Chapter

Rice False Smut: An Increasing Threat to Grain Yield and Quality

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Abstract

Rice false smut (RFS) is the most important grain disease in rice production worldwide. Its epidemics not only lead to yield loss but also reduce grain quality because of multiple mycotoxins generated by the causative pathogen, *Villosiclava virens* (anamorph: *Ustilaginoidea virens*). The pathogen infects developing spikelets and specifically converts individual grain into a RFS ball that is established from mycelia covered with powdery chlamydospores, sometimes generating sclerotia. RFS balls seem to be randomly formed in some grains on a panicle of a plant in the paddy field. However, epidemics differ largely among varieties, fields, and seasons. This chapter introduces current understanding on the disease, mycotoxins, the biology of the pathogen, pathogenesis of RFS, rice resistance, the disease cycle, the disease control, and assay.

Keywords: basal defense, biotroph, effector, epiphytic growth, grain filling gene, mycotoxin, sclerotium

1. Introduction

Rice production plays a crucial role in our food security. Rice security is not only an economic issue but also an important parameter to determine social and political stability [1]. Thus, rice research has to be geared up to develop strategies for alleviating losses due to pests and diseases. In the past decades, a number of minor diseases have attained the status of major importance in rice. One such disease is the rice false smut (RFS) disease that is a threat to yield and grain quality.

RFS was previously recorded as a minor disease of rice and considered as a symbol of good harvest in old times. In recent years, increasing occurrence of RFS has been reported in most major rice growing regions throughout the world, such as China, India, and USA [2–5]. The emergence of this disease is believed to be partially due to wide application of hybrid rice varieties, which are mostly susceptible to the RFS. The causative agent of RFS is an ascomycete fungal pathogen *Villosiclava virens* (anamorph: *Ustilaginoidea virens* [Cooke] Takahashi) [6], which specifically infects rice flowers and transforms the latter into RFS balls [3]. RFS balls are small at first growing slowly and enclosing the floral parts. The early balls were found to be slightly flattened and smooth and were covered by a thin membrane. As the pathogen growth intensifies, the RFS ball bursts with chlamydospores and becomes orange then later yellowish-green or greenish-black (**Figure 1A–C**). The RFS balls generate sclerotia (**Figure 1D**) when the temperature difference between day and night is large in autumn [3]. RFS ball is the only visible symptom of RFS disease.

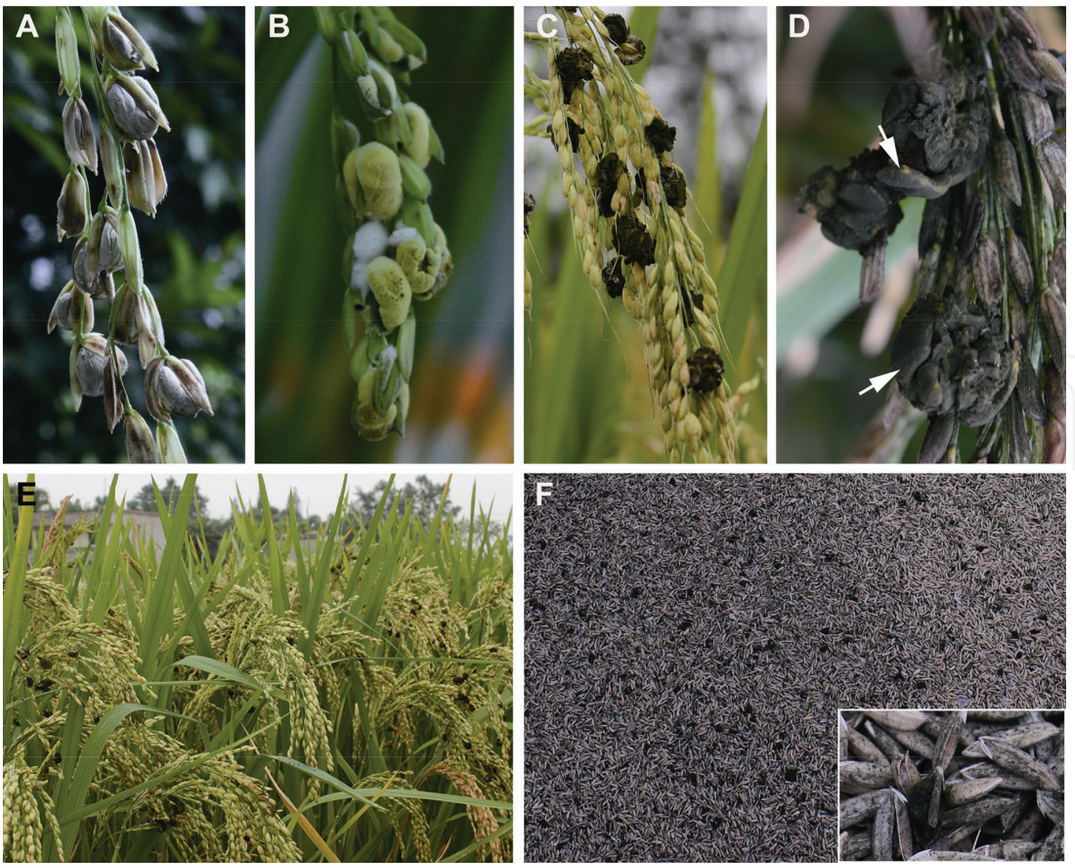

Figure 1.
Disease symptom of rice false smut: (A)–(C) white, yellow, and dark green false smut balls at early, middle, and late stages, respectively; (D) sclerotia (white arrows) are formed in false smut balls at the late stage; (E) field view of rice false smut disease; and (F) harvested rice grains are contaminated with rice false smut balls. Inset shows that rice grains are covered by chlamydospores from false smut balls.

