UvCom1 Is an Important Regulator Required for Development and Infection in the Rice False Smut Fungus *Ustilaginoidea virens*

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ABSTRACT

Ustilaginoidea virens is an economically important biotrophic fungal pathogen that causes rice false smut worldwide. However, the regulatory mechanisms of smut ball formation under *U. virens* infection remain unclear. Here, we identified an important transcription factor, *UvCom1*, from this pathogen, which regulates the formation of smut balls on rice spikelets. *UvCom1* contains two conserved internal repeat 1 (RPT) domains and is found only in fungi, with specific conservation in species of Basidiomycetes and Ascomycetes. *UvCom1* protein N- or C-terminal fusion vectors both showed transactivation activity in yeast. Deletion of *UvCom1* significantly affected the vegetative growth and conidiation of

U. virens. *UvCom1* negatively regulated the responses to oxidative, osmotic, and cell wall stresses. Remarkably, *UvCom1* was found to be essential for the formation of rice smut balls, and *UvCom1* deletion mutants lost the ability to stably utilize nutrients from the rice host. *UvCom1* was also highly expressed at the mycelial expansion stage. Transcriptomic analysis and quantitative real-time PCR revealed that *UvCom1* could affect the expression of genes significantly enriched in transmembrane transport. This study demonstrates that *UvCom1* is a key transcription factor governing smut ball formation of this biotrophic fungus.

Rice false smut, which is caused by the pathogenic biotrophic fungus *Ustilaginoidea virens* (Cooke) Takah (teleomorph: *Villosiclava virens*), is one of the most devastating grain diseases in the majority of rice-growing areas of the world (Brooks et al. 2010; Guo et al. 2012; Qiu et al. 2019). As a grain disease, it causes severe losses of yield and quality (Jiang et al. 2009; Yang et al. 2012), and threatens human or animal health by producing cyclopeptide mycotoxins such as ustiloxins in false smut balls (Koiso et al. 1994; Nakamura et al. 1994; Zhou et al. 2012). *U. virens* usually infects rice by its conidia, which are generated on the surface of rice spikelets, after which the hyphae extend randomly and enter into the inner space of spikelets through small gaps between lemma and palea to colonize floral organs (Ashizawa et al. 2012; Song et al. 2016). In floral organs, the pathogen first attacks the filament, preventing pollen from maturing and also blocking ovary fertilization, and then invades the stigma and style to simulate fertilization, resulting in the hijacking of large amounts of nutrients for the formation of rice false smut balls (Song et al. 2016). The fungus activates the grain-filling genes by simulating the filling signal, then hijacks the nutrition from rice to form smut balls (Fan et al. 2015; Song et al. 2016; Tang et al. 2013).

Genome and transcriptome analyses of *U. virens* infection of rice have indicated that this fungus might contain many transcription factors that regulate its virulence (Zhang et al. 2014). However, only *UvPRO1* and *UvHox2* in *U. virens* have been experimentally verified, and *UvPRO1* was found to play important roles in hyphal

growth, conidiation, stress response, and pathogenesis of *U. virens* (Lv et al. 2016). *UvHox2* regulates chlamydospore formation, conidiogenesis, and pathogenicity in *U. virens* (Yu et al. 2019). Here, we identified a new transcription factor, *UvCom1*, in *U. virens*. *Com1* was previously only identified in *Magnaporthe oryzae*, and deletion of *MoCom1* resulted in reduced width and increased length of conidia; furthermore, the $\Delta MoCom1$ mutants exhibited significantly reduced virulence on rice and barley seedlings (Yang et al. 2010). Proteomic data have revealed that *MoCom1* might have various effects on carbon and energy metabolism, lipid metabolism, melanin biosynthesis, structural organization of cells, and amino acid metabolism (Bhadoria et al. 2010). However, little has been known about the functions of *Com1* homologs in other fungi.

In this study, we found that the deletion of *UvCom1* caused significant decreases in mycelial growth rate, conidial production, and tolerance to oxidative, osmotic, and cell wall integrity stresses. $\Delta Uvcom1$ mutants would normally infect rice filaments, stigmas, and styles and establish a nutritional relationship with rice floral organs but could not utilize large quantities of nutrients needed to form smut balls. RNA-sequencing (RNA-seq) analyses showed a large number of differentially expressed genes enriched in functions related to carbohydrate transport and metabolism, and membrane transport and activation, indicating that the deletion of *UvCom1* should affect these functions, which resulted in a disorganized utilization of nutrients from rice. Taken together, our findings suggest that *UvCom1* is a key regulator of transmembrane transport, and is critical for fungal growth and infection-related development.

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MATERIALS AND METHODS

Fungal strains and growth conditions. The *U. virens* wild-type (WT) strain HWD-2, fully virulent on the rice cultivar Wanxian-98, was used in this study (Table 1). All transformed strains derived from the WT were cultured on potato sucrose agar (PSA) at 28°C under dark conditions. Seven-day-old mycelia collected from potato sucrose broth (PSB) shaken at 180 rpm were used for isolation of fungal DNA and RNA, and the conidia

separated from the culture were used for transformation via *Agrobacterium*-mediated transformation (ATMT), following Lv et al. (2016). Hygromycin B (final concentration of 200 µg/ml) and G418 (final concentration of 500 µg/ml) were used as selective markers for target gene deletion and complementation, respectively.

