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Molecular mechanisms of *Ustilaginoidea virens* pathogenicity and their utilization in disease control

Siwen Yu[1], Pengwei Liu[1], Jiyang Wang[2], Dayong Li[1*], Dan Zhao[1], Cui Yang[1], Dongyu Shi[1] and Wenxian Sun[1,2*]

Abstract

Rice false smut, caused by *Ustilaginoidea virens*, is one of the most important diseases in rice. The disease not only causes significant yield losses in China and worldwide but also produces multiple types of mycotoxins that pose a serious threat to the health of humans and animals. To effectively prevent and control the disease, the pathogenicity mechanisms of *U. virens* have been studied preliminarily, and some control strategies have been developed. This review focuses on recent progress in understanding the molecular mechanisms of *U. virens* pathogenicity, including virulence-related genes, transcriptional regulation of virulence genes, and effector-mediated interactions between rice and *U. virens*. Based on the molecular mechanisms underlying the rice-*U. virens* interactions, the possibilities of the pathogenicity genes in *U. virens* and host immune-related genes as potential targets for biological agents, host-induced gene silencing, and molecular design breeding are also discussed.

Keywords Rice false smut, *Ustilaginoidea virens*, Effector, Virulence genes, Molecular mechanisms

Background

The ascomycete fungus *Ustilaginoidea virens* (Cook) Takahashi (teleomorph: *Villosiclava virens*) causes rice false smut (RFS), one of the most devastating grain diseases in staple crops (Sun et al. 2020). *U. virens* spores land on rice leaves and the outer surface of rice spikes during the booting stage and then germinate under favorable conditions. Spore-forming hyphae enter the inner space of spikelets through small gaps between the lemma and palea, and infect rice florets through stamen filaments. *U. virens* infection blocks fertilization and grain filling, and thus redirecting host nutrients to promote false smut ball formation (Fan et al. 2015, 2019, 2020; Song et al. 2016). Hence, RFS causes blighted grains and a decline in 1000-grain weight (Bag et al. 2021). The increasing occurrence of RFS threatens rice production worldwide (Song et al. 2021a). *U. virens* also infects other rice organs, including young roots and coleoptiles, without causing disease symptoms. Compared with multiple phytopathogenic fungi, The pathogen encodes much fewer glycoside hydrolases, which are the major enzymes to decompose cellulose and xylan in cell walls (Zhang et al. 2014). Studies on the cell wall ultrastructure of rice florets found that stamen filaments are loosely arranged and lack cell wall components, a status that causes stamen filaments to be vulnerable to pathogen infection (Rong et al. 2017). Accordingly, RFS is a unique floret disease, and the *U. virens*-rice pathosystem might be an ideal model to investigate the invasion and specific interactions of grain diseases.

*Correspondence:
Dayong Li
lidayong@jlau.edu.cn
Wenxian Sun
wxs@cau.edu.cn
[1] College of Plant Protection, Jilin Provincial Key Laboratory of Green Management of Crop Pests and Diseases, Jilin Agricultural University, Changchun 130118, China
[2] Department of Plant Pathology, The Ministry of Agriculture Key Laboratory of Pest Monitoring and Green Management, China Agricultural University, Beijing 100193, China

RFS not only results in yield losses but also produces a large number of mycotoxins in false smut balls and threatens human and animal health (Chen et al. 2022b), including although not limited to ustiloxins, ustilaginoidins, and sorbicillinoids. Ustiloxins, cyclic peptide derivatives (Koiso et al. 1994; Shan et al. 2012), are toxic to both plants and animals (Nakamura et al. 1994; Lai et al. 2019b). Ustiloxins inhibit mitosis mainly by interfering with microtubule assembly and cytoskeleton formation (Luduena et al. 1994). A recent study found that different levels of ustiloxins A and B were detected in 240 rice samples from China and 72 rice samples from 12 other countries. Widespread contamination of ustiloxins in rice in different geographical regions highlights its potential risks to human health (Sun et al. 2022). Ustilaginoidins are bis-naphtho-γ-pyrone derivatives. Ustilaginoidin D can induce lipid metabolism disorder and hepatotoxicity in zebrafish larvae, implying that it is a potential risk to food safety (Wang et al. 2021a). Ustilaginoidin M1 is cytotoxic to all five human cancer cells (Meng et al. 2019b), but its toxicity is non-specific and inhibits the synthesis of all macromolecules in normal cells (Koyama et al. 1988). Therefore, it remains to be investigated whether ustilaginoidin M1 can be used as an anti-tumor drug. Recently, sorbicillinoids have been identified as another group of mycotoxins in *U. virens*. Sorbicillinoids induce cell cycle arrest and apoptosis and exhibit phytotoxic and antimicrobial activities (Lai et al. 2019b; Meng et al. 2019a).

The disease cycle and infection process, nutrient acquisition strategy, and identification of mycotoxin biosynthetic pathways in *U. virens* have been reviewed in recent years (Qiu et al. 2019; Fan et al. 2016; Sun et al. 2020). Therefore, this review focuses on advances in understanding the molecular mechanisms of *U. virens* pathogenicity, including virulence-related genes and transcriptional regulation thereof, and effector-mediated interactions between rice and *U. virens*. The possibilities of these genes as potential targets for biological agents, host-induced gene silencing (HIGS), and molecular design breeding are also discussed.

Molecular mechanisms of *U. virens* pathogenicity

Functional genomics and transcriptome analyses predicted that more than 1000 genes are involved in the virulence and pathogenicity of *U. virens*. These putative virulence factors are closely related to mitogen-activated protein kinase (MAPK) cascade pathways, secondary metabolism, and transcriptional regulation of *U. virens* (Zhang et al. 2014; Bao et al. 2021). Proteomic analysis identified 650 proteins in the exudate of *U. virens* (Wang et al. 2021b). Protein–protein interaction (PPI) network analysis and gene annotation suggest that exudate proteins are involved in pathogenicity, sporulation, antioxidant effects, and fungal cell wall construction and remodeling (Zhang et al. 2017). The publicly available *U. virens* genome and a high-efficiency gene targeting system greatly expedite the identification of virulence factors (Liang et al. 2018; Zhang et al. 2021). MAPK cascade pathways, transcription factors, post-translational modification, autophagy, mycotoxin biosynthesis, and some other virulence factors have been identified to be implicated in *U. virens* pathogenicity, mycelial growth, conidiation, and stress tolerance.