The disease induces considerable losses both in yield and quality [7, 8], due to the occurrence of RFS balls and increased sterility of kernels adjacent to the balls [9]. Moreover, RFS balls produce two types of mycotoxins, i.e., ustiloxin and ustilaginoidin, which are poisonous to both humans and animals and impose significant health hazards by contaminating rice grains and straws [10–12]. For example, ustiloxin A causes kidney and liver damage in mice, due to its inhibition activity on microtubule assembly and skeleton formation of the eukaryotic cells [11, 13].

RFS balls seem to be randomly formed in some grains of rice panicles in the paddy field and are inevitably collected during harvest (**Figure 1E, F**). The disease spread varies within a field or between fields and is considered to be more severe in the proximity of drainage channels [14]. Epidemics of RFS disease tend to occur when rice booting and heading stages meet with rainfall periods. However, epidemics differ largely among varieties, fields, and seasons. This chapter describes our current knowledge on the mycotoxins, biology of the pathogen, pathogenesis of RFS, rice resistance, disease cycle, disease control, and disease assay.

2. Mycotoxins

V. virens produces two kinds of mycotoxins, ustiloxins and ustilaginoidins. Ustiloxins are water-soluble cyclic peptides, each including a 13-membered ring with an ether linkage [10]. The structures of six ustiloxins, including A–D, F, and G, have been identified so far [10, 15, 16]. A gene cluster has been suggested to be responsible for the ribosomal biosynthesis of ustiloxins [17]. Ustiloxins A and B are

among the most abundant ustiloxins in RFS ball and are mainly contained in the middle layer of mycelia and immature chlamydospores at early maturity stage [18]. Recently, rapid qualitative or quantitative detection methods, such as monoclonal antibody-based enzyme-linked immunosorbent assay and colloidal gold-based lateral flow immunoassay, have been established for detecting ustiloxins A and B in rice and feed samples [19, 20]. Ustilaginoidins are bis-naphtho-γ-pyrones and can be easily dissolved in organic solvent. Twenty-five ustilaginoidins (A-W) have been isolated from *V. virens* [21–25]. Ustilaginoidins can be isolated from RFS balls and solid rice media culturing *V. virens*. Several tested ustilaginoidins are mainly distributed in the layers embracing chlamydospores of yellowish-green or dark-green RFS balls [26].

Ustiloxins could inhibit polymerization of microtubule proteins and cause abnormal mitosis resembling, which would result in acute necrosis of renal tubular cells and hepatocytes in mice [10]. Ustiloxins also show phytotoxicity, inhibiting elongation of radicle and germ and inducing swelling of seedling root in rice [10, 16]. Cytotoxic activities of ustiloxins have been demonstrated on human tumor cell lines, such as A375, A549, BGC-823, HCT116, and HepG2 [10, 16]. Similar phytotoxic and cytotoxic activities have been detected for ustilaginoidins [25, 27, 28]. In addition, ustilaginoidins show antibacterial activities against several human or plant pathogens, such as *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Pseudomonas lachrymans*, *Ralstonia solanacearum*, *Staphylococcus haemolyticus*, and *Xanthomonas vesicatoria* [24]. Due to their anti-tumor and/or antibacterial abilities, ustiloxins and ustilaginoidins may be used as potential clinical medications. As a result, chemical synthesis of ustiloxins and their analogs has been carried out [29, 30].

3. Biology of the pathogen

The RFS pathogen belongs to the kingdom: Fungi, phylum: Ascomycota, class: Ascomycetes, subclass: Sordariomycetes, order: Hypocreales, family: Clavicipitaceae, genus: *Villosiclava,* and species: *virens* and its anamorphic stage is *Ustilaginoidea virens* [6]. The colony growth of *V. virens* in culture medium PSA (potato-sucrose-agar) is very slow, with a growth rate of approximately 20 mm in diameter per week [31]. *V. virens* produces pigments during culture in PSA and is prone to generate small colonies and plenty of conidia in PSB (potato-sucrose-broth) (**Figure 2A**–**D**). The conidia are elliptical with diameters ranging from 3 to 5 μm (**Figure 2E**). Upon maturation or under unfavorable conditions, conidia may develop to rounded chlamydospores with prominent spines on the surface (**Figure 2F**) [32–34]. One or two sclerotia, which are the sexual structure of *V. virens*, can be formed in a RFS ball (**Figure 1D**). Sclerotia are horseshoe-shaped and the length ranged from 2 to 20 mm (**Figure 2G**). After several months of dormancy, sclerotia could germinate and produce fruiting bodies with stromata (**Figure 2F**), which ultimately generates ascospores with length reaching 50 μm and width 1 μm [35].

Numerous efforts have been undertaken to optimize the culture media and culturing conditions for *V. virens*. PSA and PDA (potato-dextrose-agar) are suitable for culturing *V. virens* in solid media [31]. Moreover, stachyose is a preferential carbon source for *V. virens* and could significantly promote hyphal growth and conidia germination of *V. virens*, much better than other sugars, such as sucrose, glucose, and starch [36]. Stachyose can be also applied in optimization of culture medium for other filamentous fungi. Ammonium chloride, ammonium sulfate, and ammonium nitrate are the suitable nitrogen sources for *V. virens* growth [31]. The optimal growth of *V. virens* can be achieved at 28°C and pH 6–7 [37].

