Pathogen Profile

Current understanding on *Villosiclava virens*, a unique flower-infecting fungus causing rice false smut disease

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SUMMARY

Villosiclava virens (Vv) is an ascomycete fungal pathogen that causes false smut disease in rice. Recent reports have revealed some interesting aspects of the enigmatic pathogen to address the question of why it specifically infects rice flowers and converts a grain into a false smut ball. Comparative and functional genomics have suggested specific adaptation of Vv in the colonization of rice flowers. Anatomical studies have disclosed that Vv specifically infects rice stamen filaments before heading and intercepts seed formation. In addition, Vv can occupy the whole inner space of a spikelet embracing all floral organs and activate the rice grain-filling network, presumably for nutrient acquisition to support the development of the false smut ball. This profile provides a general overview of the rice false smut pathogen, and summarizes advances in the Vv life cycle, genomics and genetics, and the molecular Vv–rice interaction. Current understandings of the Vv–rice pathosystem indicate that it is a unique and interesting system which can enrich the study of plant–pathogen interactions.

Taxonomy: *Ustilaginoidea virens* is the anamorph form of the pathogen (Kingdom Fungi; Phylum Ascomycota; Class Ascomycetes; Subclass Incertae sedis; Order Incertae sedis; Family Incertae sedis; Genus *Ustilaginoidea*). The teleomorph form is *Villosiclava virens* (Kingdom Fungi; Phylum Ascomycota; Class Ascomycetes; Subclass Sordariomycetes; Order Hypocreales; Family Clavicipitaceae; Genus *Villosiclava*).

Disease symptoms: The only visible symptom is the replacement of rice grains by ball-shaped fungal mycelia, namely false smut balls. When maturing, the false smut ball is covered with powdery chlamydospores, and the colour changes to yellowish, yellowish orange, green, olive green and, finally, to greenish black. Sclerotia are often formed on the false smut balls in autumn.

Identification and detection: Vv conidia are round to elliptical, measuring 3–5 µm in diameter. Chlamydospores are ornamented with prominent irregularly curved spines, which are 200–500 nm in length. The sclerotia are black, horseshoe-shaped and irregular oblong or flat, ranging from 2 to 20 mm. Nested polymerase

chain reaction (PCR) and quantitative PCR have been developed to specifically detect Vv presence in rice tissues and other biotic and abiotic samples in fields.

Host range: Rice is the primary host for Vv. Natural infection by Vv has been found on several paddy field weeds, including *Digitaria marginata*, *Panicum trypheron*, *Echinochloa crusgalli* and *Imperata cylindrica*. However, the occurrence of infection in these potential alternative hosts is very rare.

Life cycle: Vv infects rice spikelets at the late rice booting stage, and produces false smut balls covered with dark-green chlamydospores. Occasionally, sclerotia form on the surface of false smut balls in late autumn when the temperature fluctuates greatly between day and night. Both chlamydospores and sclerotia may serve as primary infection sources. Rainfall at the rice booting stage is a major environmental factor resulting in epidemics of rice false smut disease.

Disease control: The use of fungicides is the major approach for the control of Vv. Several fungicides, such as cuproxat SC, copper oxychloride, tebuconazole, propiconazole, difenoconazole and validamycin, are often applied. However, the employment of resistant rice cultivars and genes has been limited, because of the poor understanding of rice resistance to Vv.

Useful websites: *Villosiclava virens* genome sequence: <http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=JHTR01#contigs>

Keywords: biotroph, effector, epiphytic growth, flower-infecting, grain-filling, rice false smut ball, *Ustilaginoidea virens*.

INTRODUCTION

Villosiclava virens (Vv) is an ascomycete fungus, which possesses a teleomorphic state producing sexual ascospores and an anamorphic state generating asexual chlamydospores. The anamorph and teleomorph forms of the fungus have been given the names *Ustilaginoidea virens* (Cooke) Takahashi and *Villosiclava virens*, respectively (Tanaka *et al.*, 2008). Both sexual and asexual spores can infect the rice spikelet and convert a rice grain into a ball of mycelium (Ikegami, 1960, 1963a; Wang, 1995; Yoshino and Yamamoto, 1952).