MAPK and Ras/cAMP signaling pathways

U. virens might possess five MAPK signaling pathways, including Fus3, Slt2, Hog1, Kss1, and Smk1, which are highly conserved in fungal species (Zhang et al. 2014). Except for Smk1, the other four MAPK signaling pathways have been identified in *U. virens*. *Hog1* homologous genes in phytopathogenic fungi regulate plant infection, fungicide resistance, and responses to various environmental stresses (Saito and Tatebayashi 2004; Zhao et al. 2007; Turra et al. 2014). In *U. virens*, the Δ*Uvhog1* mutant shows a reduction in growth rate, conidiation, and expression of the stress response genes *UvATF1* and *UvSKN7*. These results suggest that *UvHog1* may play an important role in regulating mycelial growth, conidiogenesis, and stress responses in *U. virens* (Zheng et al. 2016). Deletion of the *UvSlt2* MAP kinase gene increases sensitivity to cell wall stresses, and enables the tolerance to hyperosmotic or oxidative stresses (Liang et al. 2018). UvPmk1 shares a high sequence identity with Fus3/Kss1 MAPKs in *Saccharomyces cerevisiae.* Both *UvPmk1* and *UvCDC2* knockout mutants show defective hyphal growth and attenuated virulence, indicating that both MAP kinases are essential for the pathogenicity of *U. virens* (Tang et al. 2020). Altogether, MAPK cascades play essential roles in *U. virens* virulence and pathogenicity.

UvSMEK1 is a suppressor of MAPKK or ERK kinase (MEK) null in *U. virens* and is conserved among filamentous fungi. Compared with the wild-type strain, the *UvSMEK1* deletion mutants show higher tolerance to oxidative, osmotic, and cell wall stresses, and are defective in pathogenicity, indicating that UvSMEK1 positively regulates *U. virens* pathogenicity (Yu et al. 2021a).

Post-translationally modified Ras proteins can regulate the activity of Ras-dependent effectors and the recruitment of effectors to the cytoplasmic membrane (Shima et al. 2000). Cyclase-associated proteins (CAPs) bind to adenylyl cyclases to promote post-translational modification of Ras proteins, which is essential for adenylyl cyclase activity. CAPs also promote the synthesis of cyclic AMP (cAMP) (Yi et al. 2011; Zacharioudakis et al. 2017). Cyclic AMP production and protein kinase

A (PKA) activation are necessary and sufficient for activating MAPK cascades (Maizels et al. 1998). In *U. virens*, UvCAP1, as a bridge node for protein–protein interaction, interacts with adenylyl cyclase UvAc1, GTP-binding proteins UvRas1 and UvRas2, and the adaptor protein UvSte50 (Cao et al. 2021). UvCAP1 regulates the intracellular cAMP level and plays an important role in the development and pathogenicity of *U. virens* (Cao et al. 2021). Ste50 is also involved in the MAP kinase cascades (Sharmeen et al. 2019; Sumita et al. 2020; Wang et al. 2021c). As an adaptor protein, UvSte50 interacts with multiple components of the MAPK and Ras/cAMP signaling pathways, thus playing a critical role in *U. virens* infection (Cao et al. 2022). In *U. virens*, the adenylate cyclase UvAc1 and phosphodiesterase UvPdeH, which are conserved components of the cAMP pathway, regulate intracellular cAMP levels. Both Δ*UvPdeH* and Δ*UvAC1* mutants show reduced pathogenicity, suggesting that both enzymes play important roles in regulating the pathogenicity of *U. virens* (Guo et al. 2019). Collectively, UvCAP1, UvAC1, and UvPdeH synergistically co-regulate the intracellular cAMP level, MAPK signaling pathways, and activation of Ras-dependent effectors, thereby playing a crucial role in the development and infection process of *U. virens* (Fig. 1).

Transcription factors (TFs) and their virulence functions

TFs play essential roles in the virulence, growth, and development of plant pathogens. Identification of transcription factors and their binding sites greatly facilitates the elucidation of the functions and regulatory networks of virulence factors. The *U. virens* genome is predicted to encode more than 300 transcription factors (Zhang et al. 2014). ATAC-seq (assay for transposase accessible chromatin sequencing) identified 285 transcription factors associated with gene regulation, 553,907 binding sites of transcription factors, and 33,052 protein interaction pairs in *U. virens* (Chen et al. 2021b). An increasing number of transcription factors have been experimentally verified to play important roles in *U. virens* virulence.

It has been predicted that the *U. virens* genome encodes 57 families of transcription factors, with Zn_2Cys_6–type as the largest family (Zhang et al. 2014). A total of 40 Zn_2Cys_6 fungal-specific transcription factors (ZnFTFs) containing Zn_2Cys_6 binuclear cluster and fungal_trans domains have been identified in *U. virens*. Among them, UvZnFTF1 affects mycelial growth, pigment biosynthesis and pathogenicity by regulating nutrient metabolic pathways, secondary metabolism, and the expression of pathogen-host interaction genes and secreted protein-coding genes (Song et al. 2021b). In addition, the deletion of *UvPRO1* encoding a C6 zinc finger transcription factor leads to reduced mycelial growth. After inoculation,