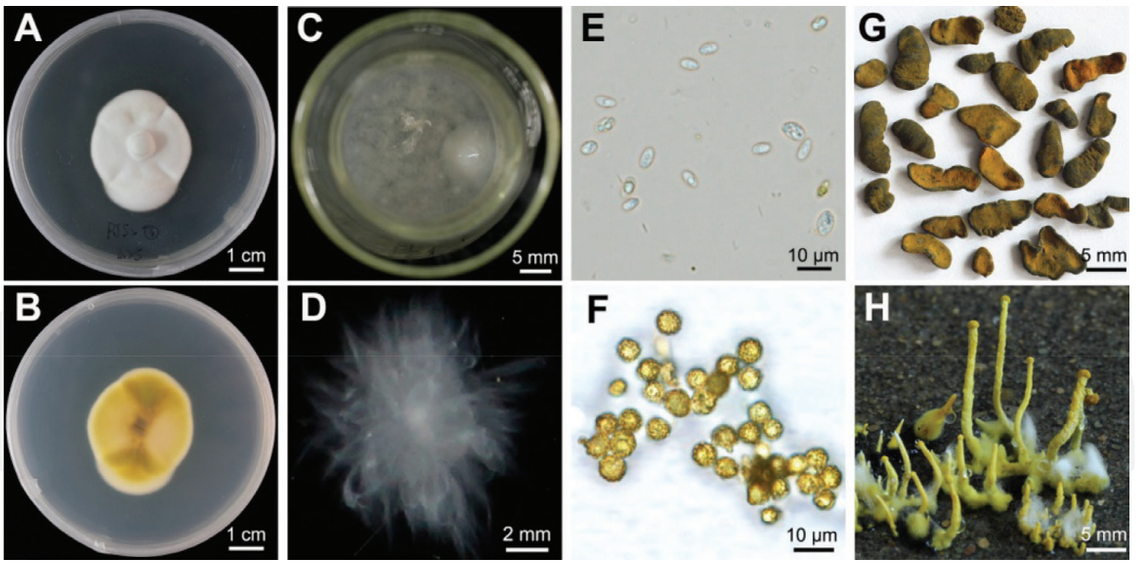

Figure 2.
Morphology of the rice false smut pathogen. Colony of Ustilaginoidea virens (anamorph) in PSA medium from top view (A) and back view (B). (C) Colonies of U. virens in PSB. (D) A single colony of U. virens in PSB. (E) Conidia of U. virens in PSB. (F) Chlamydospores of U. virens from false smut balls. (G) Sclerotia of Villosiclava virens (telemorph). (H) Sclerotia of V. virens germinate to produce fruit bodies. Images (G) and (H) are courtesy of Prof. Dongwei Hu from Zhejiang University, China.

4. Pathogenesis of RFS

4.1 Infection process of *V. virens*

The RFS pathogen *V. virens* specifically attacks rice flowers to form RFS balls, causing economically important disease. The infection process of *V. virens* in rice flower has been identified cytologically as follows: at late booting stage of rice, spores of *V. virens* come into contact with developing spikelets and germinate on their surface, or epiphytically grown hyphae reach the surface of developing spikelets. The hyphae could not penetrate the spikelet but extend into the inner space of a spikelet via the gap between the lemma and the palea [38]. After entering, the pathogen primarily infects the stamen filaments intercellularly [39], probably due to loose alignment of cells and flexible cell walls [40]. Lodicules and stigma could also be attacked, although to a lesser extent [39, 41]. However, no infection structures, such as appressorium and haustorium, can be detected during infection. Along with time, mycelia grow to enclose all the floral organs, then protrude out of the spikelet, and ultimately form a ball-shape colony covered with chlamydospores. At late stage of infection or in a RFS ball, stamen filaments are replaced by mycelia, but the ovary and lodicules remain intact, suggesting that they may contribute to the formation of RFS ball [42]. In addition, the hyphae of *V. virens* could not extend into pedicles and stems connecting the spikelets, and no anatomic changes are detected in pedicles [39].

Although RFS disease symptoms are observed at rice grains due to pathogen infections of spikelets, *V. virens* also grows on other rice organs without obvious symptoms. At the germination stage of rice seed, chlamydospores of *V. virens* could germinate on coleoptiles and the hyphae are able to extend intercellularly between epidermal cells [43]. At seedling stage, chlamydospores could also germinate on the surface of roots and grow in the intercellular space of root epidermal cells [44, 45]. More recently, a detailed observation on *V. virens*-infected rice roots indicates that the cellulose microfibrils of epidermal cell wall are very loose, similar to those of stamen filaments, and thus are prone to be infected [46]. However, the hyphae of *V. virens* are stopped by sclerenchyma layer from entering into endodermis and phloem tissues [46]. Again,

no appressorium or haustorium can be detected when *V. virens* infects tender coleoptiles and roots. To date, contribution of the infections of the coleoptiles and roots at the vegetative stage to RFS disease symptoms has not been determined if any, and the evidence of systemic infection of *V. virens* is currently lacking.

4.2 Genetic transformation of *V. virens*

Genetic manipulation is essential to clarify the pathogenicity of *V. virens* and its interaction with rice. Several transformation techniques have been successfully applied to *V. virens*. Electroporation, the process in which a strong electric pulse is applied to an organism in order to transiently increase membrane permeability, has the advantages of being rapid and inexpensive. Through electroporation on conidia, an enhanced green fluorescence protein (eGFP)-expressing *V. virens* strain was obtained, which was able to infect rice flowers and form RFS balls [47]. Polyethylene glycol (PEG)-mediated approach is typically more efficient than electroporation and generally yields a higher percentage of stable transformants. Ashizawa and colleagues [38] engineered a GFP-tagged *V. virens* strain via PEG-mediated transformation on protoplasts, and identified the infection route of *V. virens* in rice spikelets with this strain. *Agrobacterium tumefaciens*-mediated transformation (ATMT) is a fast and easy way to transfer foreign DNA into fungal cells. A few reports have recorded the establishment and optimization of ATMT procedure on *V. virens* conidia, and construction of T-DNA mutant libraries [48, 49], which facilitate characterizing virulence factors in this pathogen. For example, Yu et al. [48] obtained a T-DNA insertion library with 5600 hygromycin-resistant transformants, and identified 37 mutants with impaired pathogenicity. Targeted gene knockout with PEG- or ATMT-mediated transformation has been tried on *V. virens*; however, the homologous gene replacement frequency was very low [50]. Very recently, Liang and colleagues [51] established a CRISPR-Cas9 system, which remarkably increased the frequency of homologous gene replacement. The knockout efficiency could be as high as 50–90% for some *V. virens* genes.