DNA and RNA manipulation, Southern blot and quantitative real-time PCR. Genomic DNA was extracted from vegetative hyphae using cetyltrimethylammonium bromide protocols. Southern blotting followed the protocol of Amersham Gene Images with Alkphos Direct Labeling and Detection System (GE Healthcare). Total RNA was extracted with the TRIzol reagent (Vazyme Biotech). To detect gene expression levels at different developmental stages, the total RNA of mycelia was collected from cultures grown for 7 days on PSA, or from infected rice spikelets at different days (1, 3, 6, 9, 13, or 20 days). Reverse-transcription assays were performed using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech). The β -tubulin gene (*UV8b_900*) was used as an internal control for quantitative real-time PCR (qRT-PCR). qRT-PCR was conducted using the TransStart Tip Green qPCR SuperMix (TransGen Biotech). Primer pair P7/P8 was designed for the detection of *UvCom1* gene expression levels (Supplementary Table S1). All results were normalized by β -tubulin expression, and relative changes in gene expression levels were calculated by the comparative cycle threshold method (Applied Biosystems). The expression of genes associated with grain filling was the same as that used by Song et al. (2016) and the GAPDH gene was used to normalize the data, as documented previously (Fan et al. 2015; Song et al. 2016). For each gene, data were taken from three independent biological replicates.

Target gene deletion and complementation in *U. vires*. To generate *UvCom1* gene deletion mutants, approximately 1.5 kb of upstream and downstream flanking sequences of the gene were amplified with primers F1F/R and F2F/R, respectively. The resulting PCR products were ligated with pGKO to generate the final deletion vector pGKO-*UvCom1*. The construct was inserted into the *Agrobacterium tumefaciens* strain EHA105, then transformed into conidia of the WT via the ATMT method, following Lv et al. (2016). Hygromycin-resistant transformants were isolated and subsequently screened by PCR with primers P1 and P2, then confirmed by RT-PCR and Southern blot.

For complementation assays, an approximately 4-kb fragment containing a 2 kb of native promoter region and a full-length *UvCom1* gene-coding region was amplified from the genomic DNA of the WT using primers P11 and P12 and cloned into vector p3300neo. The EHA105 strain with the p3300neo-*UvCom1* vector was transformed by coculturing with conidia of the mutant $\Delta Uvcom1-2$ using the ATMT method (Lv et al. 2016). Transformants were selected on PSA supplemented with antibiotic G418 at 500 µg/ml. Complementation strains were screened by conventional PCR with primers P1 and P2, then confirmed by

RT-PCR. All primers used in this study are listed in Supplementary Table S1.

Vegetative growth, conidiation, and fungal morphology. To determine whether the function of *UvCom1* was associated with the growth and morphogenesis of *U. vires*, the growth rate and colony morphology of *U. vires* transformants were investigated on PSA. For vegetative growth, mycelial plugs were transferred from 14-day-old PSA plates and grown on fresh PSA medium at 28°C. After 14 days of incubation, the radial growth of vegetative mycelia was measured. Mycelial morphology of the strains was examined by Zeiss Axioplan light microscopy (Carl Zeiss). Because conidial production is an important process in many phytopathogenic fungi, conidiation of *U. vires* transformants was assessed in PSB. Strains were grown in PSB medium at 28°C. After shaking at 180 rpm for 7 days, the cultures were filtered through four layers of gauze, and conidial production was measured using a hemocytometer. Conidial morphology of the strains was examined by microscopy based on at least 500 conidia per feature. Each treatment was repeated three times.

Stress adaptation assays. To evaluate the role of *UvCom1* in mediating *U. vires* adaptation to morphogenesis- or pathogenesis-associated stresses, the radial growth rates of the WT, $\Delta Uvcom1$ mutants, and complementation strains were compared on PSA containing the oxidative stress agent H₂O₂, osmotic stress agents NaCl or Sorbitol, cell-wall-disturbing agent sodium dodecyl sulfate (SDS), Congo red (CR), or Calcofluor white (CFW). Mycelial plugs

Fig. 1. Functional domain identification and phylogenetic tree. **A**, Predicted domain of internal repeat 1 (RPT) (box) of *UvCom1* in *Ustilaginoidea virens*; aa = amino acids. **B**, Neighbor-joining tree of putative Com1 homologs from 12 fungal genomes generated with MEGA 7.0. Numbers at branch nodes are bootstrap percentages out of 1,000 replicates. **C**, Three-dimensional (3D) structure model of *UvCom1* generated by Phyre2 server. **D**, Transactivation analysis of *UvCom1* and its deletion derivatives in yeast. The full-length *UvCom1* and its deletion derivatives were fused with pGBKT7. The vectors pGBKT7 or pGBKT7-53 and pGADT7-T were expressed in yeast as negative or positive control. The β -galactosidase activity was measured with suitable X- α -Gal.

