Infection of *Ustilaginoidea virens* intercepts rice seed formation but activates grain-filling-related genes

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**Abstract** Rice false smut has become an increasingly serious disease in rice (*Oryza sativa* L.) production worldwide. The typical feature of this disease is that the fungal pathogen *Ustilaginoidea virens* (*Uv*) specifically infects rice flower and forms false smut ball, the ustiloxin-containing ball-like fungal colony, of which the size is usually several times larger than that of a mature rice seed. However, the underlying mechanisms of *Uv*-rice interaction are poorly understood. Here, we applied time-course microscopic and transcriptional approaches to investigate rice responses to *Uv* infection. The results demonstrated that the flower-opening process and expression of associated transcription factors, including ARF6 and ARF8, were inhibited in *Uv*-infected spikelets. The ovaries in infected spikelets were interrupted in fertilization and thus were unable to set seeds. However, a number of grain-filling-related genes, including seed storage protein genes, starch anabolism genes and endosperm-specific transcription factors (*RISBZ1* and *RPBF*), were highly transcribed as if the ovaries were fertilized. In addition, critical defense-related genes like *NPR1* and *PR1* were downregulated by *Uv* infection. Our data imply that *Uv* may hijack host nutrient reservoir by activation of the grain-filling network because of growth and formation of false smut balls.

**Keywords:** Flower-infecting; grain filling; microscopy; rice false smut; RNA-Seq

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**INTRODUCTION**

Rice false smut is a destructive grain disease in rice production throughout the world. The typical symptom is the formation of a false smut ball that is attributable to the growth of a white fungal mass in a spikelet, protruding out from the gap between the palea and the lemma, and eventually forming a ball-like colony, which produces numerous yellow or greenish-black chlamydospores and sometimes is covered by sclerotia (*Guo et al. 2012*). False smut not only reduces grain yield but also affects grain quality. Moreover, the pathogen produces ustiloxins with antimitotic activities poisonous to both human and animals (*Koiso et al. 1994; Nakamura et al. 1994*). In recent years, the disease has been reported with increasing frequency in rice-producing areas worldwide (*Rush et al. 2000; Atia 2004; Tsuda et al. 2006; Brooks et al. 2009; Ladhalakshmi et al. 2012*). The increasing disease incidence is experimentally attributed to heavy application of chemical fertilizers and widespread planting of high-yielding hybrid rice, and the epidemics of the disease are associated with rainy weather at rice booting stage (*Wang et al. 2004; Fan et al. 2014*).

The causative pathogen is an ascomycete fungus, which possesses an anamorphic state named *Ustilaginoidea virens* (Cooke) Tak. (*Uv*) and a teleomorphic state called *Villosiclava virens* (*Tanaka et al. 2008*). Chlamydospores and sclerotia of *Uv* are considered as the primary sources of infection, and both can produce conidia as direct inoculum to attack rice spikelets at late booting stage (*Fan et al. 2010; Guo et al. 2012*). It has been reported that *Uv* infects rice roots at the seedling stage (*Ikegami 1962; Schraud and TeBeest 2005*), and coleoptiles at the germination stage (*Zheng et al. 2009*). Most recently, we have found that under wet conditions *Uv* could epiphytically colonize leaf surfaces of paddy field weeds and abiotic surfaces such as Parafilm and cellophane (*Fan et al. 2014*). This finding implies that during the season when developing rice spikelets are not available, *Uv* may undergo an epiphytic stage producing a large number of conidia, which at rice booting stage could enter the rice sheath with rainwater and infect the developing spikelets (*Fan et al. 2014*).

A general infection process of *Uv* has been reported that *Uv* conidia land and germinate on the outer surface of rice spikelets, and the hyphae extend into the inner space of spikelets through the small gap between the lemma and the...
palea and invade/cover the floral organs (Ashizawa et al. 2012). Further observations reveal that the primary infection sites of Uv hyphae are the upper parts of the three stamen filaments between the ovary and the lodicules, and that the hyphae intercellularly extend along the filament base without killing the host cells. The ovary cannot be infected, and the hyphae fail to extend to the pedicels and stems of the panicles (Hu et al. 2013b; Tang et al. 2013). Transcriptome analysis of rice spikelets infected with Uv at asymptomatic stages reveals that the smut pathogen largely modulates genes involved in defense responses and gene regulation, and that a number of genes are uniquely responsive to Uv infection, such as genes specifically expressing during pollen development (Chao et al. 2014).

Interestingly, Uv hyphae not only infect flower organs of rice but also those of barley; however, no ball-like colonies could be formed in the latter (Hu et al. 2013a). Thus, the formation of false smut ball must be a result of the specific interaction between Uv and rice flower. As false smut ball is the only visible symptom of the smut disease, deciphering the Uv-rice interaction leading to smut ball formation is of particular importance to fully understand and effectively control the rice interaction. The ovary cannot be infected, and the hyphae extend intercellularly along the floral organs (Ashizawa et al. 2012).

RESULTS

Observation of Uv infection process in rice spikelets

According to field observations and previous reports on the infection process of Uv, no clear symptoms could be seen on Uv-infected rice plants until the macroscopic fungal mass emerges at approximately 15 d post-inoculation (dpi), and not all the spikelets from a panicle could be infected by Uv (Ashizawa et al. 2012; Tang et al. 2013). In addition, the appearing time of false smut ball is variable among different Uv isolates on different rice accessions. To determine the accurate time of sampling spikelets that are discernable to be infected by Uv for transcriptional analysis, we repeated examination of the infection process of Uv towards the formation of mature false smut balls in our experimental conditions.

Developing panicles of a susceptible rice accession (i.e. Pujilang 6) were inoculated with the pathogen isolate Uv-10 at rice booting stage. Ustilaginoidea virens hyphae were detected on the outer surface of spikelets at 1 dpi (Figure 1A). Hyphae extended rapidly and formed membrane-like mycelia around the trichomes and at the gap between the palea and lemma at 2–9 dpi (Figure 1B–D), and were detected on the surface of the inner organs such as anthers, filaments, and lodicules at 9 dpi (Figure 1E). After its colonization on the floral organs, Uv started massive growth inside spikelets, and macroscopic white mycelia covered all floral organs at 17 dpi (Figure 1G, H). A few infected spikelets (<10%) had damaged anthers and floral organs including anthers were alive in most (>90%) infected spikelets at 17 dpi. Spikelets with damaged organs were excluded in subsequent gene expression studies. Fungal mass continued to expand and protruded out from the gap between the palea and lemma at approximately 20 dpi (Figure 1I). The Uv-infected rice flowers never opened and the ovaries remained small (Figure 1H, I). These observations suggest that the pollination and fertilization of flowers are interrupted by Uv infection and the infected spikelets can be easily discernable at 17 dpi. Eventually, false smut balls covered with yellow and greenish-black chlamydospores emerged at approximately 23 and 30 dpi, respectively (Figure 1J, K).

As demonstrated above, the infection process of Uv was very long and included at least two quite different phases. Phase 1: growth on the outer surface of rice spikelets; this phase could last for more than 1 week (under our experimental conditions). Phase 2: Uv hyphae extended into the inner space of spikelets and infected inner floral organs; this phase directly determined the formation of false smut balls. In this study, false smut balls at very young age were found at approximately 17 dpi. Thus, this time point was suitable for investigating gene expression profiling related to smut ball formation.