4.3 Genome and pathogenicity of *V. virens*

The availability of *V. virens* genome provides a good basis for charactering its pathogenicity in rice flower. As reported, the genome of UV-8b is approximately 39.4-Mb, encoding 8426 putative proteins [52]. The strain IPU010 possesses a genome of 33.6-Mb, which encodes 6451 predicted proteins [53]. Genome analysis reveals that *V. virens* is evolutionarily closest to the entomopathogen *Metarhizium spp.*, suggesting host jumping from animal kingdom to plant kingdom [52]. Moreover, genome information provides evidence supporting that *V. virens* specifically infects rice flower and has a biotrophic lifestyle, since genes responsible for secreted proteins and secondary metabolism are enriched, while genes associated with polysaccharide degradation and nutrient uptake are diminished [52]. A web-based protein-protein interactive database for *V. virens-Oryza sativa* interaction has been released, greatly facilitating investigation of *V. virens* pathogenicity [54]. Putatively, 628 secreted proteins are encoded by *V. virens* genome, 193 of the secreted proteins are predicted to be effectors [52].

Effectors are powerful weapons possessed by pathogens to manipulate host immune system and metabolisms for successful colonization. Characterizing their roles is important for understanding pathogen-host interactions. In *V. virens* genome, a number of genes encoding effector proteins, such as UV_1261, UV_2508, and UV_2286, have been identified to suppress *Burkholderia glumae*-induced cell death [52], whilst UV_5823 shows ability to suppress plant RNA silencing [55]. On

the contrary, some effectors of *V. virens* could induce cell death or defense response in rice protoplast. For example, UV_44 induces cell death, and this ability relies on the serine peptidase active sites. UV_1423 could be *N*-glycosylated, which affects its ability to trigger cell death [56]. However, so far, no effectors have been characterized to function in the flower infection of *V. virens*.

Nevertheless, several virulence factors have been identified in *V. virens*. UvSUN2 is a SUN domain protein; loss of function of this factor results in inability of infecting rice flower, as well as abnormal stress responses and mycelium growth [57]. Mutation in *UvPRO1* increases sensitivity to abiotic stresses and attenuates virulence, in addition to impaired growth rate and sporulation [49]. In contrast, a low-affinity iron transporter encoded by *Uvt3277* negatively regulates virulence in *V. virens* [58].

4.4 Host compatible interaction with *V. virens*

Monitoring host responses to *V. virens* infection could help to uncover the pathogenesis of RFS. In an earlier transcriptome study, a series of differentially expressed genes have been identified in a susceptible rice cultivar 93-11 infected with a field *V. virens* isolate [59]. Among them, genes regulated by Ca^{2+} or abscisic acid are down-regulated, while genes regulated by Myb or WRKY transcription factors are up-regulated. *OsSWEET11* and *OsSWEET14*, which may be involved in disease susceptibility [60], are also up-regulated by *V. virens* infection. Specifically, many pollen development associated genes are down-regulated by *V. virens* infection, but not responsive to other biotic and abiotic stresses, suggesting that these genes may play unique roles in rice-*V. virens* interaction [59]. Additionally, several transcriptome analyses on compatible rice-*V. virens* interactions have been reported. Genes involved in hydrolase, transporter, and flower development tend to be down-regulated in susceptible cultivar Huang-Xiu-Zan upon infection [61]. Expression of many defense-related genes such as *PAL* and *PR* genes could be suppressed in susceptible rice cultivars infected with *V. virens* [42, 62].

As a successful pathogen, *V. virens* should have abilities to set up colonization in rice floral organs and acquire abundant nutrients for propagation, in addition to subvert rice immunity. Transcriptome analysis reveals that genes associated with flower opening, such as *ARF6* and *ARF8* homologs, are down-regulated by *V. virens* infection [42]. This may contribute to inhibition of flower opening during RFS pathogenesis [42]. Furthermore, *V. virens* infection causes failure of ovary fertilization. However, a number of grain-filling-specific genes, such as seed-specific starch synthesis related genes and those encoding seed storage proteins, are activated for high expression in *V. virens*-infected rice spikelets [42]. It is suggested that *V. virens* may be able to mimic fertilization and hijack rice grain-filling system for nutrient supply to pathogen growth and RFS ball formation. This finding is further supported by an independent study [63]. Although the underlying mechanism needs further investigation, the observation of *V. virens* activating rice grain filling could provide a promising explanation why mild *V. virens* infection enhances rice yield traits, including grain weight and filled grain number [9]. Identification and characterization of *V. virens* factors that manipulate rice grain filling should be an interesting research area in the future.