TABLE 1. Wild-type and mutant strains of *Ustilaginoidea virens* used in this study

Strain	Description	Reference
Wild type	Wild-type strain of <i>U. vires</i> HWD-2	Jia et al. 2015
$\Delta Uvcom1-1$	<i>UvCom1</i> deletion mutant of HWD-2	This study
$\Delta Uvcom1-2$	<i>UvCom1</i> deletion mutant of HWD-2	This study
$C\Delta Uvcom1-2$	<i>UvCom1</i> complementation strain from $\Delta UvCom1-2$	This study
<i>UvCom1-6</i>	<i>UvCom1</i> ¹⁻⁶⁵⁹ -green fluorescent protein (GFP) subcellular localization strain	This study
<i>UvCom1-5</i>	<i>UvCom1</i> ¹⁻²¹⁰ -GFP subcellular localization strain	This study
<i>UvCom1-3</i>	<i>UvCom1</i> ²¹¹⁻⁶⁵⁹ -GFP subcellular localization strain	This study

were inoculated onto PSA plates containing exogenous 0.3 M NaCl, 0.5 M Sorbitol, 0.03% H₂O₂, 0.03% SDS, CR at 120 µg/ml, or CFW at 120 µg/ml. Stress adaptation was assessed by measuring the colony diameter of 14-day-old cultures. Each treatment was repeated three times.

Virulence and plant infection assays. To investigate the function of *UvCom1* in *U. virens* infection, pathogenicity and plant infection assays of $\Delta Uvcom1$ mutants were conducted on a susceptible rice cultivar (Wanxian-98). The virulence of *U. virens* strains was tested based on the inoculation method of Lv et al. (2016). The WT, $\Delta Uvcom1$ mutant, and complementation strains were cultured in PSB medium at 28°C. After shaking at 180 rpm for 7 days, the cultures were homogenized in a blender, and rice plants were inoculated with 2 ml of mycelial and spore suspensions using a syringe in the middle section of distal internodes at the eighth stage of panicle development. The treated rice plants were placed in a greenhouse at relative humidity of 95 ± 5% and temperature of 28 ± 2°C. The experiment was repeated three times.

After inoculation of rice panicles with *U. virens*, samples were taken 1 day and 6 days after inoculation, fixed with 2.5% (vol/vol) glutaraldehyde in 50 mM phosphate buffer (pH 7.2) for 6 to 8 h at 4°C, then dehydrated with 30, 50, 70, 80, and 90% gradient alcohol

and acetone. The samples were critical point dried, mounted on stubs, sputter coated with gold-palladium, and viewed using a JEOL JSM-6390LV scanning electron microscope operating at 10 kV.

Generation of green fluorescent protein fusion constructs and subcellular localization analysis. A full-length *UvCom1* gene-coding region with native promoter was amplified from the genomic DNA of the WT using primers *UvCom1*¹⁻⁶⁵⁹ F and R (Supplementary Table S2), and cloned into vector p3300neo-green fluorescent protein (GFP). A full-length *UvCom1* gene-coding region was cloned into vector p3300neo-RP27-GFP. The sequences of the two exons were ligated into vector p3300neo-RP27-GFP to obtain the *UvCom1*¹⁻²¹⁰- or *UvCom1*²¹¹⁻⁶⁵⁹-GFP fusion construct under the control of the RP27 promoter. The EHA105 strain with the GFP fusion vectors was transformed using ATMT by coculturing with the conidia of the $\Delta Uvcom1$ -2 or WT. The transformants were selected on PSA supplemented with antibiotic G418 at 500 µg/ml. For subcellular localization analysis of *UvCom1*, the GFP signal was observed by a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss), and imaged using a 488-nm diode laser for excitation and a 500- to 550-nm band-pass emission filter.

Transactivation activity assay in yeast. To examine transcriptional activity of *UvCom1*, yeast one-hybrid assays were performed using the Matchmaker pGBKT7 (Clontech) and the full length of *UvCom1* was amplified and inserted into pGBKT7 for construction of vector pGBKT7-*UvCom1*¹⁻⁶⁵⁹. To further determine which region of the protein is involved in transcription activation, the two exons of *UvCom1* were separately amplified and inserted into pGBKT7. These two constructed vectors were termed pGBKT7-*UvCom1*¹⁻²¹⁰ (N-terminal fusion vector) and pGBKT7-*UvCom1*²¹¹⁻⁶⁵⁹ (C-terminal fusion vector). The interaction between pGBKT7-53 and pGADT7-T was taken as the positive control and pGBKT7 was taken as the negative control. The three constructs

Fig. 2. Targeted gene deletion of *UvCom1* in *Ustilago virens*. **A**, Knockout strategies of the *UvCom1* gene and restriction sites for Southern blot at the *UvCom1* genomic region. K = *KpnI*. **B**, Southern blot analysis of the wild type (WT) and $\Delta Uvcom1$ mutants. **C**, Real-time PCR analysis of the *UvCom1* mutants. **D**, Colony morphology of the *UvCom1* mutants on potato sucrose agar (PSA) after 14 days of darkness at 28°C. **E**, Colony diameter on PSA after 14 days at 28°C. Data were collected from three independent experiments for each treatment, and analyzed by Fisher's least significant difference test. Asterisks represent significant differences between the mutant and WT at $P = 0.05$. **F**, Conidial production of the *UvCom1* mutants in potato sucrose broth at 28°C and 180 rpm for 7 days.