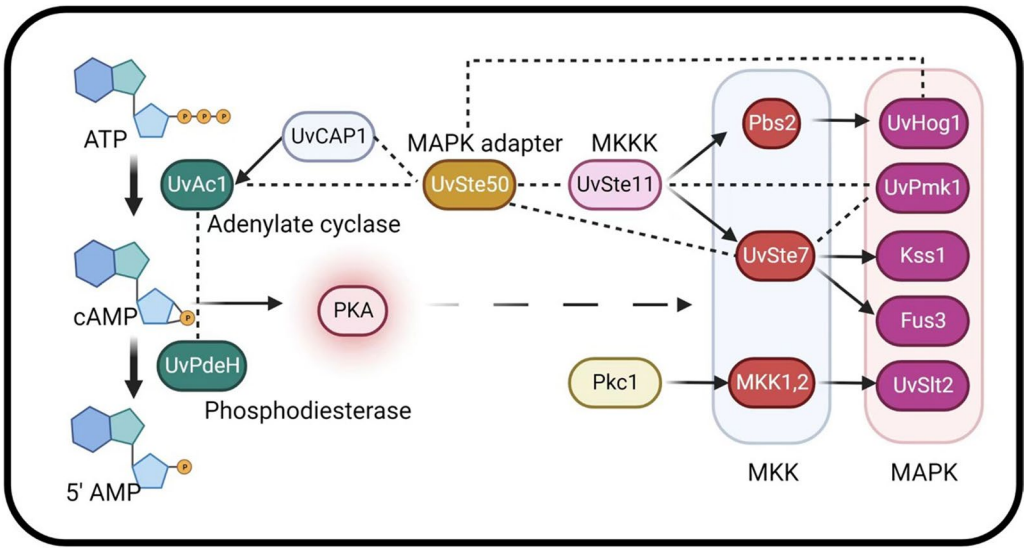

Fig. 1 The network of cAMP-MAPK pathways in *Ustilaginoidea virens*. The signal molecule cAMP is generated by the adenylate cyclase UvAc1 but is degraded by the phosphodiesterase UvPdeH. UvAc1 interacts with UvCAP1 and UvPdeH. Cyclic AMP activates the PKA pathway. UvSte50, as a MAPK adapter, interacts with UvCAP1, UvAc1, UvHog1, UvSte7, and UvSte11. UvSte7 and UvSte11 interact with UvPmk1. In addition, UvSte7 and UvSte11 function upstream of UvHog1, Kss1, and Fus3. Pkc1 activates MKK1 and MKK2, while MKK1 and MKK2 in turn phosphorylate UvSlt2. The MAPK cascades involving UvHog1, UvPmk1, and UvSlt2 play important roles in the regulation of hyphal growth, conidiogenesis, stress responses, and/or pathogenicity in *U. virens*. ATP, Adenosine triphosphate; cAMP, Cyclic adenosine monophosphate; 5′AMP, Adenosine 5′-monophosphate; MAPK, Mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MKK kinase. Created with BioRender (www. BioRender.com)

the hyphae of the Δ*Uvpro1* mutant extending along the surface of spikelets shriveled at 4 days post-inoculation (dpi), and ultimately lost the ability to infect spikelets (Lv et al. 2016). UvMsn2 belongs to the second largest family of C2H2-type zinc finger transcription factors. Similar to MoMsn2 in *Magnaporthe oryzae*, UvMsn2 regulates the vegetative growth, conidiation and conidial germination, stress responses, mitochondrial morphology, and virulence of *U. virens* (Xu et al. 2021). Seven GATA-type zinc finger transcription factors that contain one or two highly conserved class IV zinc finger DNA binding motifs in the form of Cys-X_2-Cys-$X_{17\text{-}20}$-Cys-X_2-Cys have been systemically identified in *U. virens*. UvGATA1 may be involved in host recognition and light response, whereas UvGATA3 functions in conidiation and secondary metabolism. UvGATA2 and UvGATA4 are different from those of other species in the second pair of Cys residues, suggesting that UvGATA2 and UvGATA4 might have different DNA-binding specificities with anonymous functions. In contrast to UvGATA1–UvGATA4, which are more closely related to their counterparts in plants, the three other GATA TFs (UvGATA5–UvGATA7) are clustered with GATA TFs from animals and humans. UvGATA5 and UvGATA7 may regulate nitrogen catabolite repression in fungi positively or negatively. UvGATA6 with two GATA domains might be involved in iron sensing and regulating siderophore production (Yu et al. 2019b). In addition, seven CCHC-type zinc finger transcription factors have also been functionally characterized. These CCHC-type TFs containing the conserved CCHC-box 'Cys-X_2-Cys-X_4-His-X_4-Cys' regulate stress responses, vegetative growth, conidiation, and virulence of *U. virens*. UvCCHC4 affects the expression of genes involved in mitochondrial biogenesis, ribosome activity and biogenesis, and transporter activity. The Δ*UvCCHC5* mutant infects rice spikelets without the formation of false smut balls. Interestingly, this transcription factor has no impact on the expression of grain-filling-associated genes in rice (Chen et al. 2021e).

In addition to UvCCHC5, three other types of transcription factors positively regulate the formation of false smut balls. UvCom1 contains two conserved internal repeat 1 domains found only in fungi. UvCom1 affects the expression of genes significantly enriched in transmembrane transport and negatively regulates the response to oxidation, osmosis and cell wall stresses. In particular, UvCom1 positively regulates vegetative growth and conidial formation of *U. virens* and controls the formation of false smut balls on rice panicles (Chen et al. 2020a). UvVEA belongs to a class of fungal transcription factors containing a conserved velvet domain. Interestingly, the absence of *UvVEA* in *U. virens* does not affect the virulence of this fungus but causes smaller false smut balls and a reduced number of chlamydospores, which might be associated with its deficiency in the utilization of glucose (Yu et al. 2022b). UvbZIP6, a basic leucine zipper (bZIP) transcription factor, belongs to an evolutionarily conserved transcription factor family. The *UvbZIP6* knockout mutants have a reduced conidiation capacity and can infect rice florets without forming false smut balls (Qu et al. 2022). Next, it is crucial to investigate how these transcription factors regulate the formation of false smut balls.

In addition, some other families of transcription factors play important roles in *U. virens* virulence. The homeobox transcription factor UvHox2 is essential for chlamydospore generation and *U. virens* virulence because the *UvHox2* deletion mutants produce no chlamydospores and show attenuated virulence. UvHox2 and a Myb-like DNA-binding protein (FlbD) likely co-regulate chlamydospore development through the BRLA-ABAA-WETA signaling cascade (Yu et al. 2019a). Recently, a novel transcription factor UvCGBP1 (cutinase G-box binding protein) in *U. virens* has been identified to be unique to ascomycetes (Chen et al. 2021a). Deletion of *UvCGBP1* affects the development and virulence of *U. virens*. ChIP-seq revealed that 865 downstream target genes of UvCGBP1 are enriched in MAPK signaling pathways. Approximately 36% of target genes contain AGGGG (G-box) motifs in their promoters. These findings will facilitate understanding the molecular mechanisms of the G-box binding transcription factor UvCGBP1 in regulating the MAPK pathways.

Collectively, different types of transcription factors have been identified to be involved in regulating virulence-associated traits and pathogenicity. However, the majority of studies mainly focus on gene knockout and phenotypic and transcriptome analyses. It is important to elucidate molecular mechanisms underlying how these transcription factors regulate virulence gene expression in the future.