5. Resistance of rice

5.1 Sources and inheritance of RFS resistance

Various attempts have been made to screen rice cultivars resistant to RFS. Screening of 186 rice hybrids to RFS resistance was done by Liang and

colleagues [64], which identified few hybrids with low disease incidence. They screened the commercial hybrids that had lower rates of diseased panicles and infected florets at Xindu and Qionglai (Sichuan Province, China) in 2011 together with newly registered varieties. Lore et al. [65] evaluated some hybrids and inbred cultivars growing across India for susceptibility/tolerance to RFS. Artificial inoculation of false smut was done by Kaur et al. [66], which identified nine hybrids resistant to RFS among 125 rice genotypes screened. More detailed evaluation of RFS resistance was performed by Huang and colleagues [67]. A total of 843 rice accessions were screened in disease nurseries in 3 years although some of those accessions were planted in different locations and on different dates. Finally, 36 accessions were found to show no disease incidence. A highly susceptible accession Pujiang 6 was identified in this study. Polymorphism analysis determined several resistant accessions which could be used for crossing with Pujiang 6 to construct gene mapping populations [67].

Resistance of genes against *V. virens* has not been identified yet, but numerous efforts have been undertaken to study the inheritance of the resistance. Earlier, using 266 near-isogenic introgression lines derived from susceptible cultivar Teqing and resistant Lemont and natural infection data in the field, Xu et al. [68] identified two RFS resistance-contributing QTLs, *QFsr10* and *QFsr12*, located on chromosome 10 and 12, respectively. Later, the same group further identified 10 QTLs for RFS resistance [69]. Li et al. [70] developed a population of 157 recombinant inbred lines (RILs) from crossing a susceptible landrace Daguandao (*O. sativa* subsp. japonica) and a resistant cultivar IR28 (*Oryza sativa* subsp. indica). Subsequently, different RILs and parents were evaluated following effective artificial inoculation under field conditions. Genetic analysis showed that the RFS resistance was controlled by two major genes with equal effect of 11.41 and polygenes with minor effects. Further work identified seven QTLs for RFS resistance on chromosomes 1, 2, 4, 8, 10, 11, and 12, and the phenotypic variance ranged from 9.8 to 22.5% [71]. The inheritance of RFS resistance in two-line hybrid rice was investigated using natural infection technique. When a moderate susceptible sterile line TGMS 33S was crossed with susceptible restorer lines, the F1 was susceptible to RFS; when TGMS 33S was crossed with resistant restorer lines, 87% of the F1 showed dominant or incomplete dominant inheritance of RFS resistance, the rest showed recessive inheritance [72]. It should be noted that natural infection of RFS varies among fields and seasons, efficient artificial inoculation method is highly recommended to validate the disease phenotype. Although some progresses on screening for resistant materials and determining the resistance inheritance have been achieved, genes responsible for RFS resistance are still unknown.

5.2 Molecular basis of RFS resistance

Since many rice cultivars with high RFS resistance or high susceptibility have been identified, comparative transcriptome analysis is a promising method to mine resistance- or susceptibility-related genes in rice. For instance, time-course RNA-seq was carried out on susceptible cultivar LYP9 and resistant cultivar IR28 upon *V. virens* infection [62]. Data analysis revealed that many defense-related genes were only up-regulated in the resistant cultivar IR28, but not in LYP9. Particularly, phytoalexin biosynthetic pathway genes such as *OsCPS2*, *OsMAS*, and *OsKSL11* were significantly induced in IR28 at early infection stages, indicating that phytoalexins may contribute to rice resistance against RFS. *PR* family genes, such as β-1,3-glucanase and chitinase genes, were specifically up-regulated in IR28, while generally down-regulated in LYP9. Moreover, a chitinase gene cluster was found close to a RFS resistance QTL on chromosome 11 [71], and nine genes in this cluster were

activated by *V. virens* infection. These data suggest that chitinase genes are potential candidates of RFS resistance. In addition, genes encoding receptor-like kinases and WRKY transcription factors may also play roles in RFS resistance. Interestingly, the resistant cultivar IR28 seemed to suppress *V. virens* genes that are associated with pathogenicity and fungal reproduction [62]. Another comparative transcriptome study demonstrated that peroxidase and flavin-containing monooxygenase genes, and genes involved in hormone metabolism were regulated differently in resistant and susceptible cultivars in response to *V. virens* infection [61].

Accumulation of H_2O_2 is a typical plant basal defense fighting against pathogen infections [73]. During a compatible interaction between rice and *V. virens*, obvious H_2O_2 accumulation was detected on the lemma and the palea of infected spikelet. H_2O_2 also enriched in the anthers, stamen filaments, and lodicules of the infected spikelets [74]. However, it needs to be further investigated whether H_2O_2 accumulation pattern is different between resistant and susceptible rice cultivars upon *V. virens* infection. A preliminary study described that higher contents of lignin and polyphenolic compounds were detected in spikelets of resistant rice variety Shuijing 3 than in those of susceptible variety 9522 [75], suggesting the role of these secondary metabolites in RFS resistance. To engineer rice resistant to RFS, an elicitor gene *hrf1* from *Xanthomonas oryzae* pv. *oryzae* was ectopically expressed in R109, which is susceptible to RFS. Artificial inoculation and natural infection both supported that *hrf1* conferred high resistance to the RFS pathogen, presumably through enhancing the expression of defense-related genes, including *OsPR1a*, *OsPR1b,* and *PAL* [76].

Based on the current findings that *V. virens* possesses intercellular infection strategy and biotrophic life style, and that resistant rice cultivars show up-regulation of pathogenesis-related genes and accumulation of secondary metabolites upon infection, basal defense of rice should play a dominant role in resistance against *V. virens*.