Fig. 3. Sensitivity of *UvCom1* deletion mutants to various stresses. **A**, Growth on potato sucrose agar (PSA) with various amendments—0.3 M NaCl, 0.5 M Sorbitol, 0.03% H₂O₂, 0.03% sodium dodecyl sulfate (SDS), Congo red (CR) at 120 µg/ml, or Calcofluor white (CFW) at 120 µg/ml—after 14 days at 28°C. **B**, Inhibition of colony growth by various stress inducers for *UvCom1* mutants and the wild type (WT). Data were collected from three independent experiments for each treatment and analyzed by Fisher's least significant difference test. Asterisks represent significant differences between the mutant and WT at $P = 0.05$.

were separately transformed into the yeast strain Y2H. All transformants were isolated with SD-Trp, and the β -galactosidase activity was measured on SD-Trp-His medium with suitable X- α -Gal (Clontech). All primers used for *UvCom1* transactivation activity assay in the yeast are presented in Supplementary Table S1.

RNA sequencing. To investigate the regulatory mechanism of *UvCom1*, deep transcriptome sequencing was used to compare the gene expression profiles between the WT and $\Delta Uvcom1-2$ mutant. Total RNA was extracted from 7-day-old mycelia of the two strains using TRIzol reagent, and each strain set three biological replicates. RNA-seq transcriptome libraries were prepared with the TruSeq RNA Sample Preparation Kit (Illumina) using 5 μ g of total RNA. Briefly, messenger RNA was first isolated according to a polyA selection method by oligo(dT) beads, then fragmented by fragmentation buffer. Next, double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA Synthesis Kit (Invitrogen) with random hexamer primers (Illumina). Then, the

synthesized cDNA was subjected to end repair, phosphorylation, and 'A' base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 200 to 300 bp on 2% low-range ultra agarose followed by PCR. After being quantified by TBS380, paired-end RNA-seq libraries were sequenced on the Illumina HiSeq4000 SBS Kit (300 cycles) with 150-bp paired-end reads. The library preparation and Illumina sequencing were performed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd.

Accession numbers. The RNA-seq data used in this study have been deposited in the NCBI Sequence Read Archive database with accession numbers SRR10101940 to SRR10101945.

Bioinformatics analysis. The full sequence of *UvCom1* was downloaded from the *U. virens* database (<https://www.ncbi.nlm.nih.gov/nucore/JHTR000000000>). Protein-conserved domains were predicted with SMART (<http://smart.embl-heidelberg.de/>). The three-dimensional (3D) structure modeling of proteins was

Fig. 4. Virulence assay and infection development of *UvCom1* deletion mutants on rice spikelets. **A**, Virulence of the wild type (WT) and *UvCom1* mutants at 21 days postinoculation (dpi) on rice cultivar Wanyan-98. **B**, Average number of smut balls per panicle. Data were collected from three independent experiments for each treatment and analyzed by Fisher's least significant difference test. Asterisks represent significant differences between the mutant and WT at $P = 0.05$. **C**, Infection progress of rice spikelets by the WT and $\Delta Uvcom1$. Scale bar = 50 μ m.

carried out using the Protein Homology/analogy Recognition Engine v 2.0 (Phyre2) program (<http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>). Com1 homologous protein sequences from 11 other organisms were obtained from the NCBI and their accession numbers are listed as follows: *Metarhizium acridum* (XP007812583.1), *Claviceps purpurea* (CCE35119.1), *Fusarium graminearum* (XP011316619.1), *Magnaporthe oryzae* (XP003714109.1), *Neurospora crassa* (XP011392982.1), *Botrytis cinerea* (EMR80337.1), *Pochonia chlamydosporia* (RZR60511.1), *Ophiocordyceps sinensis* (EQL01507.1), *Tolypocladium paradoxum* (POR32726.1), *Trichoderma arundinaceum* (RFU74606.1), and *Verticillium dahliae* (XP009648180.1). After sequence alignments with ClustalW v2.1, the phylogenetic tree was generated by the Mega7.0 Beta program using the neighbor-joining algorithm, and the bootstrap analysis with 1,000 replicates was performed to test the confidence of topology.

For RNA-seq data analysis, the raw paired-end reads obtained from sequencing were trimmed and quality controlled by SeqPrep (<https://github.com/jstjohn/SeqPrep>) and Sickle (<https://github.com/najoshi/sickle>) with default parameters. Clean reads were then separately aligned to the reference genome with orientation mode using TopHat software (version 2.0.0; <http://tophat.cbcb.umd.edu/>) (Trapnell et al. 2009). The library construction quality and sequencing quality of each sample are shown in Supplementary Tables S2 and S3. Based on fragments per kilobase of exon per million mapped reads, the expression level of each transcript was calculated. The RSEM software (<http://deweylab.biostat.wisc.edu/rsem/>) was used to quantify gene abundances (Li and Dewey 2011). R statistical package software edgeR (Empirical Analysis of Digital Gene Expression in R; (<http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>) (Robinson et al. 2010) was utilized for differential expression analysis. Functional-enrichment analyses, including gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), were performed to identify which differentially expressed genes (DEGs) were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected P value ≤ 0.05 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools (<https://github.com/tanghaibao/Goatools>) and KOBAS (<http://kobas.cbi.pku.edu.cn/>) (Xie et al. 2011).