Expression stability analysis of rice housekeeping genes during Uv-rice interaction

Normalization with a transcriptionally stable reference gene is important for accurate evaluation of gene expression. Housekeeping genes such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Actin, and Ubiquitin (Ubi) are commonly used as reference genes. However, these genes are not always suitable for transcriptional analysis, because their expression levels could vary under different conditions and in different tissues (Lee et al. 2002; Czechowski et al. 2005). The transcription stability of a set of housekeeping genes in rice have been assessed in different tissues/organ systems under various developmental stages and stresses (Jain et al. 2006; Li et al. 2010), but not in samples related to Uv-rice interaction.

To identify a gene whose expression can be mostly stable and thus suitable as an internal reference in our transcriptional analysis, we used geNorm version 3.5 to analyze the expression of six frequently used rice reference genes in eight spikelet samples before and after Uv infection (see Materials and Methods). As depicted by geNorm, the lowest M value represents the most stable in transcription. When considering all eight samples (Uv-infected and uninfected samples at four developmental stages of rice spikelets) together, OsGAPDH and OsUbc were the most stable with the lowest M values, while OsTubβ1 was the least stable in expression (Figure 2A). When only four uninfected samples were included for analysis, OsGAPDH and OsUbc were the most stable in transcription. When four Uv-infected samples were taken into account, OsUbi and OsGAPDH displayed the most stable expression (Figure 2B, C). Altogether, among the six tested reference genes, OsGAPDH showed the most stable expression in different developmental stages of rice spikelets before and after Uv infection. Therefore, OsGAPDH was selected as the reference gene for evaluating relative expression of rice genes involved in Uv-rice interaction in subsequent experiments.

Suppression of putative flower-opening genes in rice spikelets upon Uv infection

As demonstrated in Figure 1H, I, Uv-infected rice flowers never opened. We speculated that expression of genes related to
flower opening may be repressed. It has been reported that Arabidopsis auxin response factor (ARF)6, ARF8, and MYB21 play important roles in flower development and opening (Nagpal et al. 2005; Mandaokar et al. 2006). Rice genes Os02t0164900-01, Os06t0677800-01, and Os11t0684000-01 were highly homologous to AtARF6, AtARF8, and AtMYB21, respectively, with identities ranging 58%–74% (Figure S1).

Temporal expression profiles of the three rice homologs were examined across different stages of spikelet development before and after Uv infection. In mock-inoculated samples, transcripts of OsARF6 (Os02t0164900-01) accumulated along with spikelet development before pollination (i.e. 1–10 dpi), and the accumulation decreased at 17 dpi (4–6 d after pollination). A similar expression pattern was observed for OsARF8 (Os06t0677800-01) and OsMYB21 (Os11t0684000-01) (Figure 3). Upon Uv infection, the transcriptional levels of OsARF6 and OsARF8 were suppressed approximately 2-fold at 10 and 17 dpi compared with the mock-inoculated controls at corresponding stages, although upregulation was detected at 1 dpi (Figure 3A, B). OsMYB21 was repressed 2.3-fold at 17 dpi, though no obvious difference was detected at 10 dpi (Figure 3C).

Failure of ovary fertilization in rice spikelets infected with Uv Interruption of rice flower opening could lead to non-pollination and infertility of ovaries. As expected, the ovaries inside the false smut balls remained small and could not be stained by KI-I2 (Figures 1I, 4A), indicating no endosperm

Figure 1. Infection process of Ustilaginoidea virens (Uv) in rice spikelets
(A, B) Uv-10 isolate was inoculated into sheaths of Pujiang 6 at late booting stage. Environmental scanning electron microscope (ESEM) and digital camera were used to monitor the entire infection process of the pathogen. Ustilaginoidea virens hyphae were evident on the outer surface of spikelets at 1 d post-inoculation (dpi) (A) and formed membrane-like mycelia at 2 dpi (B). (C, D) Mycelia were observed around the gap between the palea and lemma at 9 dpi. (E, F) At 9 dpi, the pathogen extended to the floral organs inside spikelets, such as filaments (fi), anthers (an), and lodicules (lo). (G, H) The pathogen continued to grow and covered most of the floral organs with macroscopic white mycelia at 17 dpi. (I) At approximately 20 dpi, the fungal mass protruded out from the gap between palea and lemma. (J, K) Yellow and greenish-black false smut balls were found at approximately 23 dpi (J) and 30 dpi (K), respectively. Arrows and arrowheads indicate Uv mycelia and rice smut balls formed by the fungus, respectively. Stars represent the greenish ovaries in Uv-infected spikelets. Scale bars = 100 μm (A, F), 200 μm (B, D, E), or 500 μm (C, G).

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development and starch accumulation. By contrast, ovaries from the uninfected spikelets expanded and were stained black-blue by KI-I2, indicating successful pollination and endosperm development (Figure 4A).

To determine whether seed storage proteins (SSPs) accumulated in ovaries from Uv-infected rice spikelets, immunoblot analysis was carried out using two antibodies against glutelin type-A2 (Os10t0400200-01) and 11-S plant SSP (Os03t0336100-01), respectively. As displayed in Figure 4B, no obvious accumulation of glutelin and 11-S seed protein were detected in Uv-infected rice spikelets at 17 dpi and in false smut balls at 23 dpi. Together with the findings of KI-I2 experiment, our data indicate that ovaries in Uv-infected spikelets are unfertilized and in turn do not accumulate grain-filling substances such as starch and SSPs.

Activation of grain-filling-related genes in rice spikelets infected with Uv

Many pale-looking infected spikelets were found at approximately 17 dpi, which is attributed to the white fungal mass inside spikelets, and were distinguishable from non-infected green spikelets (Figure 1H). To investigate the influence of Uv infection on the transcription profile of rice spikelets, we performed RNA-Seq analysis on pale-looking infected spikelets at 17 dpi, with spikelets from comparable positions of mock-inoculated panicles as controls. Illumina RNA-Seq generated 26 and 28 million clean reads from mock-inoculated and Uv-infected spikelets, respectively. Differentially expressed genes (DEGs) were identified using the reads per kilobase transcriptome per million mapped reads (RPKM) data under the criteria of false discovery rate (FDR) of 0.001 or less and absolute log2-fold change of 1 or more. As a result, 4,109 (12.3%) out of 33,417 detected rice genes were differentially expressed upon Uv infection, of which 1,590 genes were upregulated and 2,519 were downregulated (Table S1).

Surprisingly, a large number of grain-filling-related genes were coordinately upregulated in Uv-infected spikelets, compared with mock-inoculated controls. For instance, a set of genes (32) encoding rice SSPs, such as prolamin, glutelin, and globulin, were greatly induced upon Uv infection (Table 1). Critical starch metabolism genes, such as those encoding large and small subunits of ADP-glucose pyrophosphorylase (AGPase), starch synthase, and branching enzyme, also accumulated more transcripts in Uv-infected spikelets than in the controls. In addition, two transcription factors, OsRISBZ1 and OsRPBF which have been demonstrated to regulate starch and storage protein synthesis in rice seed (Onodera et al. 2001; Yamamoto et al. 2006; Kawakatsu et al. 2009), displayed elevated transcriptional levels in spikelets infected with Uv (Table 1). Expression data in the database of RicePLEX revealed that the above grain-filling-related genes were specifically or inductively expressed in rice maturing seeds but showed only detectable or much lower levels in other organs and in spikelets before pollination (Figure S2).