6. Disease cycle

V. virens attacks rice flowers and forms RFS balls covered with chlamydospores and/or generate sclerotia, which are considered as primary inocula of RFS disease (**Figure 3**). As to the sexual cycle, a large number of sclerotia can be produced when RFS balls develop in autumn [35]. Sclerotia cannot germinate immediately, requiring a dormancy period of 2–5 months at room temperature or 4°C. They overwinter in the field and could survive up to 10 months with maintaining germination ability to generate ascospores under 25°C and high humidity [77, 78]. Even more, sclerotia can survive with high germination rate up to 5 years when stored in a dry environment at 2–4°C. In the next spring, sclerotia start to germinate, and the germination time varies among different sclerotia. Theoretically, a sclerotium could produce up to 21 million ascospores [35]. Although sclerotia are easy to rot in paddy fields under natural conditions, a limited number of sclerotia can still produce plenty of ascospores. This is supported by the fact that ascospores could be trapped 60 cm above ground in paddy fields between May and September, coinciding with rice booting stage and *V. virens* infection time [35]. Ascospores are able to infect rice flowers to form RFS balls [78, 79]. Therefore, it is believed that sclerotia act as primary inocula of RFS and play an important role in the disease cycle.

With regard to the asexual cycle, chlamydospores from RFS balls are easily transmitted by wind and rainfall, and attack developing rice spikelets of late ripening rice cultivars. This is supported by the fact that fresh chlamydospores have high germination rate and could successfully infect rice flowers to form RFS balls [79, 80]. Chlamydospores can overwinter in soil and on dead plants, or on harvested RFS balls and rice seeds, and survive up to several months; however, the germination rate

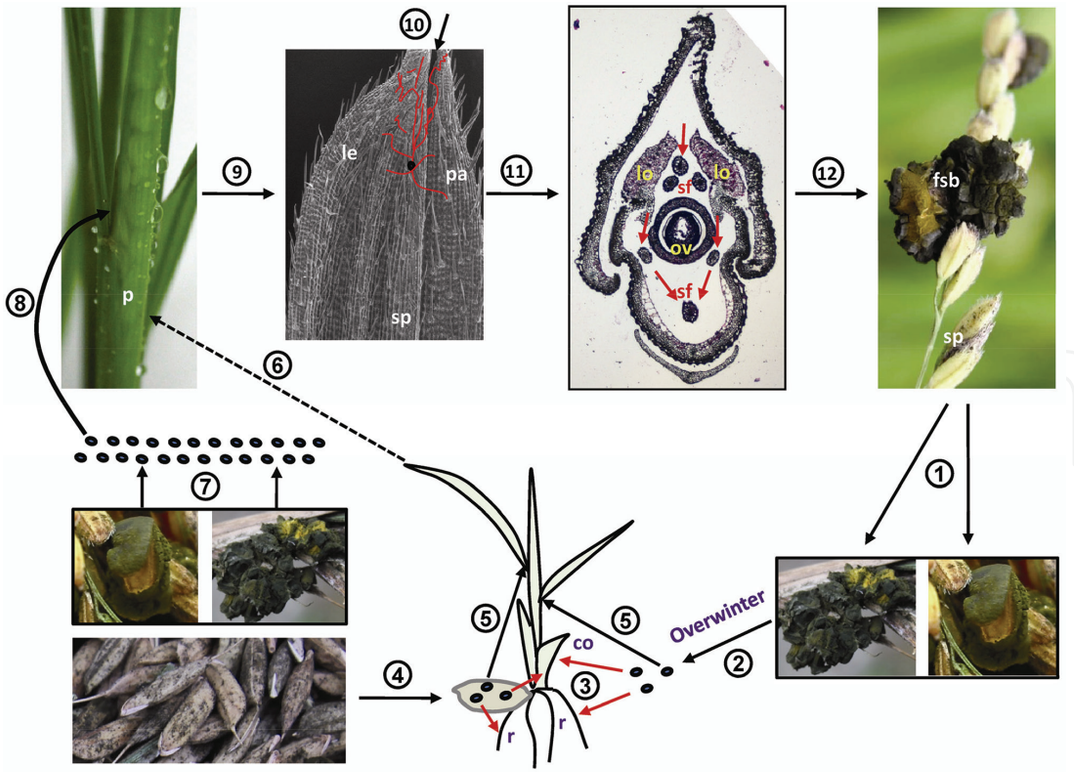

Figure 3.
Disease cycle of rice false smut. Rice false smut balls with chlamydospores and sclerotia are formed in rice spikelets (①), and overwinter in field (②). Next spring, spores in soil (③) and on contaminated rice grains (④) germinate and attack rice roots and coleoptiles when rice seeds are germinating. Hyphae grow intercellularly in roots and coleoptiles, but could not infect seedlings systemically. Instead, hyphae may grow epiphytically on leaf surface or leaf sheath, and reach the external surface between tiller buds at the late vegetative stage (⑤) or even the surface of elongated stems at the heading stage. It is possible that the pathogen hyphae reach the inner space of rice panicles and initiate infection at the late booting stage (⑥). Meanwhile, conidia produced by chlamydospores and/or ascospores from sclerotia (⑦) also initiate attack on rice spikelets in developing panicles (⑧). Spores could firstly germinate on the surface of a spikelet (⑨), and the hyphae extend into the inner space of the spikelet via the gap between the lemma and the palea (⑩). Stamen filaments are the major infection sites for the pathogen (⑪). After successful colonization in floral organs, a large amount of fungal mass are formed and eventually grow into a false smut ball (⑫). The route of infection is indicated by arrows and numbers. Arrows with dotted lines are the steps needing further exploration. Red arrows indicate the main infection sites of the pathogen. Red curve lines represent pathogen hyphae. co, coleoptile; p, panicle; sp, spikelet; le, lemma; pa, palea; sf, stamen filament; lo, lodicule; ov, ovary; and fsb, false smut ball.