Protein post-translational modifications (PTMs) and autophagy

Proteins are subject to many types of PTMs, including protein phosphorylation and ubiquitination, histone acetylation and deacetylation. Protein PTMs play important roles in the regulation of gene transcription, metabolism, enzyme activity and cell multiplication in eukaryotes (Smith et al. 2013; Woodsmith and Stelzl 2014). The methyltransferase UvKmt6, a core component of Polycomb I complex 2 (PRC2), is responsible for the trimethylation of lysine 27 of histone H3 (H3K27me3), which mediates transcriptional silencing. ChIP-seq and RNA-seq revealed that disruption of UvKmt6 derepresses the transcription of 629 genes, ultimately leading

to a reduction in growth, conidiation and pathogenicity. These include effector genes, secondary metabolism genes and stress response-related genes (Meng et al. 2021). Interestingly, quantitative proteomics based on a tandem mass tag has identified a novel type of post-translational modification, lysine 2-dihydroxyisobutylation (K_{hib}). A total of 3426 K_{hib} sites have been identified in 977 proteins in *U. virens* (Chen et al. 2021c). UvSlt2 2-hydroxyisobutyrylation increases the hydrophobic solvent-accessible surface area, thereby altering the binding affinity between the enzyme and its substrates. Subsequently, this PTM activates phosphorylated protein activity to promote infection (Chen et al. 2021f).

Protein phosphorylation and dephosphorylation mediated, respectively, by kinases and phosphatases, regulate many biological functions and processes. As a regulator of the salt stress response in yeast (Kaida et al. 2002), plasma membrane-bound phosphatase Psr1 homologs are widely distributed in fungi and affect the growth, development, and virulence in phytopathogenic fungi (Yun et al. 2015; Timpano et al. 2016). The function of UvPsr1 has also been characterized in *U. virens*. Deletion of *UvPsr1* affects mycelial growth, sporulation, and stress responses, and leads to a complete loss of pathogenicity. Meanwhile, the Δ*Uvpsr1* deletion mutants exhibit lower hyphal filtrate toxicity to inhibit the elongation of germinated seeds. These data indicate that UvPSR1 is a virulence factor in *U. virens* (Xiong et al. 2020).

Autophagy is an important process related to cell differentiation and development in various organisms (Liu et al. 2016; Hofius et al. 2017). In yeasts, the molecular bases of autophagy have been well studied, and 43 autophagy-related genes (ATGs) have been functionally characterized thus far (Furukawa et al. 2019; Fukuda and Kanki 2021). Disruption of autophagy leads to impaired pathogenesis in some plant fungal pathogens (Deng et al. 2009; Nadal et al. 2010; Ren et al. 2018). The autophagy-related proteins UvATG8, UvATG7 and UvATG14 have been demonstrated to play important roles in the growth, stress responses, asexual reproduction, and pathogenicity of *U. virens* (Meng et al. 2020; He et al. 2022; Yu et al. 2022a). Deletion of UvATG14 blocks GFP-UvATG8 transport and autophagic digestion and significantly reduces hyphal growth, asexual reproduction, and virulence of *U. virens*, implying the importance of autophagy in fungal pathogenicity (He et al. 2022).

Mycotoxin biosynthesis genes

The biosynthesis pathways of ustiloxins, ustilaginoidins, and sorbicillinoids have been identified. The *ugs* gene cluster has been demonstrated to be responsible for ustilaginoidin biosynthesis and modifications (Li et al. 2019). The polyketide synthase UvPKS1 catalyzes the first step of ustilaginoidin biosynthesis, i.e., the formation of the primary product nor-rubrofusarin from one molecule of acetyl-CoA and six molecules of malonyl-CoA. The *ugsT*, *ugsO*, *ugsJ*, and *ugsL* genes encoding major facilitator superfamily transporter, oxidoreductase, methyltransferase, and laccase are responsible for mycotoxin transport, redox modification, methylation of ustilaginoidin derivatives, and monomer dimerization, respectively. Different ratios of ustilaginoidin derivatives might have a regulatory role in *U. virens* mycelial growth (Li et al. 2019). Meanwhile, the virulence of *U. virens* is almost completely lost in the *ΔUvPKS1*, *ΔugsO*, *ΔugsH*, *ΔugsJ*, and *ΔugsT* mutants, indicating that ustilaginoidins are crucial for *U. virens* pathogenicity (Li et al. 2020b).

The sorbicillinoid biosynthetic gene cluster contains six conserved genes (*UvSorA*, *UvSorB*, *UvSorR1*, *UvSorR2*, *UvSorC*, and *UvSorT*) in *U. virens*. *UvSorA* and *UvSorB* were confirmed as the PKS genes. Mutations within the *usorA* and *usorB* genes eliminate the production of any sorbicillinoids but cause a significant increase in hyphal growth and sporulation (Lai et al. 2019a). Sorbicillinoids can inhibit mycelial growth and sporulation but can promote cell wall integrity of *U. virens* to cope with abiotic and biotic stresses (Zhang et al. 2022).

Based on the published genome and homology comparison, the toxic cyclic tetrapeptide ustiloxins are predicted to be ribosomally synthesized in *U. virens* (Tsukui et al. 2015). The predicted ribosomal peptide biosynthetic gene cluster includes a gene encoding a protein containing five repeats of Tyr-Val-Ile-Gly and three repeats of Tyr-Ala-Ile-Gly sequences for precursors of ustiloxins A and B, respectively (Tsukui et al. 2015). The highly repetitive structure of the ustiloxin precursor protein not only increases the production of ustiloxins but also contributes to the stability of its own transcripts (Umemura et al. 2022). Recently, the ustiloxin B biosynthetic pathway has been unveiled in *Aspergillus flavus*, revealing that nine genes are responsible for ustiloxin biosynthesis and modification (Ye et al. 2016). The production of ustiloxins occurs prior to the formation of false smut balls. Transcriptome analysis indicates that Ustiloxin A may affect disease resistance in host plants, which may promote the infection and development of *U. virens* (Hu et al. 2020). However, it is yet to be determined how these mycotoxins are involved in *U. virens* virulence.

Other virulence factors

Many unclassified protein families are also associated with *U. virens* virulence and pathogenicity. A Pal1 (*p*ears *a*nd *l*emons) protein has been identified to interact with the endocytic proteins UvSla2 and UvEde1, but UvPal1 does not require receptor-mediated endocytosis in *U. virens* (Chen et al. 2020b). Meanwhile, UvPal1 interacts

with the septin protein UvCdc11 in vivo and in vitro, and the loss of *UvPal1* leads to a change in the subcellular localization of UvCdc11. UvPal1 and UvCdc11 have similar effects on cell morphology, oxidative stress response, and virulence. Septins are involved in cell division, cell polarization, vesicle transport, and membrane remodeling (Sohn et al. 2022; Toth et al. 2022). These results suggest that UvPal1 may interact with UvCdc11 to mediate septin complex maintenance of cell morphology and virulence in *U. virens*. A recently discovered putative ester cyclase (UvEC1) contains a SnoaL-like cyclase domain of a polyketone compound. The Δ*UvEC1* knockout mutants exhibit an increased vegetative growth rate and elevated conidial generation ability but attenuated virulence. Quantitative proteomics analysis revealed that UvEC1 might have multiple effects on protein localization, catalytic activity, binding, toxin biosynthesis, and the spliceosome (Chen et al. 2021d).