Using quantitative real-time polymerase chain reaction (qPCR), we validated the expression patterns of four SSPs, five starch metabolism genes, and two transcription factors (OsRISBZ1 and OsRPBF) in the mock-inoculated and Uv-infected spikelets at 17 dpi. In addition, their expression in the ovaries excised from the infected spikelets was also examined. As shown in Figure 4C, these genes were expressed at comparable or higher levels in Uv-infected (non-pollinated) spikelets than in the mock-inoculated controls (4–6 d after pollination), except for OsSSI and OsRPBF. On the contrary,
much lower expression levels were detected in the ovaries excised from infected spikelets; the amount of reduction varied 446–65,536-fold for the SSPs, 2,6–82-fold for the starch metabolism genes, and 28–111-fold for the two transcription factors. Both RNA-Seq and qPCR data demonstrated that grain-filling-related genes were transcriptionally activated in infected non-pollinated spikelets at 17 dpi. Furthermore, time-course transcriptional analysis confirmed that three selected SSPs, including OsGlutln1 (Os02t0248800-01), OsGlutln2 (Os02t0453600-01), and OsProIm1 (Os06t0507100-01) displayed high expression levels in both the mock-inoculated and Uv-infected spikelets at 17 dpi, with more transcripts in the latter (Figure 4D). By contrast, their transcripts failed to be detected at 1 and 10 dpi (data not shown) and were barely detected at 5 dpi (Figure 4D). Additionally, OsGlutln1, OsGlutln2, and OsProIm1 were not expressed in the ovaries excised from the infected spikelets at 17 dpi (data not shown). These data imply that Uv infection may mimic pollination and fertilization to trigger expression of grain-filling genes in unfertilized rice spikelets, and this activation does not occur in the ovaries.

**Expression changes of rice defense/stress-related genes in response to Uv infection**

Many defense-related genes have been reported to be critical in plant innate immunity. RNA-Seq data demonstrated that a set of defense genes were coordinately downregulated in rice spikelets upon Uv infection (Table 2). Two homologs of the NPR1 gene, which is a key regulator of salicylic acid-mediated disease resistance in Arabidopsis (Cao et al. 1997), displayed a 2–4-fold reduction in Uv-infected rice spikelets compared with those in the mock-inoculated controls (Table 2). Five PR1 homologs were differentially expressed, three of which were remarkably downregulated (Table 2). The expression of three CNGC homologs involved in the plant pathogen response to mock inoculation (Os-CK) and Uv-infected (Os-Inf) rice spikelets at 1, 5, 10, and 17 d post-inoculation (dpi). The rice GAPDH was used as a reference gene. The C_2 value in mock-inoculated spikelet sample at 1 dpi was set as a calibrator for each gene. Student’s t-test was performed to determine the significance of difference between Os-CK and Os-Inf at each time point (*P < 0.05, **P < 0.01). Data were means ± SD of three biological replicates. Similar results were obtained in at least two individual experiments.
Figure 4. Examination of ovary development and expression patterns of grain-filling-related genes in rice spikelets upon *Ustilaginoidea virens* (*Uv*) infection (A) Ki-I2 staining of ovaries from mock-inoculated and *Uv*-infected rice spikelets at 17 d post-inoculation (dpi). (B) Western blot analysis of seed storage protein accumulation in *Uv*-infected rice, using antibodies against GluA-2 and 11-S seed protein. Lane 1, *Uv*-infected rice spikelets at 17 dpi; lane 2, rice spikelets with false smut balls at 23 dpi; lane 3, mature rice seeds as positive control. (C) Expression changes of *OsGlut1n3* (*Os02t0249000-01), *OsGlut1n4* (*Os10t0400200-01), *OsProm1n2* (*Os05t0332000-01), *OsSSIIa* (*Os06t0160700-01), *OsSSIIa* (*Os08t0191433-00), *OsAGPL2* (*Os10t0633100-01), *OsAGPS2b* (*Os08t0345800-01), *OsRISBZ1* (*Os07t0182000-01), and *OsRPBF* (*Os02t0252400-01) in mock-inoculated and *Uv*-infected spikelets at 17 dpi. The Ct value in the ovary sample excised from *Uv*-infected spikelets at 17 dpi was set as a calibrator for each gene. (D) Time-course expression profiles of *OsGlut1n1* (*Os02t0248800-01), *OsGlut1n2* (*Os02t0453600-01), and *OsProm1n1* (*Os07t0206400-01) across different stages of *Uv* infection. Note that no transcripts were detected in the control and infected samples at 1 and 10 dpi, and in the ovaries excised from *Uv*-infected spikelets at 17 dpi. Thus, the Ct value in mock-inoculated spikelet sample at 5 dpi was set as a calibrator for each gene. For quantitative real-time polymerase chain reaction experiments in (C) and (D), the rice GAPDH was used as a reference gene. Data were means ± SD of three biological replicates. Student’s t-test was performed to determine the significance of difference between mock-inoculated and *Uv*-infected rice spikelets at 17 dpi (*P < 0.05, **P < 0.01). Similar results were obtained in at least two individual experiments.
were examined in rice spikelets across different developmental stages before and after Uv infection. Time-course transcriptional analysis showed that OsPR1#051, OsPR1#121, and OsPR1#012 accumulated transcripts in mock-inoculated samples along with spikelet development before pollination (i.e. at 1, 5, and 10 dpi) and peaked at 10 dpi, then the accumulation began to decline after pollination (i.e., at 17 dpi), indicating their flower-preferential expression patterns (Figure 5A–C).