decreases rapidly [81]. In the next rice planting season, chlamydospores overwintered in fields and on rice seeds may germinate with hyphae to infect coleoptiles of germinating rice seeds and roots of seedlings [43–45]. Since chlamydospores could not be trapped in fields until RFS balls appear [35], it is unclear how chlamydospore germination time couples with rice booting stage for infecting rice flowers. Studies suggest that coleoptile and root infections may lead to asymptomatic colonization of the pathogen in rice plants at subsequent stages. Sensitive PCR methods have been applied to successfully detect *V. virens* in various tissues of rice before panicle heading [82, 83], suggesting the presence of pathogen in rice plants. Furthermore, colorimetric *in situ* hybridization reveals that *V. virens* mycelia are present on the surface of tiller buds enclosed by young leaf sheaths at vegetative stage, and also on the surface of elongated stems around leaf axils at the heading stage. As *V. virens* infection is not systemic, epiphytic growth could explain how the presence of mycelia in rice plants lasts from the germinating stage to the heading stage of rice [84]. Preset of pathogen mycelia in rice plants especially in leaf sheaths should greatly increase chances of attacking flowers.

Epiphytic growth of *V. virens* is not only found in rice plants, but also detected on leaf surface of various paddy field weeds and on abiotic surfaces (e.g., cellophane and parafilm) [33]. Under wet conditions, *V. virens* conidia are capable

of blastogenesis and could produce a large number of secondary conidia on these surfaces in several days. Chlamydospores and ascospores both germinate to produce conidia [78, 81], and the blastogenesis and epiphytic growth greatly increase the amount of inocula under continuing rainy conditions. Therefore, epidemics of RFS disease usually occur when rice booting stage meets with rainy days.

Alternative hosts of a pathogen commonly play an important role in disease cycle. Earlier, paddy field weeds such as *Digitaria marginata* [85], *Panicum trypheron* [86], *Echinochloa crus-galli*, and *Imperata cylindrica* [87] have been reported as alternative hosts of *V. virens*. However, a recent survey demonstrated that infection in these potential alternative hosts is very rare in nature [88]. Still, the presence of *V. virens* in weeds as confirmed by PCR detection [82] and epiphytic colonization on weed leaf surface [33] suggests that paddy field weeds contribute to RFS disease cycle in an unconventional way.

7. Disease control

In recent years, the RFS disease has become a severe threat to rice production due to its epidemics. In order to minimize direct economic loss, suitable management practices have to be made to manage the disease. Breeding and utilization of resistant cultivar is the most effective and economical way to control RFS disease and ensure the high yield of rice. Attempts have been made to identify sources of resistance against *V. virens* (see above). As inheritance of RFS resistance is not well understood, breeding for resistant rice is hindered. Late ripening rice cultivars with large panicle and high grain density are prone to RFS and should be carefully chosen for wide application.

Culture managements have been studied to reduce incidence of RFS. Early planted rice has less RFS balls rather than the late planted rice. Excess application of nitrogenous fertilizer should be avoided. Since high rate of nitrogen increases the disease incidence, sensible use of nitrogen is recommended. Fertilizer ratio is often a reasonable parameter for growers to adjust, so as to enhance the stress tolerance of rice plants, and ultimately reduce the RFS incidence. Field ridges and irrigation channels should be kept clean to eliminate alternative hosts. Conservation tillage and furrow irrigation have some effects on suppressing the disease index [2, 89]. Using suitable plant spacing and utilizing uncontaminated rice seeds are also recommended.

Chemical control, i.e., fungicide application, can be effective but is often not economical and environment-friendly. Using fungicides with high efficiency, low toxicity, and low residue is currently the best choice to control RFS disease. Fungicides, such as Wenquning (a suspension of *Bacillus subtilis* in a solution of validamycin), cuproxat SC, simeconazole, tebuconazole, difenoconazole, and hexaconazole, are effective to reduce RFS disease incidence [64, 90, 91]. It is noteworthy that the timing of spraying fungicides is critical. Application of fungicides after panicle heading should be prevented, as the pathogen infects rice flowers at late booting stage and already successfully colonizes the inner floral organs after heading. As supporting evidence, simeconazole is found to be more effective against RFS when applied 3 weeks before rice heading [92].

8. Disease assay

8.1 Natural infection

To evaluate RFS sensitivity of rice under natural infection, several classification standards of disease incidence have been reported. For example, in 1996, the

International Rice Research Institute (IRRI) [93] classified RFS into 6 scales based on incidence of severely infected tillers or infected spikelets, i.e. 0, no incidence; 1, less than 1%; 3, 1–5%; 5, 6–25%; 7, 26–50%; and 9, 51–100%. Later on, Tang and colleagues [94] established a new classification standard, and developed Disease Index to determine RFS incidence. The classification standard was based on aspect ratio and 100-weight of RFS ball, grain weight, seed setting rate, and yield loss of single diseased panicle. Six scales were classified: 0, no RFS ball; 1, one RFS ball; 2, two RFS balls; 3, 3–5 RFS balls; 4, 6–9 RFS balls; and 5, ≥10 RFS balls. Disease index = $\sum$ (Disease scale value × Diseased plant number)/(Total plant number × Highest disease scale) × 100. Note that only the highest disease scale value is adopted for each plant. This classification standard has been widely applied in recent studies [70–72]. When using natural infection method, disease incidence should be evaluated for multiple years at multiple locations, with multiple sowing dates.