The highly conserved SUN protein family containing a Cys-X_5-Cys-X_3-Cys-X_{24}-Cys motif is a unique protein family in ascomycetes (Firon et al. 2007). Two genes *UvSun1* and *UvSun2* encoding SUN domain proteins are present in *U. virens*. Mutation of *UvSun1* not only causes changes in morphology, mycelial growth, conidiation, and virulence but also leads to mis-regulation of a subset of genes involved in signal recognition and transduction, cell wall integrity, and secondary metabolism (Yu et al. 2021b). The *Uvsun2* knockout strains exhibit altered cell wall structure, abnormalities in fungal growth and stress responses, and an inability to infect rice (Yu et al. 2015). These data indicate that both genes are required for the growth, cell wall integrity, and pathogenicity of *U. virens*. Recently, *UvWHi2*, a homologous gene of *WHi2* in *S. cerevisiae*, has been identified to be involved in the responses to oxidation, hyperosmosis, cell wall stress, and nutrient limitation, thus affecting *U. virens* pathogenicity (Meng et al. 2022). In contrast, the mutation of *Uvt3277*, a low-affinity iron transporter-encoding gene with high homology in other fungi, leads to increased virulence. The role of Uvt3277 in the pathogenesis of *U. virens* remains elusive (Zheng et al. 2017).

Long non-coding RNAs (lncRNAs) (> 200 nt) lack functional open reading frames and do not belong to other well-defined non-coding RNAs (Wierzbicki et al. 2021). In plants, lncRNAs are not only involved in chromatin modification, transcriptional activation and interference, nuclear transport and other important regulatory processes but also balance plant growth and defense by regulating salicylic acid synthesis (Liu et al. 2022). RNA-seq analysis showed that *U. virens* encodes 1724 lncRNAs, including 1084 long intergenic non-coding RNAs (lincRNA), 51 intronic RNA (incRNA), 566 natural antisense transcripts (lncNATs), and 23 sense transcripts. Gene ontology enrichment analysis of differentially expressed lincRNAs and lncNATs indicates that these genes were mainly involved in transport-related regulation (Tang et al. 2021). This study provides an important basis for further revealing the function of lncRNAs in regulating fungal life processes.

Many identified virulence factors play indispensable roles in the growth, development and pathogenicity of *U. virens* (Table 1). Knowledge of fungal virulence factors provides important ideas for exploring the pathogenicity mechanisms and molecular targets for the effective management of false smut disease in rice.

Interactions between *U. virens* effectors and rice proteins

In long-term evolutionary interactions with pathogens, plants develop two layers of the immune system. Pattern recognition receptors on plant cell surfaces recognize pathogen-associated molecular patterns (PAMPs) to trigger basal defense, known as pattern-triggered immunity (Yu et al. 2017). The nucleotide-binding domain and leucine-rich repeat receptors monitor specific effector proteins and activate high levels of immune responses, called effector-triggered immunity (Jones and Dangl 2006; Chang et al. 2022). To overcome plant immunity, pathogens secrete a large number of effector proteins. The effectors disable plant surveillance systems and facilitate pathogen invasion and proliferation by acquiring nutrients and interfering with physiological and biochemical processes, including regulation of gene transcription, the stability, subcellular localization and secretion of immune-related proteins, manipulation of hormone signaling pathways, and so on (Shen et al. 2018; Tariqjaveed et al. 2021).

Understanding the interactions between *U. virens* effectors and target proteins in rice will provide theoretical bases for controlling RFS disease and creating resistant germplasms. Based on the gap-free *U. virens* genome, Zhang et al. (2021) predicted 256 conventional effectors that are mainly small cysteine-rich effectors (SCREs), and 165 atypical effectors that might be secreted into host cells via a non-conventional secretion pathway. Through a high-throughput screening, dozens of *U. virens* effectors have been demonstrated to inhibit non-host cell death in *Nicotiana benthamiana* (Zhang et al. 2014). Guided by these findings, at least ten effectors in *U. virens* have been functionally characterized (Fig. 2). The protein SCRE2/UV_1261 is the first effector identified to be essential for *U. virens* virulence (Fan et al. 2019; Fang et al. 2019). When SCRE1, SCRE2, SCRE4 and SCRE6 are heterologously expressed in *M. oryzae*, these proteins are secreted and translocated into plant cells during infection. Transient expression of these SCRE proteins in