### Table 1. Expression changes of rice grain-filling-related genes in response to Ustilaginoidea virens (Uv) infection

| Gene IDa | Gene description                                      | Log$_2$-fold change (Os-Inf/Os-CK) | FDRc |
|----------|--------------------------------------------------------|-----------------------------------|------|
| **Seed storage protein** |                                                        |                                   |      |
| Os02t0248800-01 | Similar to glutelin type-B2 precursor | 2.67                             | 0.00E + 00 |
| Os02t0453600-01 | Similar to glutelin | 2.60                             | 0.00E + 00 |
| Os03t0427300-01 | Glutelin type-A III precursor | 2.54                             | 0.00E + 00 |
| Os02t0249000-01 | Glutelin, seed storage protein | 2.49                             | 0.00E + 00 |
| Os02t0268100-01 | Similar to glutelin (fragment) | 2.17                             | 0.00E + 00 |
| Os02t0268300-00 | Similar to glutelin (fragment) | 2.11                             | 0.00E + 00 |
| Os01t0400200-01 | Glutelin type II precursor | 2.06                             | 0.00E + 00 |
| Os01t0761800-00 | Similar to glutelin type-A3 | 1.92                             | 2.50E – 01 |
| Os02t0249600-01 | Similar to glutelin | 1.73                             | 8.22E – 65 |
| Os01t0762500-00 | Glutelin subunit mRNA | 1.68                             | 0.00E + 00 |
| Os02t0456100-00 | Similar to glutelin | 1.30                             | 6.39E – 29 |
| Os03t0188500-01 | Glutelin family protein | 1.27                             | 2.51E – 81 |
| Os12t0472500-00 | Glutelin family protein | 1.16                             | 1.71E – 02 |
| Os07t0219300-00 | Prolamin precursor (13 kDa prolamin) | 0.97                             | 5.20E – 02 |
| Os05t0332000-01 | Similar to prolamin precursor | 4.94                             | 1.32E – 38 |
| Os05t0331532-01 | Similar to prolamin | 3.98                             | 0.00E + 00 |
| Os05t0329200-00 | Similar to prolamin | 3.92                             | 2.93E – 04 |
| Os05t0330600-00 | Similar to prolamin | 3.62                             | 0.00E + 00 |
| Os05t0331800-00 | Similar to prolamin | 2.98                             | 2.53E – 16 |
| Os05t0331266-00 | Similar to prolamin | 2.85                             | 0.00E + 00 |
| Os05t0328466-00 | Similar to prolamin | 2.66                             | 2.52E – 02 |
| Os05t0328800-00 | Prolamin 7 | 2.66                             | 1.09E – 03 |
| Os05t0328333-00 | Similar to prolamin | 2.51                             | 2.25E – 09 |
| Os06t0507100-01 | Similar to prolamin | 2.00                             | 2.56E – 43 |
| Os07t0206400-00 | 13 kDa prolamin precursor | 1.84                             | 0.00E + 00 |
| Os07t0220000-00 | Similar to prolamin | 1.76                             | 0.00E + 00 |
| Os07t0219400-01 | Prolamin precursor | 1.74                             | 0.00E + 00 |
| Os07t0206500-00 | 13 kDa prolamin precursor | 1.53                             | 0.00E + 00 |
| Os12t0269200-01 | Similar to prolamin precursor | 1.13                             | 0.00E + 00 |
| Os07t0219300-00 | 11S plant seed storage protein family protein | 1.39                             | 1.21E – 02 |
| Os04t0499000-01 | 26 kDa globulin (alpha-globulin) | 1.14                             | 0.00E + 00 |
| **Starch metabolism** |                                                        |                                   |      |
| Os01t0631000-01 | AGPase large subunit | 1.03                             | 0.00E + 00 |
| Os08t0345800-01 | AGPase small subunit | 2.32                             | 5.55E – 210 |
| Os06t0160700-01 | Starch synthase I | 0.26                             | 4.02E – 02 |
| Os06t0229800-01 | Starch synthase IIa | 1.73                             | 6.39E – 86 |
| Os08t01474c3-00 | Starch synthase IIa | 1.46                             | 8.23E – 173 |
| Os06t0726400-01 | Branching enzyme I | 1.17                             | 3.24E – 75 |
| Os02t0528200-01 | Branching enzyme IIb | 1.10                             | 0.00E + 00 |
| Os08t0526000-00 | Isoamylase | 0.91                             | 2.78E – 163 |
| Os06t0133000-01 | Granule-bound starch synthase I | 1.88                             | 0.00E + 00 |
| **Transcription factor** |                                                        |                                   |      |
| Os07t0182000-01 | bZIP transcription factor | 1.29                             | 6.47E – 60 |
| Os08t0252400-01 | DOF zinc finger transcription factor | 1.29                             | 1.90E – 82 |

Genes selected for quantitative real-time polymerase chain reaction analysis are underlined and presented in Figure 4. aGene accession number for rice gene in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). bThe log$_2$-fold change of gene expression in Uv-infected rice spikelets versus mock-inoculated rice samples from RNA-Seq experiment. cFalse discovery rate (FDR) as calculated according to Benjamini and Yekutieli (2001). dThese starch metabolism genes have been implicated in grain filling during rice endosperm development (Zhou et al. 2013).
Table 2. Expression changes of rice defense/stress-related genes in response to *Ustilaginoidea virens* (*Uv*) infection

| Gene IDa | Homolog name | Gene description | Log2-fold change (Os-Inf/Os-CK)b | FDRc |
|----------|--------------|------------------|-----------------------------------|------|
| Os01t094300-01 | NPR1 | Similar to NPR1 | -1.96 (−0.73 ± 0.11) | 1.04E-79 |
| Os11t041900-00 | NPR1 | Ankyrin domain containing protein | -1.58 | 4.12E-04 |
| Os05t0595000-00 | PR1 | Allergen V5/Tpx-1 related family protein | -7.78 (−5.64 ± 0.38) | 0.00E + 00 |
| Os12t0633400-01 | PR1 | Similar to Pathogenesis-related protein PR-1 precursor | -6.11 (−5.07 ± 0.54) | 1.02E-121 |
| Os31t0382400-01 | PR1 | Similar to Pathogenesis-related protein PRB1-2 precursor | -1.88 (−0.82 ± 0.28) | 5.05E-157 |
| Os10t0191300-01 | PR1 | Similar to PR-1a pathogenesis related protein (Hv-1a) precursor | 1.43 | 4.75E-05 |
| Os01t0382000-01 | PR1 | Similar to Pathogenesis-related protein PRB1-2 precursor | 2.77 (2.45 ± 0.37) | 1.36E-32 |
| Os03t0646300-01 | CNGC | Similar to Cyclic nucleotide-gated channel A (Fragment) | -10.30 | 2.88E-04 |
| Os06t0256600-01 | CNGC | Ankyrin domain containing protein | -1.78 (−1.02 ± 0.10) | 1.96E-71 |
| Os07t088500-01 | CNGC | Disease resistance protein domain containing protein | -1.06 | 7.02E-04 |
| Os02t0265500-01 | COI1 | Similar to Coronatine-insensitive 1 | -2.30 (−2.54 ± 0.14) | 4.55E-08 |
| Os05t0449500-01 | COI1 | Similar to Coronatine-insensitive 1 | -1.48 | 1.08E-79 |
| Os10t0391400-01 | JAZ | Tify domain containing protein | 3.47 (3.95 ± 0.15) | 2.98E-15 |
| Os04t0177300-01 | MIN7 | Similar to H2o27B04.10 protein | -2.52 (−2.08 ± 0.26) | 8.54E-05 |
| Os07t0674750-00 | MIN7 | Conserved hypothetical protein | -2.10 | 3.82E-05 |
| Os04t0626000-01 | RIN4 | Similar to NOI protein | -4.47 (−3.68 ± 0.28) | 1.25E-61 |
| Os06t0661000-00 | RIN4 | Hypothetical conserved gene | -3.66 | 8.67E-14 |
| Os08t0243900-01 | TLP | Similar to Thaumatin-like protein | -1.83 | 1.90E-27 |
| Os05t086000-01 | GH3-5/JAR1 | GH3 auxin-responsive promoter family protein | -1.29 | 1.13E-46 |
| Os05t037400-01 | COI1 | Coronatine-insensitive 1 | 1.48 | 1.62E-28 |
| Os05t091400-01 | JAZ | Tify domain containing protein | 3.47 (3.95 ± 0.15) | 2.98E-15 |
| Os04t0177300-01 | MIN7 | Similar to H2o27B04.10 protein | -2.52 (−2.08 ± 0.26) | 8.54E-05 |
| Os07t0674750-00 | MIN7 | Conserved hypothetical protein | -2.10 | 3.82E-05 |
| Os04t0626000-01 | RIN4 | Similar to NOI protein | -4.47 (−3.68 ± 0.28) | 1.25E-61 |
| Os06t0661000-00 | RIN4 | Hypothetical conserved gene | -3.66 | 8.67E-14 |
| Os03t0156401-00 | Unknown | Hypothetical gene | 1.17 | 1.40E-08 |