Statistical analysis. Data were subjected to analyses of variance using SPSS 13.0 software (SPSS Inc.). Mean comparisons were conducted using Fisher's least significant difference test ($P = 0.05$).

RESULTS

Identification and characterization of *UvCom1* in *U. virens*. Com1 was identified through homology search of the *U. virens* genome database by using the BLASTP algorithm with a sequence of Com1 from *M. oryzae* as query, and named *UvCom1* in this study. The *UvCom1* gene contained two exons, and its exon-intron structure was verified by RT-PCR (data not shown). Functional domain analysis demonstrated that *UvCom1* contains two conserved internal repeat 1 (RPT) domains (Fig. 1A). Phylogenetic analysis of *Com1* homologs from different fungi revealed that *UvCom1* is the most similar to the homolog from *C. purpurea* and is specifically conserved in species of the divisions Basidiomycetes and Ascomycetes (Fig. 1B). The 3D structure modeling showed that *UvCom1* protein has putative helix-loop-helix structures (Fig. 1C). In trans-activation activity assays, yeast transformants containing vector pGBKT7-*UvCom1*¹⁻⁶⁵⁹, pGBKT7-*UvCom1*¹⁻²¹⁰-N, or pGBKT7-*UvCom1*²¹¹⁻⁶⁵⁹-C could grow normally on SD-Trp-His plates and colonies appeared blue (Fig. 1D). These results indicate that *UvCom1* is a transcription factor, and both N-terminal and C-terminal domains of the protein are involved in transcription activation.

***UvCom1* deletion significantly affected vegetative growth and conidiation of *U. virens*.** In total, 20 transformants were obtained from the *UvCom1* knock-out library, which was constructed by homologous recombination strategy (Fig. 2A), and eight independent deletion mutants with similar phenotypes were identified by PCR analyses (Supplementary Fig. S1), with primers listed in Supplementary Table S1. We then randomly selected two *UvCom1* deletion mutants, $\Delta Uvcom1-1$ and $\Delta Uvcom1-2$, to further confirm the *UvCom1* gene deletion event by Southern blot and RT-PCR analyses (Fig. 2B and C). The Southern blotting showed that an approximately 7.2-kb band was detected in the mutants $\Delta Uvcom1-1$ and $\Delta Uvcom1-2$, in contrast with an approximately 9.8-kb band in the WT, indicating that the *UvCom1* gene was deleted in these two mutants. RT-PCR results demonstrated that *UvCom1* was not expressed in the selected two mutants. Hence, the deletion mutants $\Delta Uvcom1-1$ and $\Delta Uvcom1-2$, serving as two independent repetitions, were further chosen for functional characterization of *UvCom1*. In a complementation assay, the complemented transformant C $\Delta Uvcom1-2$ recovering normal phenotypes was confirmed by RT-PCR and the results showed that the *UvCom1* gene could be expressed in mycelia of C $\Delta Uvcom1-2$ (Fig. 2C).

Compared with the WT, the mycelial growth rate of $\Delta Uvcom1$ mutants was significantly reduced (Fig. 2D and E) ($P < 0.05$). We also observed the mycelial morphology under microscopy but found no apparent differences in cells and cellular structures between $\Delta Uvcom1$ mutants and the WT (Supplementary Fig. S2A). Conidial production was also significantly reduced in $\Delta Uvcom1$ mutants (Fig. 2F) ($P < 0.05$) but no morphological defects were observed in the conidia of $\Delta Uvcom1$ mutants (Supplementary Fig. S2B). These results indicated that *UvCom1* plays important roles in the vegetative growth and conidiation of *U. virens*.

Fig. 5. Expression and subcellular localization of *UvCom1* in *Ustilaginoidea virens*. **A**, Expression of *UvCom1* relative to β -tubulin in conidia (Co) and hyphae (Hy) in potato sucrose broth (PSB) and at different infection stages on rice (1 to 20 days) by quantitative real-time PCR. Data were collected from three independent experiments for each treatment and analyzed by Fisher's least significant difference test. Asterisks indicate significant differences compared with Hy ($P = 0.05$). **B**, Subcellular localization of *UvCom1*²¹¹⁻⁶⁵⁹ in *U. virens*. Vegetative hyphae and conidia of the transformant expressing *UvCom1*²¹¹⁻⁶⁵⁹-GFP were observed under epifluorescence microscopy. DIC = differential interference contrast, GFP = green fluorescent protein, and scale bar = 20 μ m.

***UvCom1* negatively regulated responses to oxidative, osmotic, and cell wall stresses.** Among all tested stress chemicals, 0.3 M NaCl and 0.5 M sorbitol showed no inhibitory effect on the $\Delta Uvcom1$ mutants but significantly inhibited the mycelial growth of the WT ($P < 0.05$). Furthermore, the stress agents of 0.03% H_2O_2 , 0.03% SDS, CR at 120 $\mu\text{g/ml}$, or CFW at 120 $\mu\text{g/ml}$ had fewer significant inhibitory effects on the $\Delta Uvcom1$ mutants than on the WT (Fig. 3A and B) ($P < 0.05$). These results suggested that *UvCom1* may negatively regulate the responses to oxidative or osmotic stresses and cell wall integrity of *U. virens*.