Table 1 Genes involved in the pathogenesis of *Ustilaginoidea virens*

Gene	Encoding protein	Pathogenic features (Δ)	References
UvHog1	The ortholog of yeast HOG1 MAP kinase	Reduced pathogenicity	Zheng et al. (2016)
UvSlt2	The mitogen-activated protein kinase (MAPK)	Reduced pathogenicity	Liang et al. (2018)
UvPmk1	CMGC kinase	Reduced pathogenicity	Tang et al. (2020)
UvCDC2	CMGC kinase	Reduced pathogenicity	Tang et al. (2020)
UvSMEK1	A suppressor of MEK Null	Reduced pathogenicity	Yu et al. (2021a)
UvCap1	Cyclase-associated protein	Reduced pathogenicity	Cao et al. (2021)
UvAc1	Adenylate cyclases	Reduced pathogenicity	Guo et al. (2019)
UvPdeH	Phosphodiesterases	Reduced pathogenicity	Guo et al. (2019)
UvPRO1	C6 zinc finger transcription factors	Reduced pathogenicity	Lv et al. (2016)
UvMsn2	C2H2 zinc finger transcription factors	Reduced pathogenicity	Xu et al. (2021)
UvGATA1-7	GATA-binding zinc finger transcription factors	Not determined	Yu et al. (2019b)
UvCCHC1	CCHC-type zinc finger proteins	No change	Chen et al. (2021e)
UvCCHC2	CCHC-type zinc finger proteins	No change	Chen et al. (2021e)
UvCCHC3	CCHC-type zinc finger proteins	No change	Chen et al. (2021e)
UvCCHC4	CCHC-type zinc finger proteins	Reduced pathogenicity	Chen et al. (2021e)
UvCCHC5	CCHC-type zinc finger proteins	Reduced pathogenicity	Chen et al. (2021e)
UvCCHC6	CCHC-type zinc finger proteins	Enhanced pathogenicity	Chen et al. (2021e)
UvCCHC7	CCHC-type zinc finger proteins	Enhanced pathogenicity	Chen et al. (2021e)
UvCom1	Transcription factor	Reduced pathogenicity	Chen et al. (2020a)
UvVEA	Transcription factor	Reduced pathogenicity	Yu et al. (2022b)
UvbZIP6	Transcription factor	Reduced pathogenicity	Qu et al. (2022)
UvZnFTF1	Zn_2Cys_6 class fungal-specific transcription factors	Reduced pathogenicity	Song et al. (2021b)
UvHox2	Transcription factor	Reduced pathogenicity	Yu et al. (2019a)
UvCGBP1	G-box binding transcription factor	Reduced pathogenicity	Chen et al. (2021a)
UvKmt6	Catalytic subunit of Polycomb I complex 2	Reduced pathogenicity	Meng et al. (2021)
UvPsr1	Plasma membrane phosphatase	Reduced pathogenicity	Xiong et al. (2020)
UvEC1	Putative ester cyclase	Reduced pathogenicity	Chen et al. (2021d)
UvATG8	Autophagy-related protein	Reduced pathogenicity	Meng et al. (2020)
UvATG7	Autophagy-related protein	Reduced pathogenicity	Yu et al. (2022a)
UvATG14	Autophagy-related protein	Reduced pathogenicity	He et al. (2022)
UvPal1	Homologue of a cellular morphogenetic protein	Reduced pathogenicity	Chen et al. (2020b)
UvCdc11	Septin protein	Reduced pathogenicity	Chen et al. (2021d)
UgsT	Major facilitator superfamily transporter	Not determined	Li et al. (2019)
UgsO	FAD binding domain protein	Reduced pathogenicity	Li et al. (2019, 2020b)
UgsJ	Methyltransferase	Reduced pathogenicity	Li et al. (2019, 2020b)
UgsL	Multicopper oxidase	Reduced pathogenicity	Li et al. (2019, 2020b)
UvSorA	Polyketide synthase	Not determined	Zhang et al. (2022)
UvSorB	Polyketide synthase	Not determined	Zhang et al. (2022)
UvSun1	SUN family protein	Reduced pathogenicity	Yu et al. (2021b)
UvSun2	SUN family protein	Reduced pathogenicity	Yu et al. (2015)
UvWHi2	General stress response factor	Reduced pathogenicity	Meng et al. (2022)
Uvt3277	Homology with low-affinity iron transporter protein	Enhanced pathogenicity	Zheng et al. (2017)

N. benthamiana suppresses necrosis-like defense symptoms triggered by mammalian BAX and oomycete elicitin INF1. Convincingly, ectopic expression of these effectors in transgenic rice plants significantly inhibits pattern-triggered immunity, including flg22- and chitin-induced defense gene expression and oxidative burst (Fang et al. 2019; Zhang et al. 2020; Qiu et al. 2022; Zheng et al. 2022). Interestingly, a small peptide region of SCRE1 that contains an important 'cysteine-proline-alanine-arginine-serine' motif retains its immunosuppressive ability