*Plant–pathogen interaction*

*Induced systemic resistance*

*Dehydration-responsive-element-binding protein*

Genes selected for quantitative real-time polymerase chain reaction (PCR) analysis are underlined. aGene accession number for rice gene in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/). bThe log2-fold change of gene expression in *Uv*-infected rice spikelets versus mock-inoculated rice samples from RNA-Seq experiment. Data in parentheses represent expression changes (mean ± SD) determined by quantitative real-time PCR. cFalse discovery rate (FDR) as calculated according to Benjamini and Yekutieli (2001).
that the pollination of uninfect ed spikelets occurred at approximately 12 dpi. Upon Uv infection, their transcriptional levels were downregulated at 5, 10, and 17 dpi (Figure 5A–C). Remarkably, the expression of OsPR1#121 and OsPR1#051 were reduced by 33- and 50-fold, respectively, compared with their corresponding controls at 17 dpi (Figure 5A, B). On the contrary, OsPR1#011 displayed upregulation during the spikelet development and further induction upon Uv infection at 10 and 17 dpi (Figure 5F). Distinct expression patterns between OsPR1#011 and the other three PR1s (i.e., OsPR1#051, OsPR1#121, and OsPR1#012) suggest that they play different roles in spikelet development and Uv-rice interaction. The CNGC homolog Os03t0646300-01 showed a similar expression profile with OsPR1#121 (Figure 5D). Compared with mock-inoculated controls, the transcriptional level of the DREB1E homolog Os02t0676800-01 was 47, 27, and 261-fold higher at 1, 5, and 17 dpi, respectively, although approximately 2-fold lower at 10 dpi (Figure 5E).

**DISCUSSION**

Flower is an important sink organ with abundant nutrients allocated to it and can produce many kinds of nutrient-rich secretions, thus serving as an excellent habitat for microorganisms. Meanwhile, risks exist for pathogens specializing in flower infection, due to the ephemeral nature of floral organs. For instance, flower production is highly seasonal in most plant species, and floral organs are prone to abscise upon completion of pollination or under stresses (Sun et al. 2004; Rogers 2006). These challenges require that flower-infecting pathogens should have phenologies tightly coupled with those of their hosts, and have the ability to maintain flowers living long enough until they propagate offspring. Nevertheless, flower-infecting fungi widely exist in nature and cause many economically important plant diseases, such as Fusarium head blight caused by Gibberella zeae in wheat and Ergot disease caused by Claviceps purpurea.
in rye (Ngugi and Scherm 2006). Anatomical, physiological, and/or molecular evidence has been demonstrated for fungi infecting ovaries (Ngugi and Scherm 2004; Tudzynski and Scheffer 2004). As an example, C. purpurea infects rye ovary by first penetrating the cuticle of stigmatic hairs, then growing directly towards the rachilla at the base of the ovary probably mimicking pollen tubes, and tapping the vascular tissue for acquiring nutrients (Tudzynski and Scheffer 2004). Ustilaginoidea virens displays a different organ specificity, that is, infecting stamen filaments exclusively (Tang et al. 2013). In the present work, our data suggest that Uv may colonize rice flowers and hijack the rice nutrient reservoir through modulating host defense responses, flower opening, and grain-filling network.

Successful pathogens should have the abilities to evade or subvert host defense, to colonize host tissues/organs, and to propagate within the host and exit the host eventually. In this work, we found that Uv could suppress expression of rice defense-related genes homologous to NPR1, PR1, CNGC, and AtMIN7 in Arabidopsis (Table 2; Figure 5). The Arabidopsis NPR1 protein is an important regulatory component in plant immunity, controlling the onset of systemic acquired resistance (SAR). Loss-of-function mutation in NPR1 leads to increased susceptibility to pathogens and little expression of PR genes (Cao et al. 1997). Expression of three rice NPR1 homologs has been identified to be induced by the rice bacterial blight pathogen Xanthomonas oryzae pv. oryzae and the blast fungus Magnaporthe grisea, and one (Os01t0194300-01) of the homologs is proved to be the ortholog of Arabidopsis NPR1 (Yuan et al. 2007). PR1 genes are widely used as marker genes for SAR. There are 12 PR1 members in the rice genome, all of which are upregulated in compatible and/or incompatible rice-blast fungus interactions (Mitsuhara et al. 2008). By contrast, the expression of the rice NPR1 ortholog (Os01t0194300-01) and three PR1 genes preferentially expressed in rice flowers (Mitsuhara et al. 2008) were suppressed in Uv-infected spikelets (Table 2; Figure 5), which suggests that the SAR pathway in rice spikelets may be suppressed upon Uv infection.

In plant defense response signaling cascades, 
\[ Ca^{2+} \] elevation in cytosol is a critical early event. Plant CNGCs are channels for 
\[ Ca^{2+} \] conductance across plasma membrane, and thus are important in activating downstream components of defense signaling (Ma and Berkowitz 2011). Mutation in the Arabidopsis CNGC2 leads to failure of hypersensitive response to Pseudomonas syringae DC3000 and impaired cytosolic 
\[ Ca^{2+} \] elevation; meanwhile, PAMP-induced nitric oxide (NO) is also impaired (Clough et al. 2000; Ali et al. 2007; Ma et al. 2009). In this work, we detected that the expression of three CNGC homologs was suppressed in rice infected with Uv, implying that 
\[ Ca^{2+} \] -mediated defense responses may be repressed upon Uv infection. Arabidopsis AtMIN7, an interactor of effector protein HopM1 from P. syringae, plays an important role in cell wall-associated defense. Knock-out of AtMIN7 causes impairment of cell wall-associated defense, characterized by reduction of polarized callose deposition in response to the P. syringae mutant delta CEL, a strain that is defective in suppressing callose deposition (Nomura et al. 2006). Here, we showed that during Uv-rice interaction, two rice homologs of AtMIN7 were downregulated by more than 4-fold (Table 2). In addition, the expression of a gene encoding putative callose synthase 1 catalytic subunit (Os02t0832400-02) was also suppressed (Table S1). Suppression of extracellular cell wall-associated host defense should be an effective strategy for Uv to subvert host surveillance system. This speculation is consistent with a recent report that Uv is an extracellular pathogen whose hyphae extend intercellularly in rice stamen filaments (Tang et al. 2013). Taken together, Uv may suppress multiple targets in rice immunity, presumably through secreted proteins and effectors (Zhang et al. 2014).

Rice stamen filament elongates rapidly during anthesis and senesces shortly after flowering. As a stamen filament-infecting fungus (Hu et al. 2013a; Tang et al. 2013), Uv should have the ability to complete successful colonization before flowering and/or to inhibit flowering. In this work, we found that the floral organs were covered by a mass of Uv mycelia before flowering time and the infected flowers never opened, indicating that Uv may inhibit rice flowering to maintain the colonization sites (Figure 11). Arabidopsis miRNA miR167 and its targets AtARF6 and AtARF8 regulate stamen and gynoecium development in immature flowers. Double-mutant plants of AtARF6 and AtARF8 and overexpressers of miR167a produce non-opening flowers in Arabidopsis (Nagpal et al. 2005; Wu et al. 2006). The Arabidopsis mutant myb21/24 displays defects in floral development and flower opening (Mandaokar et al. 2006). Knocking-down of EOBII, a Petunia × hybrida homolog of MYB21, prevents flowers entering anthesis (Colquhoun et al. 2011). In the present study, both qPCR and RNA-Seq showed that rice OsARF6, OsARF8, and OsMYB21 were suppressed upon Uv infection (Figure 3; Table S1), suggesting that the pathogen may interfere with flower opening via modulating these transcription factors.