***UvCom1* was essential for fungal pathogenicity.** In pathogenicity assays, after 21 days postinoculation (dpi), a typical symptom of yellow smut balls was observed on rice spikelets infected by the WT and complementation strains, whereas the $\Delta Uvcom1$ mutants failed to produce such a symptom (Fig. 4A and B).

For all tested strains, at 1 dpi, hyphae were found to be elongated and extended along the surface of spikelets. At 6 dpi, hyphae were observed on the surfaces of filaments of rice colonized by the WT and $\Delta Uvcom1$ mutants. Until 18 dpi, rice grains were not found to be forming the ball-like colonies by $\Delta Uvcom1$ mutants, whereas infection by WT and complementation strains normally led to the formation of rice smut balls (Fig. 4C). These results indicated that $\Delta Uvcom1$ mutants could establish a nutritional relationship with rice spikelets but could not form smut balls.

***UvCom1* was highly expressed at the mycelial expansion stage in rice spikelets.** The gene expression profile of *UvCom1* at different developmental stages of *U. virens* during pathogenesis was examined. In comparison with the vegetative-mycelial stage, the expression of *UvCom1* was significantly increased at the stage of mycelial colonization in rice spikelets at 9 dpi (Fig. 5A) ($P < 0.05$), suggesting that *UvCom1* might be involved in mycelial expansion to form rice smut balls during *U. virens* infection.

***UvCom1*²¹¹⁻⁶⁵⁹ determines the nuclear localization of *UvCom1* protein.** In the subcellular protein localization assay of *UvCom1*, none of the transformants had detectable GFP signals in vegetative hyphae or conidia under control of its native promoter. To determine whether the native promoter of *UvCom1* affected the accumulation of GFP signals in the transformants, we also generated transformants with the p3300neo-RP27-*UvCom1*-GFP C-terminal fusion construct under the control of the RP27 promoter. However, GFP signals were not detected in vegetative hyphae and conidia of any transformants. To test whether the GFP-*UvCom1* C-terminal fusion interfered with the detection of GFP, we subsequently generated transformants with p3300neo-RP27-GFP-*UvCom1* N-terminal fusion constructs. All transformants could recover normal phenotypes but GFP signals were still not detectable in vegetative hyphae or conidia. To assess the promoter activity of *UvCom1*, a 2.0-kb fragment of the *UvCom1* promoter was cloned into the vector p3300neo-GFP. As a result, GFP signals were present in the cytoplasm of vegetative hyphae and conidia (Supplementary Fig. S3). These results suggested that the *UvCom1* promoter may be constitutively active in *U. virens*. Because the *UvCom1* protein contained two exons, we generated *UvCom1*¹⁻²¹⁰-GFP and *UvCom1*²¹¹⁻⁶⁵⁹-GFP fusion construct p3300neo-GFP under the control of the RP27 promoter, and transformed them into the WT. The *UvCom1*²¹¹⁻⁶⁵⁹-GFP transformants showed strong fluorescent signals in the nuclei of conidia and vegetative hyphae (Fig. 5B), whereas no fluorescent signals were observed in the *UvCom1*¹⁻²¹⁰-GFP transformants (Supplementary Fig. S3). These results suggested that full-length *UvCom1* protein might affect the detection or production of the GFP signals, and that *UvCom1*²¹¹⁻⁶⁵⁹ determines the nuclear localization of *UvCom1* protein.

Deletion of *UvCom1* might not affect the expression of genes associated with grain filling in rice. In previous studies,

Fig. 6. Expression of genes associated with grain filling under inoculation of $\Delta Uvcom1$ and the wild type (WT) at different stages in rice (1, 3, 6, and 9 days) by quantitative real-time PCR. A, B, C, D, E, and F represent the relative expression of genes *OsRISBZ*, *OsSSIIla*, *Os02g32660*, *OsAGPS2b*, *OsGlut1n1*, and *OsPromln2*, respectively, in rice cultivar Wanxian-98. Error bars represent standard variations of three replicates in independent experiments.

infection of *U. virens* induced the expression of genes associated with grain filling through imitated fertilization, and the rice provided large amounts of nutrients to the pathogen (Song et al. 2016). To determine whether *UvCom1* is involved in the regulation of genes related to grain filling in rice, the expression of rice genes associated with grain filling was determined after inoculation with the *UvCom1* deletion mutant or WT. The results revealed that expression of most selected grain-filling-related genes, including *OsRISBZ1*, *OsSSIIIa*, *OsAGPS2b*, and *OsGlut1n1*, seems to be equal between the WT and $\Delta Uvcom1-2$ mutant, whereas *Os02g32660* and *OsPromln2* appear to be expressed much higher in the WT and $\Delta Uvcom1-2$ mutant, respectively (Fig. 6A to F), indicating that the deletion of *UvCom1* might not affect the expression of genes associated with grain filling, and failure of smut ball formation of $\Delta Uvcom1$ mutants is likely due to deficiency of pathogen development.

Genome-wide identification of genes regulated by *UvCom1*. In RNA-seq analysis, 625 DEGs were identified in the $\Delta Uvcom1-2$ mutant, including 286 upregulated and 339 down-regulated genes, based on the $\log_2$ ratio ($\Delta Uvcom1-2$ mutant/WT) values with twofold changes (Fig. 7A). Heatmaps were used to illustrate the differential expression of the genes detected in the WT and $\Delta Uvcom1-2$ mutant (Fig. 7B). In *U. virens*, 3,602 genes (43%) were assigned to GO terms. The 625 DEGs were classified into 32 functional groups using GO analysis. Most of these DEGs were clustered into catalytic activity, metabolic process, single-organism process, and cellular process (Fig. 7C).