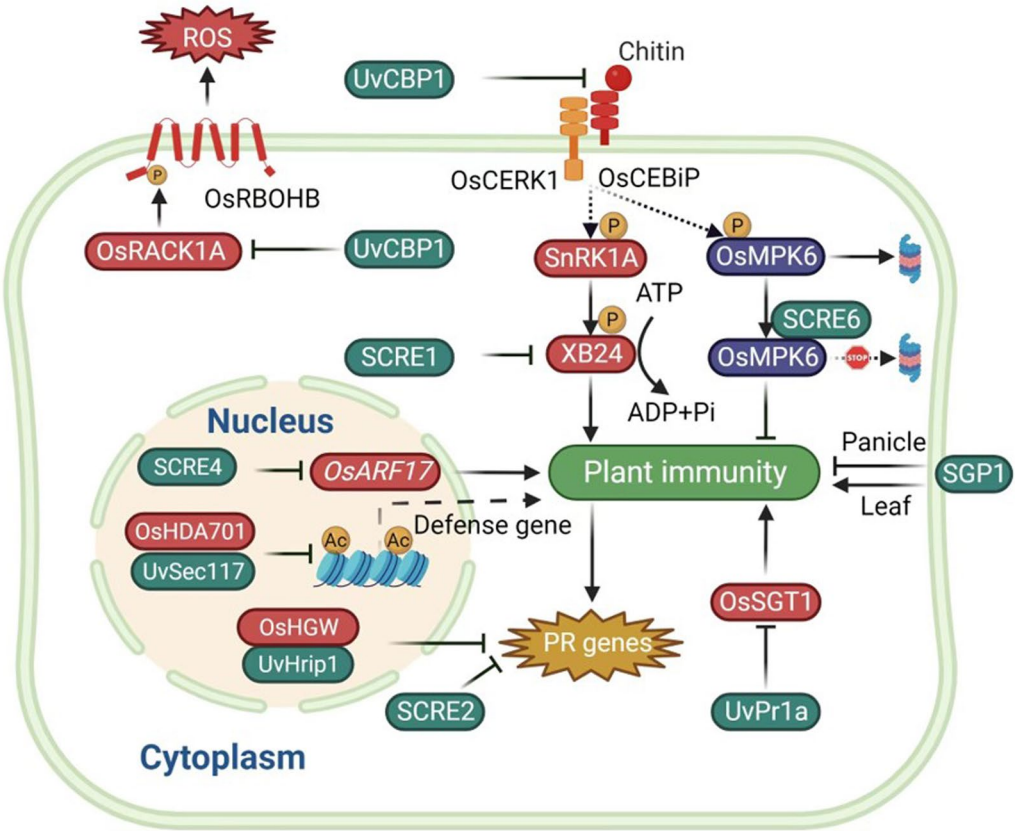

Fig. 2 Schematic representation showing the molecular mechanisms by which *Ustilaginoidea virens* effectors target host proteins and suppress plant immunity. Chitin is recognized by OsCEBiP and OsCERK1, thereby promoting the phosphorylation of SnRK1A and OsMPK6. UvCBP1 is a multifunctional protein. The effector blocks chitin access to the receptor OsCEBiP and disarms chitin-triggered immunity. Meanwhile, UvCBP1 interacts with the rice scaffold protein OsRACK1A and competes for its interaction with the NADPH oxidase OsRBOHB, thus inhibiting ROS accumulation. Activated SnRK1A phosphorylates XB24 and promotes ATPase activity to degrade ATP. SCRE1 outcompetes SnRK1A and ATP for XB24 binding, thus inhibiting ATPase activity and rice immunity. As a negative immune regulator, activated OsMPK6 is prone to 26S proteasome-mediated degradation. SCRE6 functions as a tyrosine phosphatase that dephosphorylates and stabilizes OsMPK6. UvPr1a, as a serine protease subtilase, directly degrades OsSGT1, a positive immune regulator. In the nuclei, SCRE4 inhibits the expression of OsARF17, encoding a positive immune regulator. UvSec117 recruits OsHDA701 into the nucleus and enhances its deacetylation activity. OsHDA701-mediated deacetylation of histone H3K9 inhibits the activation of defense genes and rice immunity. UvHrip1 interacts with OsHGW in nuclei and inhibits plant immunity via an unknown mechanism. Interestingly, SGP1 is recognized as a PAMP in the leaves, while the effector contributes to panicle infection. Collectively, distinct effectors promote *U. virens* infection through various molecular mechanisms. Green boxes indicate *U. virens* effectors; red boxes indicate positive immune regulators; purple boxes indicate negative immune regulators. PR, Pathogenesis-related; ROS, Reactive oxygen species; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; Ac, Adenylate cyclase; P, Phosphorus. SGP1, SUPPRESSOR OF G2 ALLELE OF SKP1; SCRE, Small cysteine-rich effector. Created with BioRender (www. BioRender.com)

(Zhang et al. 2020). Furthermore, the knockout mutants of these *SCRE* genes are greatly attenuated in *U. virens* virulence to rice. Collectively, an increasing number of effectors have been identified to be required for full virulence of *U. virens*.

Furthermore, high-throughput methods, such as yeast two-hybrid and immunoprecipitation-mass spectrometry assays, have been used to identify host targets of the *U. virens* effectors. Most recently, molecular mechanisms of virulence functions have been revealed for multiple effector proteins in *U. virens*. The essential virulence effector SCRE6 and its homologs secreted by *U. virens* have been characterized as the first ever-unidentified fungal effector phosphatases, which represent a novel family of tyrosine phosphatases (Zheng et al. 2022). The effector interacts with OsMPK6, a negative regulator of rice immunity. During pathogen infection, PAMPs induce the phosphorylation and subsequent degradation of OsMPK6, while SCRE6 can specifically dephosphorylate and stabilize OsMPK6, thus leading to the accumulation of OsMPK6 and promoting *U. virens* infection (Zheng et al. 2022). Another virulence effector UvSec117 in *U. virens* targets

the rice histone deacetylase OsHDA701 and recruits OsHDA701 into host nuclei through the nuclear localization signal of UvSec117, which enhances the deacetylation activity of OsHDA701. The OsHDA701-mediated deacetylation of histone H3K9 inhibits the activation of defense genes and rice immunity (Chen et al. 2022a). The two families of fungal effectors inhibit rice immunity by targeting negative regulators of rice immunity. On the other hand, several *U. virens* effectors have been identified to interfere with positive immune regulators. For instance, the effector SCRE4 is translocated into host nuclei during infection and inhibits the expression of *OsARF17*, encoding a positive immune regulator, thereby promoting *U. virens* infection (Qiu et al. 2022). In addition, a serine protease subtilase UvPr1a, as a virulence effector, directly degrades SUPPRESSOR OF G2 ALLELE OF SKP1 (OsSGT1), a positive regulator of innate immunity against multiple rice pathogens (Chen et al. 2022c). The study on SCRE1 function reveals a new infection mechanism in *U. virens*. Sucrose non-fermenting-1-related protein kinase 1 (SnRK1a) in rice is essential for energy homeostasis (Broeckx et al. 2016), and positively regulates plant immunity against various pathogens through phosphorylation of the ATPase XB24 (Yang et al. 2022). The effector SCRE1 outcompetes SnRK1a for XB24 binding and thereby inhibits the SnRK1a-mediated phosphorylation of XB24 and ATPase activity (Yang et al. 2022). Meanwhile, the binding of SCRE1 to XB24 also blocks XB24 access to ATP and thus inhibits ATP hydrolysis and plant immunity (Yang et al. 2022). In addition, UvCBP1, similar to the chitin-binding effectors Ecp6 of *Cladosporium fulvum* and Slp1 of *M. oryzae*, competes with the rice chitin receptor OsCEBiP for binding to free chitin, thereby blocking chitin-triggered immunity in rice and promoting *U. virens* infection (Li et al. 2021a). Interestingly, UvCBP1, as a multi-functional effector, also interacts with the rice scaffold protein OsRACK1A and competes for its interaction with the NADPH oxidase OsRBOHB, thus leading to the inhibition of reactive oxygen species (ROS) production. OsRACK1A overexpression enhances pathogen-triggered immunity in rice flowers and increases rice resistance to *U. virens* without yield penalty (Li et al. 2022).

Recently, more host targets have been identified for *U. virens* effectors. A putative effector gene *UvHrip1* is highly up-regulated during *U. virens* infection. UvHrip1 interacts with OsHGW, a key regulator of rice heading date and grain weight (Wang et al. 2020b; Wei et al. 2020). In addition, UVI_02019870, a highly conserved small-secretory hypersensitivity-inducible protein in filamentous fungi, interacts with OsCPL1, which is a putative chloroplast protein precursor and regulates the chloroplast signaling pathway (Li et al. 2020a). However, it remains unknown whether and how UvHrip1 and UVI_02019870 regulate immune responses by interacting with these target proteins in rice.

Besides functioning as virulence factors, the fungal effectors are often recognized as molecular patterns that trigger plant immunity (Tariqjaveed et al. 2021). The secreted protein Ser-Thr-rich glycosyl-phosphatidylinositol (GPI)-anchored protein SGP1 in *U. virens* is likely recognized as a microbe-associated molecular pattern and triggers innate immunity in rice leaves. On the other hand, SGP1 is required for the pathogenicity of *U. virens* during infection (Song et al. 2021c). In addition, high-throughput screening through transient gene expression in *N. benthamiana* and rice protoplasts demonstrated that multiple putative effectors induce defense responses and cell death in *N. benthamiana* and rice protoplasts (Fang et al. 2016). In other phytopathogenic fungi, many apoplastic effectors have been identified as PAMPs or damage-associated molecular patterns to trigger plant immunity (Tariqjaveed et al. 2021). Investigating how these putative *U. virens* effectors trigger plant immunity is of great interest. More interestingly, these effectors have the potential to be developed as immune elicitors that might be used for disease control.