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Fig. 1 Typical symptoms of rice false smut disease caused by *Villosiclava virens* (Vv). False smut balls are formed in Vv-infected rice panicles. During disease progression, the colour of false smut balls changes from white (a) to yellowish (b) and, ultimately, to greenish black (c). Sclerotia (inset) are usually formed on false smut balls in autumn. Arrows and arrowheads indicate false smut balls and sclerotia, respectively.

Vv is not a bona fide smut fungus and Vv-caused disease is called false smut disease (Bischoff *et al.*, 2004). This disease not only reduces rice yield and grain quality (Chib *et al.*, 1992; Jiang *et al.*, 2009; Ono and Uehara, 1949; Osada, 1995; Yang *et al.*, 2012), but also leads to the contamination of grains and straws with Vv-produced mycotoxins, which are poisonous to both humans and animals (Koiso *et al.*, 1994; Nakamura *et al.*, 1994; Zhou *et al.*, 2012). Thus, food safety requires a rapid and accurate detection of Vv mycotoxins (Fu *et al.*, 2015a,b; Ji *et al.*, 2012; Miyazaki *et al.*, 2009; Shan *et al.*, 2012, 2013, 2014). In recent years, rice false smut disease has been increasingly recorded in rice-growing areas worldwide (Brooks *et al.*, 2010; Guo *et al.*, 2012; Jecmen and TeBeest, 2015; Ladhakshmi *et al.*, 2012; Singh and Pophaly, 2010), and has attracted increasing attention from researchers and farmers.

Despite several efforts on field screening for resistant rice cultivars and quantitative resistance locus (QRL) analysis (Guo *et al.*, 2012; Huang *et al.*, 2015; Zhou *et al.*, 2014), rice resistance genes against Vv have not been identified. The inheritance of false smut resistance is still obscure, hindering breeding for resistance to false smut pathogen. The development of efficient strategies to control rice false smut disease requires a detailed understanding of the biology, genetics and pathogenicity of the pathogen. This profile provides an overview of the current knowledge on Vv, focusing on updates in the Vv life cycle, genomics and genetics, and its molecular interaction with rice.

DISEASE SYMPTOMS AND CONTROL

The typical symptom caused by Vv in rice is the formation of a white fungal mass protruding from the inner space of a spikelet at the early infection stage, followed by transformation into a light-yellow smut ball which, ultimately, changes colour to greenish-black (Fig. 1). The

outer layer of the mature smut ball consists of numerous chlamydospores, and is often covered by sclerotia in autumn (Fig. 1c) (Ikegami, 1961, 1963a). False smut ball is the only disease symptom identified so far. In a rice panicle, only some of the spikelets are randomly infected and converted into false smut balls (Fig. 1).

Currently, the control of rice false smut disease mostly relies on fungicides. Several fungicides, such as Wenquning (a suspension of *Bacillus subtilis* in a solution of validamycin), copper hydroxide, copper oxychloride, cuproxat SC, simeconazole, horizon, tebuconazole, difenoconazole and hexaconazole, have been shown to possess over 70% control against rice false smut disease (Ahonsi and Adeoti, 2003; Gao *et al.*, 2010; Liang *et al.*, 2014; Tripathi *et al.*, 2014; Tsuda *et al.*, 2006; Zhou and Wang, 2011). It should be noted that the timing of spraying with fungicides is critical for control. For instance, the fungicide simeconazole is found to be more effective against rice false smut disease when applied 3 weeks before rice heading (Tsuda *et al.*, 2006).

Alternatively, cultural management has been investigated in the control of rice false smut disease. Brooks *et al.* (2009) reported that conservation tillage, continuous rice cropping and moderate nitrogen fertility rates could lead to 69%, 88% and 34% reduction, respectively, in rice false smut disease for susceptible cultivars in Arkansas, USA. Furrow irrigation has also been shown to greatly suppress rice false smut disease in susceptible rice varieties (Brooks *et al.*, 2010). However, furrow irrigation is not practicable in all rice fields and may result in severe rice blast disease in some varieties (Bonman, 1992).

IDENTIFICATION AND DETECTION

The rice false smut ball is covered with chlamydospores, which are round to elliptical and measure from 3 to 5 μ m in diameter

(Kim and Park, 2007). Irregularly curved spines with a length of 200–500 nm can be observed on the surface of chlamydospores (Kim and Park, 2007). The mature false smut ball sometimes produces sclerotia, which are black, horseshoe-shaped and irregular oblong or flat, and measure from 2 to 20 mm in length (Fig. 1c) (Fu *et al.*, 2012). Under scanning electron microscopy, the outer sclerotial wall is rough and the interior of sclerotia is intertwined with compact hyphae (Fu *et al.*, 2012).