***UvCom1* played important roles in carbohydrate transport and metabolism, transmembrane transport, and transporter activity.** In GO and KEGG enrichment analyses, a large number of DEGs were enriched in transmembrane transport, intracellular organelle part, and intrinsic and integral components of

Fig. 7. Transcriptomic analysis to identify differentially expressed genes in $\Delta Uvcom1-2$ mutant and the wild type (WT). **A**, Number of genes with more than twofold changes in expression in $\Delta Uvcom1-2$. **B**, Heatmap of reads per kilobase of transcript per million mapped reads normalized transcripts that depicts the 625 differentially expressed genes in each pairwise comparison. **C**, Functional categorization of up- or downregulated genes in $\Delta Uvcom1-2$. DEGs = differentially expressed genes.

Fig. 8. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional annotations of unigenes in Δ Uvcom1-2 mutant and the wild-type (WT) transcriptome. The scatter diagram shows enrichment of differentially expressed genes (DEGs) in the signaling pathways. **A**, GO enrichment analysis. FDR = false discovery rate. **B**, KEGG enrichment analysis. The X axis represents enrichment ratio (enrichment ratio = amount of DEGs enriched in the pathway/amount of all genes in annotation gene set) and the Y axis represents the pathway (P value < 0.05). **C**, Quantitative real-time PCR to validate differentially expressed genes related to transmembrane transporter. The relative abundance of the transcripts of each gene from Δ Uvcom1-2 was normalized by comparison with the gene from the WT (relative transcript = 1). Uv8b_6013 = major facilitator superfamily (MFS) multidrug transporter, Uv8b_6977 = sugar transporter family protein, Uv8b_6000 = putative metabolite transport protein, Uv8b_2743 = G-protein coupled receptor, Uv8b_144 = carboxylic acid transport protein, Uv8b_7201 = MFS phospholipid transporter Git1, Uv8b_7868 = urea active transporter, Uv8b_4961 = putative sodium/phosphate symporter, Uv8b_2545 = external NADH-ubiquinone oxidoreductase, and Uv8b_53 = oligopeptide transporter OPT-like protein.

Fig. 9. Expression of 12 randomly selected genes associated with transmembrane transport of *Ustilagoidea virens* under inoculation of the wild type (WT) and $\Delta Uvcom1-2$ at different infection stages in rice by quantitative real-time PCR: A, 1 day postinoculation (dpi); B, 3 dpi; C, 6 dpi; D, 9 dpi; E, 13 dpi; and F, 20 dpi. Error bars represent standard variations of three replicates in independent experiments.

membrane pathways (Fig. 8A and B), which are related to carbohydrate transport and metabolism, transmembrane transport, and transporter activity (Supplementary Tables S4, S5, and S6). We found that many DEGs belonged to the major facilitator superfamily (MFS) (UV8b_6013, UV8b_6977, UV8b_6000, UV8b_6685, UV8b_144, UV8b_6649, UV8b_236, UV8b_3294, UV8b_4620, UV8b_2207, UV8b_1759, UV8b_7201, UV8b_857), inorganic ion transport and metabolism (UV8b_4845, UV8b_654, UV8b_2367, UV8b_2511, UV8b_5548, UV8b_8112, UV8b_366, UV8b_5308, and UV8b_4961), and amino acid transport and metabolism (UV8b_1682, UV8b_121, UV8b_5087, UV8b_6984, UV8b_6592, UV8b_8057, and UV8b_7868). To confirm the gene expression patterns, we selected 10 genes for qRT-PCR analyses. The results showed that the expression pattern of each upregulated or downregulated gene was consistent with that in the RNA-seq data (Fig. 8C). To further validate the expression patterns of transport-related genes between the WT and $\Delta Uvcom1$ -2 mutant in planta, we randomly selected 12 genes related to transmembrane transport for qRT-PCR, and expressions of these genes were significantly upregulated or downregulated at different infection stages (1, 3, 6, 9, 13, or 20 dpi) (Fig. 9) ($P < 0.05$). These results indicated that *UvCom1* plays an important role in transmembrane transport.