The process of rice grain filling involves a pronounced transportation event of nutrients, mainly sugars and amino acids; and starch and SSPs highly accumulate in the developing endosperm. Rice SSPs include glutelins, prolamins, globulins, and albumins. In mature seed, prolamins and glutelins account for 20−30% and 60−80% of total protein, respectively (Zhou et al. 2013). These proteins are specifically accumulated in maturing seed and mostly encoded by multigene families (Nie et al. 2013), which are highly expressed only after fertilization (Figure S2). Promoters of rice SSPs could drive endosperm-specific expression of the β-glucuronidase reporter gene, demonstrating their seed-specific expression (Wu et al. 1998). Several genes involved in starch anabolism have been identified to be crucial for endosperm development and grain filling in rice (Zhou et al. 2013), and these genes also display seed-specific/preferential expression patterns (Figure S2). Two transcription factors, the rice prolamin box binding factor RPBF and the basic leucine zipper factor RISBZ1, have been demonstrated to regulate the expression of rice SSPs and starch metabolism genes. Knocking-down of the two regulators leads to reduced levels of SSPs and starch in rice seed (Onodera et al. 2001; Yamamoto et al. 2006; Kawakatsu et al. 2009). In addition, rice osbZIP58 (osrBZ1) null mutants have decreased levels of total starch and amylase (Wang et al. 2013). A set of rice SSPs, starch synthesis genes, and the two seed-specific transcription factors (i.e., RPBF and RISBZ1) were highly activated in Uv-infected spikelets in which the ovaries were unfertilized (Table 1; Figure 4). It is indicated that Uv infection may activate the grain-filling system in rice spikelets.
Acquiring nutrients from the host is critical for pathogen growth and propagation. *Ustilaginoidea virens* infects rice reproductive tissues and forms a ball-shape colony, of which the size is usually several times larger than mature rice seed. It is reasonable that the smut pathogen needs to acquire large amounts of nutrients from rice. Our data suggest that *Uv* may have the ability to mimic fertilization in rice flowers, so that ample nutrients can be allocated to the colonization sites and hijacked by *Uv* for growth and smut ball formation. It should be noted that SSPs such as glutelin and 11-S seed protein were not detected in the infected spikelets (Figure 4B), although their encoding genes were highly transcribed (Table 1). Whether or where starch and SSPs are ever synthesized in smut balls needs to be further investigated. Also, it would be interesting to identify pathogen components that can upregulate the expression of grain-filling-related genes in rice, and dissect how these genes affect the formation of false smut balls.

**MATERIALS AND METHODS**

**Plant material, pathogen isolate, and artificial inoculation**

Pathogen isolate *Uv*-10 was obtained via amerosporous purification from rice (*Oryza sativa* L) false smut balls in Sichuan Province, China. Discs of *Uv*-10 mycelia growing on potato dextrose agar were inoculated into potato sucrose broth (PSB), and cultured for 7 d at 28 °C and 120 rounds/min. A mixture of conidia and mycelia was either collected for RNA extraction, or blended and adjusted with fresh PSB to an appropriate concentration (i.e. the density of conidia reached $1 \times 10^6$/mL) as the inoculum.

The injection inoculation method was applied as described with minor modifications (Tang et al. 2013). Plants of a susceptible rice cultivar (Pujiang 6) were grown in plastic pots under natural conditions. At a late booting stage (~1 week before heading), inoculum of *Uv*-10 was injected into rice sheaths with a sterile syringe until the inoculum suspension dripped. At least 30 sheaths from six pots were inoculated with the pathogen suspension, and mock inoculation was carried out using PSB on another set of plants. Inoculated plants were kept at 25 °C with 85% relative humidity (RH) for 5 d, and then transferred to 28 °C with 80% RH. Spikelet samples were collected at an interval of 1–3 dpi for subsequent experiments.

**Microscopic observation**

To monitor the infection process of *Uv*-10 in rice spikelets, samples collected at multiple time points after inoculation were examined directly under an environmental scanning electron microscopy (ESEM) (FEI Quanta 450; FEI, Hillsboro, OR, USA) with the low vacuum model (70 Pa). Macroscopic images of the spikelets and false smut balls were acquired with a Canon EOS Rebel Tzi digital camera (Canon, Tokyo, Japan).

**Quantitative real-time polymerase chain reaction**

Using TRizol reagent (Life Technologies, Carlsbad, CA, USA), total RNAs were isolated from mock-inoculated and *Uv*-inoculated rice spikelet samples at 1, 5, 10, and 17 dpi. The QuantTect Reverse Transcription Kit (QIAGEN, Hilden, Germany) was used for reverse transcription of total RNAs following the manufacturer’s instruction. Contaminated DNA was eliminated by the Kit-supplied gDNA Wipeout Buffer. Quantitative real-time polymerase chain reaction was carried out using a QuantiTect SYBR Green PCR Kit (QIagen). Gene expression analysis was conducted by the comparative $C_{\text{t}}$ method $2^{\Delta\Delta C_{\text{t}}}$ according to Fan et al. (2012). Three biological replicates were used, each of which was extracted from mixed spikelets collected from at least six panicles; and at least two individual experiments were carried out to confirm the results. Primer sequences are included in Table S2.

geNorm version 3.5 software was employed to select a stable rice reference gene for qPCR analysis according to the manufacturer’s instruction. In total, eight rice spikelet samples were used, including spikelets from mock-inoculated and *Uv*-inoculated panicles at 1, 5, 10, and 17 dpi, representing different stages of spikelet development, seed maturation, and *Uv* infection. Note that pollination occurred on mock-inoculated panicles at approximately 12 dpi. Six housekeeping genes were included: GAPDH, Actin1, Ub1, beta-tubulin (Tub2), eukaryotic elongation factor 1-alpha (eEF1α), and ubiquitin-conjugating enzyme E2 (Ubc). Primers sequences for the last three genes were adopted from Jain et al. (2006). KI-I$_2$ staining

To determine the fertilization status of ovaries, KI-I$_2$ staining was performed on mock-inoculated and *Uv*-infected spikelets at 17 dpi. Briefly, ovaries were excised from control spikelets and early false smut balls, incubated in 75% ethanol for 1–3 h until the green color faded, rinsed with distilled water, and then stained in KI-I$_2$ solution for 0.5 h. After staining, the ovaries were rinsed with 75% ethanol and incubated in 100% ethanol for 0.5 h before examined.

**Protein extraction and western blotting**

Total protein was extracted as described (Kawakatsu et al. 2008). Briefly, spikelets and false smut balls were ground into fine powder with a mortar and pestle, and extracted with buffer containing 50 mmol/L Tris-HCl (pH 6.8), 4% sodium dodecylsulfate (SDS), 8 mol/L urea, 5% 2-mercaptoethanol, and 20% glycerol for 2 h at room temperature. Total protein was obtained from the supernatant by centrifugation at 20,817 g for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and subjected to immune-blot analysis using antibodies against glutelin type-A2 (Os10t0400200-01) and 11-S plant SSP (Os03t0336100-01), which were produced in rabbits with the synthetic peptides ANAYRISREAAQR and DERWEEKKAAKQRK, respectively, and were kindly provided by Dr Guozhen Liu (Beijing Genomics Institute). An ECL kit (GE Healthcare, Pittsburgh, PA, USA) was used to detect signals.