At the molecular level, a polymerase chain reaction (PCR)-based technique has been developed for the detection of the Vv pathogen. Vv-specific primers and corresponding nested primers have been designed based on the rDNA internal transcribed spacers (ITS1 and ITS2) and the 5.8S rRNA gene (Zhou *et al.*, 2003), and successfully applied for the detection of Vv in various tissues of rice and related grasses, facilitating the early prediction of false smut disease in fields (Zhou *et al.*, 2006). Nested PCR has also been developed to detect Vv in rice panicles before and after heading; the results indicate the existence of the pathogen before heading (Ashizawa and Kataoka, 2005). Real-time PCR assays with higher sensitivity than the conventional nested PCR have also been set up and successfully applied to detect the existence of Vv in soil (Ashizawa *et al.*, 2010), rice samples (Li H. *et al.*, 2013) or air samples around susceptible rice plants in fields (Zheng *et al.*, 2012).

LIFE CYCLE

Vv ascospores and chlamydospores can infect rice spikelets (Ikegami, 1960, 1963a; Wang, 1995; Yoshino and Yamamoto, 1952). Successful infection of Vv produces white ball-shaped mycelium, which ultimately develops into a false smut ball covered with dark-green chlamydospores (Ikegami, 1961; Tang *et al.*, 2013; Zhang Y. *et al.*, 2014). Sclerotia are often developed on the surface of false smut balls in late autumn when the temperature fluctuates greatly between day and night (Ikegami, 1963a). With regard to the sexual cycle, the sclerotia may overwinter in soil and germinate to produce stromata, which then form asci, releasing ascospores (Deng, 2015; Honkura and Kimura, 1988; Singh and Dubey, 1984; Zhang Y. *et al.*, 2014). For the asexual cycle, chlamydospores on the surface of false smut balls may survive in nature and serve as an important source of inoculum between seasons (Biswas, 2001; Wang, 1988; Zhang Y. *et al.*, 2014). However, there is an open question of how the germination of chlamydospores and sclerotia couples with the rice booting stage, and initiates infection in rice flowers to form false smut balls. Recent studies have provided important insights into this question.

Status between infection seasons

Many efforts have been made to uncover the status of Vv from the end of a previous infection season to the start of the next (see

below). Generally, Vv propagules from rice false smut balls have two destinations, i.e. in fields and on harvested rice seeds.

In fields, chlamydospores and sclerotia may overwinter in soil and germinate in appropriate conditions. Studies have demonstrated that the germination rate of chlamydospores decreases rapidly with storage time and maturity of the false smut ball (Fan *et al.*, 2010; Wang, 1988; Wang and Bai, 1998), implicating chlamydospore dormancy. Nevertheless, high moisture and warm temperature (i.e. 26 °C) can break the dormancy of chlamydospores and promote their germination under laboratory conditions (Wang, 1988). Sclerotia can survive over 10 months in laboratory or field conditions, and maintain germination ability to produce ascospores under high humidity, 25 °C and light conditions (Dong and Fu, 1989; Miao, 1994; Wang, 1995). The germination conditions required for chlamydospore and sclerotia can be met by the environmental conditions at the rice booting stage. It is indicated that chlamydospores and sclerotia may serve as inocula at the rice booting stage. Chlamydospores and ascospores both germinate to produce secondary conidia (Wang, 1988, 1995). Vv conidia are capable of blastogenesis and epiphytic colonization on the surfaces of various plant leaves (e.g. rice and paddy field weeds) and on abiotic surfaces under wet conditions, producing plenty of propagules in several days (Fig. 2a–d) (Fan *et al.*, 2014). Therefore, the amount of inoculum should be greatly increased at the rice booting stage, especially under continuing rainy conditions. This might be the reason why epidemics of false smut disease occur when the rice booting stage coincides with rainy days. In addition, alternative hosts of Vv have been reported in the field, such as the common rice weeds *Digitaria marginata* (Shetty and Shetty, 1985), *Panicum trypheron* (Shetty and Shetty, 1987), *Echinochloa crusgalli* and *Imperata cylindrica* (Atia, 2004). However, infection occurrence in these potential alternative hosts is very rare in nature, indicating their limited roles in the Vv life cycle (Deng *et al.*, 2015).