8.2 Artificial inoculation

Due to uncertainty of environmental conditions under natural infection, a high efficient artificial inoculation method is desired for evaluating *V. virens* pathogenicity and rice resistance. As the pathogen specifically infects rice stamen filaments at specific rice stage to cause disease, it is difficult and complicated to optimize an efficient inoculation system. Parameters, such as inocula type, inoculation time and method, incubation conditions after inoculation, and so on, should be considered [42, 95–98]. To date, a number of studies conclude an efficient inoculation method under controlled conditions: first, culture *V. virens* in PSA at 28°C until white colony grows large enough for inoculating into PSB. Usually, 4–8 plugs of mycelia with around 6 mm diameter each are needed, and incubated in PSB at 28°C in dark, 110–150 rpm for 5–7 days. Second, a mixture of mycelia and conidia is blended as inocula, of which the conidia concentration is adjusted to around 10^6 conidia/mL with 4% potato juice. Third, at late booting stage of rice (5–7 days before heading), inocula are injected into panicles with a syringe until the inocula drip out (**Figure 4**). Fourth, the inoculated rice plants are kept at 25°C and 95% relative humidity for 5 days, and then moved to 28°C with relative humidity over 75%. Around 4 weeks post inoculation, disease incidence could be recorded. High RFS incidence (90–100%) has been obtained on susceptible rice cultivars such as Pujiang 6, Yueyou 938, and so on [42, 97]. It should be noted that the artificial inoculation method needs to be modified when applied to different *V. virens* isolates and rice cultivars. For example, the highest RFS incidence is achieved when inoculation is carried out 3–5 days before heading for Yueyou 938 [97], and that is 5–7 days before heading for Pujiang 6 [42]. The disease symptom progression also varies among different *V. virens*-rice combinations under different post-inoculation conditions. As for *V. virens* PJ52-rice Pujiang 6 interaction under artificial inoculation conditions, no obvious symptom could be found through 1 dpi to 5 dpi. At 9 dpi, white fungal biomass can be seen with the naked eye. The fungal biomass enlarges and protrudes out of rice spikelets as early as 13 dpi, and large RFS balls appear at 17 dpi (**Figure 4**).

The above-mentioned classification standard and Disease Index [94] can also be applied to evaluation of disease incidence under artificial inoculation. Alternatively, the following method can be adopted when the disease scale is reaching the highest (i.e., scale 5, ≥10 RFS balls) for each plant. This situation is often encountered when using susceptible rice cultivars to evaluate *V. virens* pathogenicity. Due to high variation of the disease incidence for RFS pathosystem, at least 100 panicles from at least 30 rice plants are recommended to be inoculated. At around 4 weeks post inoculation, each inoculated panicle is collected for counting the number of RFS balls and the number of total spikelets. The number of RFS *b*alls *p*er inoculated *p*anicle

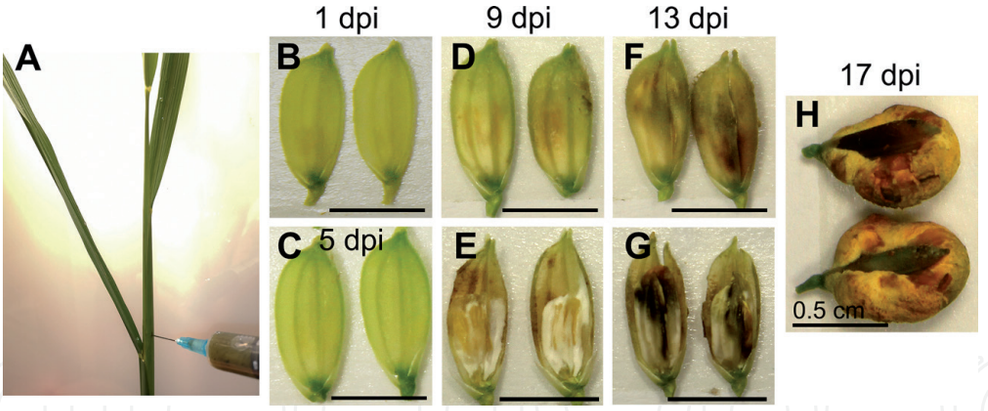

Figure 4.
Artificial inoculation of rice false smut pathogen. (A) Inocula of V. virens are artificially injected with a syringe into a rice panicle at the late booting stage (5–7 days before heading). (B)–(H) Symptom development on rice spikelets after artificial inoculation. No obvious symptom is seen at 1 dpi (day post inoculation) and 5 dpi. White fungal mass could be detected in inner space of the infected spikelets at 9 dpi (E). The fungal mass grows larger at 13 dpi (G), and at 17 dpi eventually forms a ball-shape colony, called false smut ball (H). Scale bar, 0.5 cm. Image (A) is courtesy of Dr. Junjie Yu from Jiangsu Academy of Agricultural Sciences, China.

is recorded as BPP. Percentage of diseased spikelets (PDS) for each inoculated panicle is calculated as: PDS = 100 × number of diseased spikelets/total number of spikelets. To compare pathogenicity among different *V. virens* isolates or resistance/susceptibility among different rice cultivars, BBP and PDS values are recommended to be presented as box-plot. Statistical analysis such as the *ANOVA* test is required to calculate the significance of difference among BBP or PDS datasets.

9. Future aspects

RFS is an emerging disease threatening the production safety of rice grains worldwide. Great progresses have been made to understand the RFS pathogen and its interaction with rice. However, many important questions are yet to be addressed. How much the mycotoxins produced by *V. virens* are contaminated in cooked rice and livestock feed? Whether mycotoxins play a role in pathogenicity? Are there any RFS resistance genes and how do they mediate defense against *V. virens*? How does *V. virens* activate rice grain filling system to hijack nutrient supply for the formation of RFS ball? In addition, an efficient inoculation method mimicking natural infection is highly desired for basic research and accurate evaluation of rice resistance.

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Conflict of interest

The authors declare no conflict of interest.

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