These studies not only broaden our understanding of the fungal infection strategies through which pathogens inhibit host immunity and promote infection, but also provide ideas for developing new resistant rice germplasm against various diseases through gene editing and molecular design technologies.

Molecular strategies to control false smut disease

Significant advances have been made in identifying essential virulence factors in *U. virens*. Elucidation of the molecular mechanisms underlying *U. virens* virulence and pathogenicity will facilitate the development of effective strategies to control rice false smut. Based on the identified virulence factors, host-induced gene silencing (HIGS) has been explored for the management of rice false smut. Most sRNAs function endogenously, but some can travel across organism boundaries between hosts and microbes and silence genes in trans, a mechanism called 'cross-kingdom RNAi'. During microbial infection, host plants transport sRNAs mainly through extracellular vesicles into pathogens to suppress virulence-related genes (Hua et al. 2018; Huang et al. 2019). In the HIGS system, the constructs to generate small interfering RNAs (siRNAs) are transformed into transgenic host plants to target crucial virulence genes of invading pathogens. The success of HIGS largely depends on the presence of a functional RNA silencing system in the pathogen and cross-kingdom sRNA transportation system (Weiberg et al. 2013; Cai et al. 2018). Recently, the

HIGS strategy has been developed to create transgenic rice plants with RFS resistance, indicating that an RNA interference system exists in *U. virens* (Hou and Ma 2020; Wang et al. 2020a). For example, the chitin synthase genes *UvChs2* and *UvChs5*, which are involved in chitin synthesis and pathogenicity of *U. virens* have been successfully designed as target genes for silencing through HIGS (Li et al. 2021b). In addition, three essential virulence genes, *UvCom1*, *UvPro1*, and *UvAspE* (*Uv8b_1773*), which encode specific transcription factors in *U. virens*, have also been silenced by HIGS. As expected, effective silencing of virulence genes in *U. virens* through siRNAs produced in the above-mentioned transgenic rice plants significantly alleviates disease symptoms of rice but does not cause penalty on agronomic traits, indicating that HIGS is an effective strategy to create rice germplasms with RFS resistance (Chen et al. 2022b).

Based on progress in effector biology, multiple strategies for molecular design might be developed to control false smut disease efficiently. First, a new strategy to enhance rice resistance to *U. virens* has been developed by overproducing OsRACK1A, a host target of UvCBP1, to counteract the immunosuppressive effect of UvCBP1 (Li et al. 2022). More importantly, the transgenic rice plants exhibited no adverse effect on agronomic traits. Second, some immune regulators in host plants have been uncovered as targets of virulence effectors. The genes encoding negative immune regulators will be ideal targets for gene editing. Third, an increasing number of effectors have been identified to trigger plant immunity. Investigating whether defense-inducing effectors can be utilized as immune elicitors is worthwhile.

Conclusion and perspectives

RFS has become an important fungal disease threatening rice production and quality. Although the research foundation on *U. virens* pathogenicity is relatively weak, dozens of key pathogenicity genes have been recently identified (Table 1). With the development of biotechnology, a few identified pathogenicity genes have been successfully developed as HIGS targets for disease control. It is also speculated that some pathogenicity factors might be ideal targets for the molecular design of green pesticides when the structures and properties of these proteins are uncovered.

The effectors secreted by phytopathogenic fungi target host proteins in different subcellular compartments and suppress plant immunity through various mechanisms (Tariqjaveed et al. 2021). The *U. virens* genome encodes 421 putative conventional and non-conventional effectors; only a few effectors have been functionally characterized. The majority of identified fungal effector proteins inhibit plant immunity, including ROS generation, expression of pathogenicity-related (PR) genes, and MAPK activation. Because rice false smut is a unique floret disease, we speculate that the most important aspects of *U. virens* effector biology might be as follows. Firstly, previous studies suggest that *U. virens* hijacks the grain-filling system to acquire nutrients for the development of false smut balls. It is an important topic to investigate whether and how *U. virens* effectors function in acquiring nutrients through a grain-filling system. SCRE4 has been identified to suppress the expression of the *OsARF17* gene, which might be involved in flower development and grain filling (Qiu et al. 2022). It is worthwhile to elucidate the molecular mechanisms of SCRE4 and other effectors in regulating the transportation of nutrients. Secondly, the GPI-anchored protein SGP1 triggers immune responses in leaves but contributes to *U. virens* infection of rice florets (Song et al. 2021c). It is interesting to identify more effectors that specifically target floret immunity and understand the underlying molecular mechanisms. Thirdly, plant hormones play an important role in coordinating growth and immunity. We need to pay more attention to the effectors that modulate plant hormone signaling and promote *U. virens* infection. Elucidation of specific protein–protein interactions between *U. virens* and rice will facilitate revealing disease resistance and susceptibility genes (Fan et al. 2015). In particular, these resistance and susceptibility genes can provide potential targets for molecular design and breeding and gene editing. The summarized progresses provide important scientific bases for creating new germplasm with RFS resistance in rice.

Deployment of resistant cultivars is considered the most economical, effective and environmentally friendly approach to control RFS, but the development of RFS-resistant rice varieties faces significant challenges due to the lack of resistant germplasms. Although gene-for-gene resistance has not been reported for RFS resistance, a number of RFS-resistant QTLs have been recently identified (Neelam et al. 2022). Therefore, screening, identification and pyramiding of RFS-resistant QTLs will also be a promising way to control false smut disease in rice.

Abbreviations

ATAC-seq	Assay for transposase accessible chromatin sequencing
HIGS	Host-induced gene silencing
lncRNAs	Long non-coding RNAs
MAPK	Mitogen-activated protein kinase
MEK	MAPKK or ERK kinase
PAMPs	Pathogen-associated molecular patterns
PKA	Protein kinase A
PTM	Post-translational modifications
RFS	Rice false smut

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Authors' contributions
WXS conceived the concept. SWY, DYL, PWL, DZ, CY, and DYS participated in writing the manuscript. SWY and JYW submitted pictures. WXS and DYL revised and finalized the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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