Harvested rice seeds from Vv-diseased fields are contaminated with false smut balls containing chlamydospores (Ditmore and TeBeest, 2005). Ditmore *et al.* (2006) confirmed the presence of Vv by PCR in rice plants grown from Vv-contaminated seeds, but not in control plants. Three-week-old seedlings grown from healthy seeds in Vv-contaminated soils were also detected as Vv positive by PCR (TeBeest *et al.*, 2010). Nevertheless, no false smut balls were found on panicles produced from contaminated seeds or grown in contaminated soils. Miao (1993) found that Vv-contaminated rice seeds planted in sterile soil under controlled environmental conditions led to the formation of false smut balls in panicles, whereas panicles produced from 0.2% CuSO₄-treated seeds were not diseased. However, whether the pathogen forming the false smut balls is the same on contaminated seeds needs to be further clarified. Using a green fluorescence protein (GFP)-tagged Vv strain should be a promising approach.

Fig. 2 *Villosiclava virens* (Vv) undergoes blastogenesis and epiphytic growth and can form false smut balls in a rice male-sterile mutant. (a–d) Representative epifluorescent microscopy images showing epiphytic growth and/or blastogenesis of a green fluorescent protein (GFP)-labelled Vv isolate on detached leaves of rice (a, *Oryza sativa* accession Pujiang 6), paddy field weed *Echinochloa crusgalli* (b) and abiotic surface of cellophane (c, d). White arrows indicate Vv hyphae. c, conidium; sc, secondary conidium. Bars, 50 μ m (a, b), 10 μ m (c, d). (e) A representative image showing false smut balls on the rice male-sterile mutant *abcg15* (Qin *et al.*, 2013). The Vv isolate P4 was inoculated at the late booting stage and the photograph was taken at 17 days post-inoculation. White arrows indicate the white sterile anthers in *abcg15* flowers. fsb, false smut ball.

Infection process in rice flowers

It was once believed that Vv only infected fertilized spikelets (Chen *et al.*, 2007). However, recently, we have found that the rice male-sterile mutant *abcg15* (Qin *et al.*, 2013) can be severely infected by Vv (Fig. 2e), indicating that rice flower fertilization is not essential for Vv infection and the formation of a false smut ball. In addition, detailed anatomical analyses through artificial inoculation have revealed that infection occurs at the booting stage on developing spikelets before fertilization, and the ovaries in Vv-infected spikelets are interrupted in fertilization and thus are unable to set seeds (Fan *et al.*, 2015; Tang *et al.*, 2013). Using a Vv strain that expressed a GFP, Ashizawa *et al.* (2012) demonstrated the infection route of Vv in rice panicles after artificial inoculation. After injection into rice sheaths at the late booting stage (5–7 days before heading), Vv conidia germinate on the surface of developing spikelets, and hyphae gradually grow into the inner space of spikelets through the small gap between the lemma and palea. In this phase, Vv presumably undergoes epiphytic growth, so that no infection sites are observed on the spikelet surface. Sequentially, hyphae extend into the inner floral organs, including the anthers, filaments, stigmas and lodicules, and ultimately embrace all the organs (Ashizawa *et al.*, 2012). More detailed histological examinations of false smut balls from both artificial inoculation and natural infection have revealed that Vv infects the stamen filaments and occasionally attacks the stigma and lodicules; however, no infection of the ovary could be found (Tang *et al.*, 2013). The pathogen hyphae extend intercellularly along the filament base, and no specialized fungal infection structures, such as appressoria and haustoria, were detected

(Tang *et al.*, 2013). By exploiting a GFP-tagged strain, Hu *et al.* (2014) also demonstrated that stamen filaments were the primary sites of Vv colonization.

Life style and infection type

It was once a topic of debate whether Vv is a biotroph or necrotroph and whether Vv is a systemic infection or flower-specific pathogen (Gao and Zhong, 2011). Progress made in recent years has indicated that Vv is a biotroph and flower-infecting pathogen, although it can asymptotically colonize other rice organs and other plants (Fan *et al.*, 2014, 2015; Tang *et al.*, 2013; Zhang Y. *et al.*, 2014).

