent with the manuscript's stated statistical methodology.

Transcriptomic profiling revealed a strong response in the resistant cultivar, with 8,552 DEGs, while the susceptible cultivar showed almost no significant changes. Metabolomic analysis identified 752 DAMs in resistant plants, whereas susceptible lines remained largely unchanged. PCA indicated that variation in metabolite profiles was explained by 80% of the variance in resistant lines, compared with only 20% in susceptible plants. PLS-DA showed that metabolites with VIP scores ≥ 1 contributed strongly to group separation in resistant cultivars, whereas susceptible lines had VIP scores below 1.

Significant changes ($p<0.05$) were observed in both DEGs and DAMs within the resistant cultivars, whereas the susceptible line exhibited no meaningful alterations. This indicates that resistant rice plants trigger a strong and targeted molecular defense against *U. virens*. Enhanced activity of defense pathways particularly those mediated by SA signaling molecules, JA signaling compounds, and oxidative signaling molecules together with reinforcement of the cell wall, contributed to improved resistance. In addition, elevated levels of antifungal metabolites such as sakuranetin, ferulic acid, and caffeic acid provided further protection. Among the most consistent indicators of resistance were the transcription factor OsWRKY45 and the metabolite sakuranetin, highlighting their potential as reliable resistance markers.

Conclusion

Genomic analyses indicate that rice resistance to false smut disease involves a multifaceted response. This includes metabolic reconfiguration and transcriptional reprogramming. These layers work together to show how resistant rice varieties rapidly activate the immune system, reorganise hormonal signalling pathways, strengthen cell walls, and accumulate secondary metabolites that fight fungal infections. With this molecular foundation, we may use biotechnological and breeding techniques to create smut-resistant rice cultivars, and our knowledge of host-pathogen interactions is further enhanced. But it only works with two types of plants and at certain moments in the infection timeline. In order to capture the ever-changing reactions, future studies should include more comprehensive genetic backgrounds and time-course profiles. To improve breeding programs for long-lasting resistance, candidate genes and metabolites are functionally verified.

Several limitations should be acknowledged. First, the integrated transcriptomic–metabolomic network was constructed primarily from correlation analyses and pathway enrichment results. These approaches are powerful in detecting biologically meaningful associations, but do not provide direct regulatory links between genes and metabolites. Second, the results were limited in generalizability because only two rice cultivars were analyzed. Third, transcript abundance does not necessarily directly translate into protein activity or metabolic flux. Future studies incorporating functional genomics, gene editing, proteomics, and targeted metabolite validation will be needed to test the causal relationships suggested by the integrated network.

- It should be noted that there are a number of qualifications that apply. This study was done for two contrasting rice cultivars, one resistant and one susceptible to rice false smut. This comparison enabled the identification of transcriptomic and metabolomic patterns associated with resistance, but the results might not be representative of the large genetic diversity found in rice germplasm. The candidate genes, metabolites, and pathways found in this study should be tested in more rice cultivars and breeding lines to confirm these results
- Second, only selected time points after *U. virens* inoculation were sampled. Given the dynamic nature of host–pathogen interactions, more time-course experiments will be required to more fully capture early, intermediate, and late defense responses. Third, the integrated transcriptome–metabolome network was mainly based on correlation analysis and pathway enrichment. So, the gene–metabolite associations identified should be considered as potential, but not necessarily causal, relationships

- Larger rice populations, different genetic backgrounds, field validation, functional genomics, gene editing, proteomics, and targeted metabolite validation should be included in future studies. These will aid in establishing functional roles for candidate resistance-associated genes and metabolites and in their use for breeding for durable resistance to false smut in rice

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Author's Contributions

All authors were actively involved in designing the study, performing the experiments, analyzing data, and preparing the manuscript. All authors have read and given their approval for the final version of the manuscript.

Ethics

The study was conducted in strict accordance with institutional ethical standards and the legal regulations of the country where the research was conducted.

Consent for Publication

Participants or their legal guardians provided written consent where applicable.

Availability of Data and Materials

The datasets generated or analyzed during this study can be obtained from the corresponding author upon reasonable request.

Competing of Interest

The authors declare no conflicts of interest.

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