**RNA-Seq analysis**

Total RNAs were isolated from mock-inoculated and *Uv*-inoculated rice spikelet samples collected at 17 dpi. RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and Agilent 2100 Bioanalyzer (NanoDrop Technologies, Wilmington, DE, USA). Three aliquots of rice RNA, each of which was extracted from mixed spikelets collected from at least six panicles, were equally pooled for deep transcriptome sequencing. cDNA synthesis, library preparation, and illumina
HiSeq 2000 sequencing were conducted at Beijing Genomics Institute (BGI, Shenzhen, China). For each rice sample, approximately 2 Gb of reads were generated.

After removing adaptor sequences and filtering low-quality sequences, clean reads from rice samples were mapped to the rice reference genome (http://rapdb.dna.affrc.go.jp/download/rgrsp1.html) using SOAPaligner/SOAP2 (Li et al. 2009). The RPKM method was used to calculate the normalized expression data of each rice and Uv transcript (Mortazavi et al. 2008). Differentially expressed genes were identified according to Audic and Claverie (1997), under the criteria of the absolute log2-fold change of 1 or more and FDR of 0.001 or less (Benjamini and Yekutieli 2001).

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load/rgrsp1.html) using SOAPaligner/SOAP2 (Li et al. 2009). The RPKM method was used to calculate the normalized expression data of each rice and Uv transcript (Mortazavi et al. 2008). Differentially expressed genes were identified according to Audic and Claverie (1997), under the criteria of the absolute log2-fold change of 1 or more and FDR of 0.001 or less (Benjamini and Yekutieli 2001).

REFERENCES

Ali R, Ma W, Lemtiri-Chlieh F, Tsaltas D, Leng Q, von Bodman S, Berkowitz GA (2007) Death don't have no mercy and neither does calcium: Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell 19: 1081–1095

Ashizawa T, Takahashi M, Arai M, Arie T (2012) Rice false smut pathogen, Ustilaginoidea virens, invades through small gap at the apex of a rice spikelet before heading. J Gen Plant Pathol 78: 255–259

Atia M (2004) Rice false smut (Ustilaginoidea virens) in Egypt. J Plant Dis Protect 111: 71–82

Audic S, Claverie JM (1997) The significance of digital gene expression profiles. Genome Res 7: 986–995

Benjamini Y, Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. Ann Stat 29: 1165–1188

Brooks SA, Anders MM, Yeater KM (2009) Effect of cultural management practices on the severity of false smut and kernel smut of rice. Plant Dis 93: 1202–1208

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57–63

Chao J, Jin J, Wang D, Han R, Zhu R, Zhu Y, Li S (2014) Cytological and transcriptional dynamics analysis of host plant revealed stage-specific biological processes related to compatible rice-ustilaginoidea virens interactions. PLoS ONE 9: e91591

Clough SJ, Fengler KA, Yu IC, Lippok B, Smith RK Jr, Bent AF (2000) The Arabidopsis dnd1 “defense, no death” gene encodes a mutated cyclic nucleotide-gated ion channel. Proc Natl Acad Sci USA 97: 9323–9328

Colquhoun TA, Schwieterman TA, Schwieterman ML, Wedde AE, Schimmel BC, Marciniai DM, Verdonk JC, Kim YJ, Oh Y, Galis I, Baldwin IT, Clark DG (2011) EOBII controls flower opening by functioning as a general transcriptional switch. Plant Physiol 156: 974–984

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139: 5–17

Detournay O, Schnitzler CE, Poole A, Weis VM (2012) Regulation of cridinarid-dinoflagellate mutualisms: Evidence that activation of a host TGFbeta innate immune pathway promotes tolerance of the symbiont. Dev Comp Immunol 38: 525–537

Fan J, Chen C, Yu Q, Kalaf A, Achor DS, Bransky RH, Moore GA, Li ZG, Gmitter FG Jr., (2012) Comparative transcriptional and anatomical analyses of tolerant rough lemon and susceptible sweet orange in response to ‘Candidatus Liberibacter asiaticus’ infection. Mol Plant Microbe Interact 25: 1396–1407

Fan J, Guo X, Huang F, Li Y, Liu Y, Li L, Xu Y, Zhao J, Xiong H, Yu J, Wang W (2014) Epiphytic colonization of Ustilaginoidea virens on biotic and abiotic surfaces implies the widespread presence of primary inoculum for rice false smut disease. Plant Pathol 63: 937–945

Fan RH, Wang YQ, Liu B, Zhang JZ, Hu DW (2016) The process of asexual spore formation and examination of chalmydospore germination of Ustilaginoidea virens. Mycosystema 29: 188–192 (in Chinese).

Guo X, Li Y, Fan J, Li L, Huang F, Wang W (2012) Progress in the study of false smut disease in rice. J Agric Sci Tech A 2: 1211–1217

Hu DW, Tang YX, Yong ML (2013a) Rice false smut is a stamen filament-infected disease. In: Peng YL, ed. The 10th International Congress of Plant Pathology. Chinese Society for Plant Pathology, Beijing, pp. 123

Hu M, Luo L, Wang S, Liu Y, Li J (2013b) Infection processes of Ustilaginoidea virens during artificial inoculation of rice panicles. Eur J Plant Pathol doi: 10.1007/s10658-10013-10364-10657

Hwang JE, Lim CJ, Chen H, Je J, Song C, Lim CO (2012) Overexpression of Arabidopsis dehydration-responsive element-binding protein 2C confers tolerance to oxidative stress. Mol Cells 33: 135–140

Ikegami H (1962) Seedling inoculation with the chlamydospores of the false smut fungus. Ann Phytopathol Soc Japan 27: 16–23 (in Japanese)

Jain M, Nihawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 345: 646–651

Kawakatsu T, Yamamoto MP, Hirose S, Yano M, Takaiwa F (2008) Characterization of a new rice glutelin gene GluD-1 expressed in the starchy endosperm. J Exp Bot 59: 4233–4245

Kawakatsu T, Yamamoto MP, Touno SM, Yasuda H, Takaiwa F (2009) Compensation and interaction between RISSB1 and RPBF during grain filling in rice. Plant J 59: 908–920

Koiso Y, Li Y, Iwasaki S, Hanaoka K, Kobayashi T, Sonoda R, Fujita Y, Yaegashi H, Sato Z (1994) Ustiloxins, antimitotic cyclic peptides from false smut balls on rice panicles caused by Ustilaginoidea virens. J Antibiot (Tokyo) 47: 765–773

Ladhalakshmi D, Laha GS, Singh R, Karthikeyan A, Mangrauthia SK, Sundaram RM, Thukkaiyannan P, Virakatamath BC (2012) Isolation and characterization of Ustilaginoidea virens and survey of false smut disease of rice in India. Phytoparasitica 40: 171–176

Lee PD, Sladerk R, Greenwood CM, Hudson TJ (2002) Control genes and variability: Absence of ubiquitous reference transcripts in diverse mammalian expression studies. Genome Res 12: 292–297

Li QF, Sun SSM, Yuan DY, Yu HX, Gu MH, Liu QQ (2010) Validation of candidate reference genes for the accurate normalization of real-
time quantitative RT-PCR data in rice during seed development. Plant Mol Biol Rep 28: 49–57

Li, R, Yu C, Li Y, Lam TW, Yiu SM, Kristiansen K, Wang J (2009) SOAP2: An improved ultrafast tool for short read alignment. Bioinformatics 25: 1937–1940

Ma W, Berkowitz GA (2011) Ca$^{2+}$-conduction by plant cyclic nucleotide gated channels and associated signaling components in pathogen defense signal transduction cascades. New Phytol 190: 566–572