Vv attacks rice floral organs and grows intercellularly without killing host cells (Tang *et al.*, 2013). During the formation of false smut balls, rice floral organs, such as anthers and ovaries, remain alive; even in mature smut balls, greenish and alive ovaries can be observed (Fan *et al.*, 2015; Tang *et al.*, 2013; Zhang *et al.*, 2010). However, cell death may be inevitably induced in filaments in which the host cells seem to be replaced by hyphae under microdissection at the late infection stage (Tang *et al.*, 2013). Overall, Vv shows no necrotrophic growth during the entire infection and acquires nutrients from living host tissues, whilst maintaining them alive even longer. Thus, Vv is classified as a biotroph. Similar to some biotrophs, such as *Claviceps purpurea* (Tudzynski and Scheffer, 2004), Vv can be cultivated in axenic cultures, greatly facilitating scientific research.

The pedicel connects a rice spikelet and the main stem of a panicle. Histological examinations of diseased panicles have indicated that Vv hyphae cannot extend into pedicel and stem tissues,

and no anatomical changes in the pedicel can be detected (Li *et al.*, 2013; Tang *et al.*, 2013). It is indicated that flower infection is not caused by Vv moving through vascular tissues. It has been reported that Vv also attacks rice coleoptiles intercellularly at the early germination stage and rice roots at the seedling stage, leading to asymptomatic colonization (Ikegami, 1962a,b, 1963a; Schroud and TeBeest, 2005; TeBeest, 2010). In addition, the presence of Vv in different tissues of rice plants can be detected after coleoptile and root infections (Ikegami, 1963b; TeBeest, 2010). However, the direct relation between coleoptile or root infection and the formation of false smut balls in the panicle has not been clarified. Overall, these findings indicate that Vv-induced disease is not systemic, although the pathogen can infect some other tender tissues, including rice coleoptiles (Ikegami, 1962a,b, 1963b), roots of germinated seedlings (Schroud and TeBeest, 2005) and young *Arabidopsis* leaves in occasional cases (Fan *et al.*, 2014), in addition to flower filaments (Hu *et al.*, 2013; Tang *et al.*, 2013).

GENOMICS AND GENETICS

Vv can grow in axenic cultures, although mycelial growth is very slow in solid medium, e.g. the colony diameter is approximately 20 mm after 7 days of culture in potato sucrose agar (PSA) medium (Fu *et al.*, 2013). Carbon sources, such as sucrose, glucose and starch, favour the mycelial growth of Vv, whereas ammonium sulfate, ammonium nitrate and ammonium chloride are the preferential nitrogen sources (Fu *et al.*, 2013; Wang and Chen, 2013; Zhang *et al.*, 2014). Vv conidiospores are mononuclear, whereas somatic hyphae are multinuclear (Fu *et al.*, 2012). It is still controversial whether Vv is homothallic or heterothallic (Fu *et al.*, 2014; Yu *et al.*, 2015). Owing to its ability for axenic growth, considerable advances in Vv genomics and genetics have been achieved.

Vv genome

The availability of the Vv genome can largely facilitate the investigation of this pathogen. Wang *et al.* (2013) constructed a deep-coverage Bacterial Artificial Chromosome (BAC) library of the strain UV-8b, providing general genome information for Vv. In more detail, Zhang *et al.* (2014) sequenced the same strain, UV-8b, using the Roche 454 sequencing platform, and obtained a 39.4-Mb draft genome sequence, which encodes 8426 predicted proteins. Intriguingly, Vv shares the closest evolutionary relationship with the entomopathogenic *Metarhizium* spp., indicating potential host jumping across plant and animal kingdoms (Kepler *et al.*, 2012; Zhang *et al.*, 2014). Analysis of the Vv genome sequence shows that it has relatively fewer gene inventories for nutrient uptake, polysaccharide degradation and secondary metabolism, but is enriched with genes for secreted proteins and secondary metabolism, which may be associated with its biotrophic lifestyle and flower-specific infection (Zhang *et al.*, 2014).

In addition, the Vv genome encodes 628 potentially secreted proteins, including 193 putative effectors; however, their roles in pathogen–host interactions are largely unknown (Zhang *et al.*, 2014).