DISCUSSION

Com1, which is specifically conserved in species of Basidiomycetes and Ascomycetes, has only been previously identified in *M. oryzae* (Bhadauria et al. 2010; Yang et al. 2010), and little is known about its function in fungi. In this study, deletion of *UvCom1* caused a lower growth rate of *U. virens*, which is different from the observations in the $\Delta Mocom1$ mutants. In *M. oryzae*, $\Delta Mocom1$ mutants showed a morphology of longer and narrower conidia than the WT (Yang et al. 2010) but, in *U. virens*, deletion of *UvCom1* did not affect the conidial morphology. Inhibition of conidiation was found in the $\Delta Uvcom1$ mutants, which is similar to the results obtained in *M. oryzae*. Our results indicated that *UvCom1* negatively regulates the responses of the *U. virens* to oxidative and osmotic stress and cell wall integrity. In RNA-seq data, many membrane transporters were induced significantly in the *UvCom1* deletion mutant, and the alteration of membrane transport in the mutant might play a role in its negative response to these various stresses. However, limited work has been done to investigate the relationships between membrane transporters and oxidative, osmotic, or cell wall stress responses in fungi. Biological functions of the upregulated transporters could be elucidated in the future. Transcription factor *SKN7* has been reported to be involved in the regulation of stress-related genes in *F. graminearum* (Jiang et al. 2015), and *GPD1* and *CTT1* were found to regulate the response to osmotic stress in different fungi (Albertyn et al. 1994; Schüller et al. 1994). Our RNA-seq data showed that these three genes were upregulated in *UvCom1* deletion mutants, suggesting that the deletion of *UvCom1* affects the expression of other regulators of stress response. Overall, our results indicate that Com1 homologs play species-dependent roles in morphogenesis of different fungi but have conserved functions in conidiation.

In this study, GFP-*UvCom1* or *UvCom1*-GFP fusion constructs under the control of RP27 or its native promoter were generated and transformed into the WT but no GFP signals were observed in either conidia or vegetative hyphae under confocal microscopy. Similar results were obtained in *M. oryzae* (Yang et al. 2010). We hypothesize that the full-length *UvCom1* protein might have an adverse effect on the fluorescence of the GFP fusion protein. To test this hypothesis, *UvCom1*¹⁻²¹⁰-GFP or *UvCom1*²¹¹⁻⁶⁵⁹-GFP fusion constructs were transformed into the WT. As expected, *UvCom1*²¹¹⁻⁶⁵⁹-GFP fusion construct transformants showed strong fluorescent signals in the nuclei of conidia and vegetative hyphae. In *M. oryzae*, the PPVKRPRE or PLAKKFK sequences could direct the nuclear localization of *Mocom1*³³⁵⁻⁶¹³-GFP fusion proteins

(Yang et al. 2010). By alignment, the PPVKRPRE sequence was also found to exist in the *UvCom1* protein sequence, and it might also direct the nuclear localization in *U. virens*.

The $\Delta Uvcom1$ mutants could normally infect rice filaments, stigmas, and styles, and establish a preliminary nutritional relationship with rice spikelets. However, the mutants might have lost the ability to fully utilize the nutrition from rice, and the mycelia in the spikelets could not expand to form typical false smut balls. We also tested the expression of genes related to grain filling after infection by the WT or $\Delta Uvcom1$ mutants. The results showed that all of these genes could be expressed at different stages of infection, indicating that rice grain filling was not suppressed by inoculation with $\Delta Uvcom1$ mutants. We hypothesized that the deletion of *UvCom1* might lead to the loss of sugar transport for the fungus. RNA-seq data showed that many genes enriched in membrane transport pathways were downregulated in the $\Delta Uvcom1$ mutant. In *Saccharomyces cerevisiae*, monosaccharide uptake is mediated by transporters belonging to the major facilitator superfamily (Young et al. 2013). Biotrophic phytopathogenic fungi might infect plants to obtain nutrients by redirecting carbon and nitrogen resources. Among these MFS genes, a sugar transporter family protein (UV8b_6977) was significantly downregulated in RNA-seq data. We examined the gene expression profile at different developmental stages of *U. virens* during pathogenesis by qRT-PCR, and found that the expression of UV8b_6977 was significantly increased from the stage of mycelial expansion to the formation of smut balls in rice spikelets (Supplementary Fig. S4). The functional link between *UvCom1* and UV8b_6977 in fungal infection needs to be confirmed.

RNA interference (RNAi) is a conserved regulatory mechanism of gene expression and has become a powerful tool to silence genes (Waterhouse and Fusaro 2006). RNAi starts with the initial processing or cleavage of a precursor double-stranded RNA (dsRNA) into short (21 to 25 nucleotides) small-interfering RNA or microRNA duplexes by Dicer (Baulcombe 2004; Hamilton and Baulcombe 1999). In planta, the expression of dsRNAs was recently shown to induce host plant-induced gene silencing (HIGS) in fungal cells (Koch et al. 2013). HIGS has been used to reduce the severity of fungal diseases in wheat, barley, and banana (Cheng et al. 2015; Ghag et al. 2014; Nowara et al. 2010). In wheat, HIGS of *FgChs* and *FgCYP51* could enhance resistance to *F. graminearum* (Cheng et al. 2015; Koch et al. 2013). Silencing of fungal glucanosyltransferase genes enhanced resistance to *Blumeria graminis* (Nowara et al. 2010). Com1 is conserved in fungi and is not found in plants or animals, including humans. As a virulence factor of phytopathogens, this protein could be used in the future as a specific molecular target for disease control. We speculate that *UvCom1*, which is essential for *U. virens* development and infection, is possibly a feasible RNAi target for promoting the resistance of rice plants against rice false smut.

Overall, it can be speculated that the *UvCom1* plays a key role in regulating transmembrane transport by facilitating the utilization of large quantities of nutrients to form rice false smut balls. This is the first hypothesized mechanism of the involvement of a *Com1* gene in the virulence of a biotrophic phytopathogen. More evidence is still needed to elucidate the regulatory role of *UvCom1* in transmembrane transport.

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