Ma W, Qi Z, Smigel A, Walker RK, Verma R, Berkowitz GA (2009) Ca$^{2+}$, CAMP, and transduction of non-self perception during plant immune responses. Proc Natl Acad Sci USA 106: 20995–21000

Mackey D, Belkhadir Y, Alonso JM, Ecker JR, Dangl JL (2003) Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell 112: 379–389

Mackey D, Holt BF, 3rd, Wiig A, Dangl JL (2002) RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell 108: 743–754

Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Choi G, Browse J (2006) Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J 46: 984–1008

Mitsuhara I, Iwai T, Seo S, Yanagawa H, Hirose S, Okhawa Y, Ohashi Y (2008) Characteristic expression of twelve rice PR1 family genes in response to pathogen infection, wounding, and defense-related signal compounds (121/180). Mol Genet Genomics 279: 415–427

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5: 621–628

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development 132: 4107–4118

Nakamura K, Izumiyma N, Ohtsubo K, Koiso Y, Iwasaki S, Sonoda R, Fujita Y, Yaegashi H, Sato Z (1994) "Lupinosis"-like lesions in mice caused by ustiloxin, produced by Ustilaginoidea virens: A morphological study. Nat Toxins 2: 22–28

Ngugi HK, Scherm H (2004) Pollen mimicry during infection of blueberry flowers by conidia of Monilinia vacciniicorymbosi. Physiol Mol Plant Pathol 64: 113–123

Ngugi HK, Scherm H (2006) Biology of flower-infecting fungi. Annu Rev Phytopathol 44: 261–282

Nie DM, Ouyang YD, Wang X, Zhou W, Hu CG, Yao J (2013) Genome-wide analysis of endosperm-specific genes in rice. Gene 530: 236–247

Nomura K, Debroy S, Lee YH, Pumplin N, Jones J, He SY (2006) A novel ultrafast tool for short read alignment. Bioinformatics 22: 1937–1940

Onoodera Y, Suzuki A, Wu CY, Washida H, Suzuki A, Takaiwa F (1998) A rice bacterial virulence protein suppresses host innate immunity to cause plant disease. Science 310: 220–223

Onodera Y, Ashizawa T, Sonoda R, Tanaka C (2008) Villlosiclava virens gen. nov., comb. nov., teleomorph of Ustilaginoidea virens, the causal agent of rice false smut. Mycotaxon 106: 491–501

Tang YX, Jin J, Hu DW, Yong ML, Xu Y, He LP (2013) Elucidation of the infection process of Ustilaginoidea virens (teleomorph: Villlosiclava virens) in rice spikelets. Plant Pathol 62: 1–8

Tudzynski P, Scheffer J (2004) Claviceps purpurea: Molecular aspects of a unique pathogenic lifestyle. Mol Plant Pathol 5: 377–388

Wang DW, Wang S, Fu JF (2004) Research advance on false smut of rice. Liaoning Agric Sci 1: 21–24 (in Chinese)

Wang JC, Xu H, Zhu Y, Liu QQ, Cai XL (2013) OsbZIP58, a basic leucine zipper transcription factor, regulates starch biosynthesis in rice endosperm. J Exp Bot 64: 3453–3466

Wasternack C, Hause B (2013) Jasmonates: Biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot 111: 1021–1058

Wu CY, Adachi T, Hatano T, Washida H, Suzuki A, Takaifa F (1998) Promoters of rice seed storage protein genes direct endosperm-specific gene expression in transgenic rice. Plant Cell Physiol 39: 885–889

Wu MF, Tian Q, Reed JW (2006) Arabidopsis microRNA167 controls patterns of ARF6 and ARF8 expression, and regulates both female and male reproduction. Development 133: 4211–4218

Yamamoto MP, Onodera Y, Touno SM, Takaifa F (2006) Synergism between RPPB Dof and RSB2 bZIP activators in the regulation of rice seed expression genes. Plant Physiol 141: 1694–1707

Yuan Y, Zhong S, Li Q, Zhu Z, Lou Y, Wang L, Wang X, Wang M, Li Q, Yang D, He Z (2007) Functional analysis of rice NPR1-like genes reveals that OsNPR1/NH1 is the rice orthologue conferring disease resistance with enhanced herbivore susceptibility. Plant Biotechnol J 5: 315–324

Zhang Y, Zhang K, Fang A, Han Y, Yang J, Xue M, Bao J, Hu D, Zhou B, Sun X, Li S, Wen M, Yao N, Ma LJ, Liu Y, Zhang H, Huang F, Luo C, Zhou L, Li J, Chen Z, Miao J, Wang S, Liu J, Xu JR, Hsiang T, Peng YL, Sun W (2014) Specific adaptation of Ustilaginoidea virens in occupying host florets revealed by comparative and functional genomics. Nat Commun 5: 3849

Zeng DW, He LP, Meng CM, Hu DW (2009) The cytological evidence on infection of Ustilaginoidea virens at the germination stage. In: Peng YL, Zhu YY, eds. Proceedings of the Annual Meeting of Chinese Society for Plant Pathology. China Agricultural Science and Technology Press, Beijing. pp. 118 (in Chinese)

Zhou SR, Yin LL, Xue HW (2013) Functional genomics based understanding of rice endosperm development. Curr Opin Plant Biol 16: 236–246

SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article at the publisher’s web-site.

Figure S1. Sequence alignment of ARF6, ARF8 and MYB21 homologs from rice and Arabidopsis Using DNAMAN v5.2.2 software with default parameters, amino acid sequences of OsARF6 (Os02t0164900-01), OsARF8

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Os06t0677800-01) and OsMYB21 (Os11t0684000-01) from rice were aligned with their homologs from Arabidopsis (AT1G30330, AT5G37020, AT3G27810, respectively). Identical amino acids are shaded in black. The ARF6 (A), ARF8 (B) and MYB21 (C) homologs share high identities of 62%, 58% and 74%, respectively. Conserved motifs are underlined based on BLASTP analysis in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). B3 DNA, plant specific B3 DNA binding domain; Auxin_resp, a conserved region of auxin-responsive transcription factors; AUX_IAA, a conserved domain of the AUX/IAA family; SANT, ‘SWI3, ADA2, N-CoR and TFIIB’ DNA-binding domain.

**Figure S2.** Expression profiling of rice grain-filling-related genes in various tissues/organs and at different stages of reproductive development.

Global expression data for the grain-filling-related genes listed in Table 1 were retrieved from Affymetrix microarray experiments in the RicePLEX database (http://www.plexdb.org/modules/PD_browse/experiment_browser.php). Sample information was as following: Mature leaf; Young leaf; up to 0.5 mm, shoot apical meristem and rachis meristem (SAM); 0-3 cm, floral transition and floral organ development (P1); 3-10 cm, meiotic stage (P2 and P3); 10-15 cm, young microspore stage (P4); 15-22 cm, vacuolated pollen stage (P5); 22-30 cm, mature pollen stage (P6); 0-2 days post pollination (dap), early globular embryo (S1); 3-4 dap, middle and late globular embryo (S2); 5-10 dap, embryo morphogenesis (S3); 11-20 dap, embryo maturation (S4); 21-29 dap, dormancy and desiccation tolerance (S5). **Table S1.** The complete list of differentially expressed genes from rice in response to Uv infection. *(provided as an Excel file)* **Table S2.** Primers used in this study.