Mycotoxins may be important during pathogenesis. Based on the sequenced genome, it is predicted that non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) gene clusters may be responsible for the biosynthesis of the two Vv mycotoxins, ustiloxin and ustilaginoidin, respectively (Fu *et al.*, 2015a; Koiso *et al.*, 1994; Lu *et al.*, 2015; Meng *et al.*, 2015; Nakamura *et al.*, 1994; Shan *et al.*, 2012, 2013, 2014; Zhang *et al.*, 2014). By contrast, Tsukui *et al.* (2015) suggested that ustiloxins from Vv are ribosomally synthesized, according to homology analysis with the biosynthetic gene cluster for ustiloxin B from *Aspergillus flavus*. However, experimental evidence is needed to confirm gene clusters for ustiloxin production in Vv. A recent report has indicated that the mycotoxins generated in the rice false smut balls on infected rice panicles could be different from those in the cultured fungal mass, and some novel ustilaginoidins have been identified only from the fungus on solid rice medium (Lu *et al.*, 2015). In addition, five main ustilaginoidins show differential distribution patterns together with the development of the false smut ball (Meng *et al.*, 2015). These observations raise the question of whether ustiloxins and/or ustilaginoidins play a role in epiphytic growth and pathogenicity.

Genetic diversity

Recent reports applying genomic and transcriptomic data have revealed single nucleotide polymorphism (SNP) and simple sequence repeat (SSR) markers for the identification of Vv genetic diversity (Jia *et al.*, 2015; Sun *et al.*, 2013; Yu *et al.*, 2014). Earlier studies using PCR-based approaches, such as rDNA-ITS variability (Wang *et al.*, 2009), amplified fragment length polymorphism (AFLP) (Zhou *et al.*, 2008) and random amplification of polymorphic DNA (RAPD) (Li *et al.*, 2012), have identified very limited genetic diversity of Vv. However, three SNP-rich genomic regions have been identified by comparative genomics (Sun *et al.*, 2013). Based on the analysis of the three SNP-rich genomic regions, significant genetic diversifications were detected among populations from five major rice production areas in China, and isolates from the same area showed considerable DNA composition stability (Sun *et al.*, 2013), which is consistent with the conjecture that Vv may not be an air-borne, but a water- and/or soil-borne pathogen. Consistent with this speculation, Wang *et al.* (2014) reported that geography is more important than rice cultivar in constructing the genetic diversity of Vv. Moreover, there is no significant association between rice cultivars and Vv diversity (Pan *et al.*, 2006). Nevertheless, different spikelets in a rice panicle can be infected by different Vv isolates, and one spikelet can be infected by one or multiple Vv isolates (Yu *et al.*, 2013), although it is unclear

whether the pathogenicity of genetically different isolates is different. Interestingly, genetic divergence is generally higher in isolates from inland areas than from coastal areas (Wang *et al.*, 2012). Genetic variation in north-east China is relatively low, which may be a result of less active sexual reproduction (Sun *et al.*, 2013).

Genetic transformation

Several procedures for the generation of GFP-tagged transgenic Vv strains have been reported (Ashizawa *et al.*, 2012; Tanaka *et al.*, 2011; Yu M. *et al.*, 2015; Zhang *et al.*, 2006). Principles should be applied for the examination of the Vv transformants obtained: they must be able to infect rice flowers and cause the featured disease symptoms, i.e. false smut balls. By electroporation of intact Vv conidial cells with the linearized transformation vector pCB1004eGFP, Tanaka *et al.* (2011) successfully obtained enhanced GFP (eGFP)-expressing transformants. Ashizawa *et al.* (2012) also developed a GFP-labelled Vv strain by polyethylene-glycol-mediated protoplast transformation. Using *Agrobacterium tumefaciens*-mediated transformation (ATMT), Zhang *et al.* (2006) successfully engineered a GFP-labelled Vv strain. The hygromycin resistance gene was used as a selection marker, because 150 µg/mL hygromycin B can completely inhibit Vv growth. Yu M. *et al.* (2015) also constructed a GFP-tagged Vv strain that largely facilitated the investigation of the Vv infection process (Fan *et al.*, 2014; Yu M. *et al.*, 2015). With an optimized ATMT procedure in Vv, Yu M. *et al.* (2015) generated a T-DNA insertion library of 5600 hygromycin-resistant Vv transformants, and identified 37 pathogenicity-impaired mutants. Among the 37 mutants, B20, which is disrupted in the *UvSUN2* gene, showed deficiency in virulence, stress response, conidial morphology and hyphal growth (Yu M. *et al.*, 2015).

MOLECULAR Vv–RICE INTERACTIONS

Vv biotrophically infects rice flowers and forms false smut balls, which are the only symptoms visible to the naked eye. The size of a false smut ball is usually several times larger than that of a mature rice seed, indicating that many nutrients are needed for the formation of false smut balls. Intriguingly, Vv also attacks barley flowers, but does not form a ball-like colony (Hu *et al.*, 2013). Therefore, the formation of false smut balls must require specific interactions between Vv and rice. Two aspects are discussed here: host responses to Vv infection and Vv virulence factors.

Host responses to Vv infection

Transcriptomic analyses have identified a number of rice genes modulated by Vv infection. In the compatible interaction between the rice cultivar 93-11 and the Vv isolate *Guangxi-2*, rice genes involved in phosphorylation, programmed cell death and cell wall biogenesis are differentially expressed (Chao *et al.*, 2014). WRKY-

and Myb-regulated genes are overexpressed, whereas abscisic acid (ABA)- and Ca²⁺-regulated genes are repressed (Chao *et al.*, 2014). Moreover, several genes specific to pollen development are down-regulated in Vv-infected rice spikelets, but do not respond to stresses, such as heat, cold, salt, drought, submergence, *Magnaporthe oryzae* or *Xanthomonas oryzae* pv. *oryzae* infection (Chao *et al.*, 2014). These observations suggest that pollen development genes play a role in unique Vv–rice interactions. Comparative transcriptomic analysis of resistant 'IR28' and susceptible 'HXZ' rice cultivars in response to Vv infection revealed that many genes involved in secondary metabolites and hormone metabolism, and genes encoding flavin-containing monooxygenases and peroxidases, are differentially regulated in resistant and susceptible cultivars (Yang *et al.*, 2014). Furthermore, Han *et al.* (2015) suggested that the resistant cultivar 'IR28' has the ability to suppress the expression of pathogenicity genes in Vv, and 'IR28' genes involved in the production of pathogenesis-related proteins and diterpene phytoalexins may contribute to rice false smut resistance.

Because there are only certain percentages of inoculated spikelets that are infected by Vv, responses in non-infected spikelets may be different from those in infected ones. Thus, host responses detected from samples collected after artificial inoculation could be a systemic consequence if non-infected spikelets outnumber infected ones. By sampling the spikelets that are obviously Vv infected, a transcriptomic analysis revealed that a number of grain-filling-related genes are highly activated, whereas, in non-infected spikelets, these genes are only activated after fertilization (Fan *et al.*, 2015). Moreover, preliminary transcriptional data showed over nine-fold up-regulation of the *Absciscic acid-insensitive 3 (ABI3)* gene (LOC_Os01g68370) in Vv-infected spikelets (Fan *et al.*, 2015). *ABI3* plays important roles in ABA signalling and in the regulation of seed-specific development, and ectopic expression of *ABI3* can induce the expression of a large number of seed-specific genes (Suzuki *et al.*, 2003). Thus, this gene may be involved in the activation of the grain-filling network during the Vv–rice interaction. We speculate that Vv may hijack the host nutrient reservoir by mimicking fertilization and activating the grain-filling network, extracting nutrients towards colonization sites for growth and for the formation of false smut balls. Consistent with this hypothesis, a recent study has shown that mild Vv infection (one to two false smut balls per diseased panicle) can enhance rice yield attributes, such as the filled grain number and grain weight (Dhua *et al.*, 2015). Similar results were presented in the data provided by Nessa *et al.* (2015). Dhua *et al.* (2015) also observed that small false smut balls (average ball weight of less than 40 mg) tended to be produced in tolerant rice cultivars. It will be interesting to address the underlying mechanism in the future. As a stamen filament-infecting fungus, Vv is challenged by the risk that the rice filament is short lived. Our recent work

Fig. 3 A schematic diagram showing how *Villosiclava virens* (Vv) converts a rice spikelet to a false smut ball. Vv spores landing on the top of the second leaf (l) can enter the developing panicle (p) along with rainwater (①). Subsequently, Vv spores are attached to the developing spikelet (sp) and germinate to produce hyphae (②). The hyphae extend into the inner space of the spikelet via the gap between the palea (pa) and lemma (le) (③). Primary infection sites are found in the stamen filaments (sf) (④), where the hyphae extend intercellularly in both directions (⑤). Hyphae can extend into anthers (an) and reach the rachilla (ra), and intertwine with the ovary (o) and other floral organs. Vv hyphae acquire abundant nutrients to form false smut balls (fsb), hypothetically through hijacking of the rice nutrient reservoir (⑥). This step (arrow with dotted line) is of particular interest and requires further exploration. Red curved lines represent Vv hyphae. Red arrowheads point to the primary infection sites at the stamen filaments.

shows that flower opening and related genes are suppressed in Vv-infected spikelets (Fan *et al.*, 2015), indicating that Vv may have the ability to maintain colonization sites by inhibiting rice from flowering.

Vv virulence factors

Effectors are essential virulence factors employed by pathogens for the colonization of hosts. Combining genomic and transcriptomic data, a number of genes with potential roles in Vv pathogenicity and the pathogen–host interaction have been predicted. These genes are highly enriched in the transcriptome during the early infection of rice (Zhang Y. *et al.*, 2014). The Vv genome encodes at least 193 putative effectors, 18 of which have been identified to suppress plant hypersensitive responses (Zhang Y. *et al.*, 2014). As a biotroph, Vv may need to express some of these effectors to subvert rice innate immunity for successful infection. RNA silencing is a major defence mechanism against pathogens in plants (Ding, 2010; Vance and Vaucheret, 2001), whereas pathogens can secrete effectors to suppress the RNA silencing system of plants, such as PSR1 and PSR2 from *Phytophthora sojae* (Qiao *et al.*, 2013). Effectors with similar functions may also exist in Vv. For instance, *Nicotiana benthamiana* transient expression screening reveals that Uv_5823 has an effect in the suppression of gene silencing (Fang, 2014). A T-DNA insertion library of Vv has been established and dozens of pathogenicity-deficient mutants have been identified (Yu M. *et al.*, 2015). The characterization of the

responsible genes of these mutants will add important information to the molecular mechanism of Vv pathogenicity.

CONCLUSIONS AND OUTLOOK

In the past few years, we have witnessed dramatic progress towards the understanding of the rice false smut pathogen. Cytological and molecular approaches have revealed the process of how Vv converts a rice spikelet into a false smut ball (Fig. 3). At the rice booting stage, Vv spores first need to enter the leaf sheath, and land on the developing spikelet (Fig. 3, ① ②). Spores germinate on the outer surface of the spikelet and the elongated hyphae extend into the inner space of the rice spikelet through the gap between the lemma and the palea (Fig. 3, ③) (Ashizawa *et al.*, 2012). The hyphae specifically infect stamen filaments, inhibit rice flowering and intercept seed formation (Fig. 3, ④ ⑤) (Fan *et al.*, 2015; Tang *et al.*, 2013); meanwhile, Vv infection induces the expression of rice grain-filling genes which may be pivotal for the development of the false smut ball (Fig. 3, ⑥) (Fan *et al.*, 2015). These findings lead to three important considerations for future research on false smut resistance and Vv pathogenesis: (i) the water-proof degree of the rice sheath at the booting stage; (ii) the aperture size between the lemma and palea of the rice spikelet; and (iii) the ability of Vv to acquire sufficient nutrients for false smut ball formation. In addition, current knowledge indicates the blastogenesis and epiphytic characteristics of Vv under wet conditions, which may play a role in epidemic occurrence of false smut disease when the rice booting stage coincides with rainy days

(Fan *et al.*, 2014). The available genomic information and genetic engineering tools provide an important basis to unravel the mysteries of Vv life.

Despite the above progress, some important questions need to be addressed to better understand the interactions between Vv and rice. How does Vv turn the host floral organ into a nutrient supplier to form false smut balls? Are the floral organs, such as the filament and ovary, essential for Vv infection? Are there any links between specific effectors and mycotoxins with Vv epiphytic growth or flower colonization? Are there any resistance genes in rice that can mount an immune response against Vv? The identification of pathogen factors that activate rice grain-filling genes and the application of floral mutants and resistant germplasm should be valuable in addressing some of these questions. In addition, mycoviruses infecting Vv are widespread (Jiang *et al.*, 2014, 2015; Zhang *et al.*, 2013a,b; Zhang T. *et al.*, 2014; Zhong *et al.*, 2014a,b,c; Zhu *et al.*, 2015). Whether mycoviruses affect the virulence of Vv is also an interesting question. Overall, Vv–rice interplay is an interesting specific pathosystem and any progress obtained should enrich our knowledge of host–pathogen interactions and biotrophic fungi.

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