UvHOG1 is important for hyphal growth and stress responses in the rice false smut fungus Ustilaginoidea virens

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Rice false smut caused by Ustilaginoidea virens is one of the most important diseases of rice worldwide. Although its genome has been sequenced, to date there is no report on targeted gene deletion in U. virens and no molecular studies on genetic mechanisms regulating the infection processes of this destructive pathogen. In this study, we attempted to generate knockout mutants of the ortholog of yeast HOG1 MAP kinase gene in U. virens. One Uvhog1 deletion mutant was identified after screening over 600 hygromycin-resistant transformants generated by Agrobacterium tumefaciens mediated transformation. The Uvhog1 mutant was reduced in growth rate and conidiation but had increased sensitivities to SDS, Congo red, and hyperosmotic stress. Deletion of UvHOG1 resulted in reduced expression of the stress response-related genes UvATF1 and UvSKN7. In the Uvhog1 mutant, NaCl treatment failed to stimulate the accumulation of sorbitol and glycerol. In addition, the Uvhog1 mutant had reduced toxicity on shoot growth in rice seed germination assays. Overall, as the first report of targeted gene deletion mutant in U. virens, our results showed that UvHOG1 likely has conserved roles in regulating stress responses, hyphal growth, and possibly secondary metabolism.

Ustilaginoidea virens (Cooke) Takah (Teleomorph: Villosiclava virens) is the causal agent of rice false smut that threatens rice production worldwide¹. It is an ascomycete that is closely related to Claviceps purpurea and its genome has been recently sequenced². In infected rice kernels, seed development is inhibited and replaced with the formation of so-called smut balls that contain darkly-pigmented chlamydospores. In addition to causing yield losses, U. virens produces ustiloxins that have inhibitory effects on growth of plant seedlings and are harmful to the nervous system of animals³–⁵.

Although the success rate is relatively low, false smut symptom development can be observed by inoculation with conidia at the rice booting stage⁶. It has been shown that germ tubes of U. virens can enter and grow intercellularly in the filaments of rice heads. When the fungus reaches the base of filaments, hyphal growth extends upward from basal filaments to anther apex and finally encloses the floral organs to form the smut balls⁷. Due to U. virens infection, rice seed development is completely stopped⁸. However, molecular mechanisms regulating the plant infection processes of the rice false smut fungus are not clear. In fact, although its genome has been sequenced², targeted gene deletion by homologous recombination has not been reported in U. virens. Nevertheless, U. virens is amenable to transformation and disruption mutants can be identified by random insertional mutagenesis⁹. In comparison with many other plant pathogenic fungi such as Magnaporthe oryzae, Fusarium graminearum, and Ustilago maydis¹⁰–¹², the frequency of homologous recombination and gene replacement is low in C. purpurea¹³,¹⁴. It is possible that U. virens also has a relatively low homologous recombination frequency, making targeted gene knockout, an important approach to study gene functions in pathogenic fungi, less efficient in this important rice pathogen.

In the budding yeast Saccharomyces cerevisiae, the high osmolarity glycerol (HOG) response pathway consisting of the Ssk2/Ssk22-Pbs2-Hog1 MAP kinase (MAPK) cascade is important for survival under hyperosmotic conditions¹⁵,¹⁶. In filamentous fungi, the key components of this MAP kinase pathway are well conserved but their

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biological functions are not limited to responses to high osmolarity\textsuperscript{17,18}. In general, Hog1 orthologs have been implicated in regulating plant infection, fungicide resistance, and responses to various environmental stresses, such as oxidative and cell wall stresses. However, its exact functions vary significantly among different fungal pathogens. For example, the Hog1 ortholog is important for plant infection in 	extit{Botrytis cinerea}, 	extit{Mycosphaerella graminicola}, and 	extit{F. graminearum}, but deletion of OSM1 in \textit{M. oryzae} or its ortholog in \textit{Colletotrichum lagenarium} has no effect on penetration and virulence\textsuperscript{19–23}. Whereas the \textit{Bcsk1} and sakA mutants are hypersensitive to oxidative stress in \textit{B. cinerea} and \textit{A. fumigatus}\textsuperscript{20,21,24}, the \textit{Fghog1} and \textit{Cshog1} deletion mutants are only slightly reduced in growth rate by \textit{H2O2} in \textit{F. graminearum} and \textit{Bipolaris sorokiniana}\textsuperscript{20,22}. In \textit{C. lagenarium}, \textit{Neurosora crassa}, and other fungi, the HOG pathway plays a role in resistance to dicarboximide and phenylpyrrole fungicides. In \textit{F. graminearum}, the FgHog1 MAP kinase pathway is also important for hyphal growth, the production of deoxynivalenol (DON), a trichothecene mycotoxin, and sexual reproduction\textsuperscript{20}.

Like many other filamentous fungi\textsuperscript{18}, \textit{U. virens} has the Hog1 and other two well-conserved MAP kinase cascades. However, none of them have been functionally characterized. In this study, we generated the \textit{Uvhog1} mutant and characterized its defects. As the first report on generation of a targeted deletion mutant in \textit{U. virens}, we found that this fungal pathogen has a relatively low homologous recombination frequency (< 0.2\%). The \textit{Uvhog1} mutant was reduced in vegetative growth and conidiation but had increased sensitivities to hyperosmotic and cell wall stresses. Our results showed that \textit{UvHOG1} likely has conserved roles in stress responses in \textit{U. virens} and improvements are necessary to make molecular studies more efficient and routine.

**Results**

\textbf{\textit{U. virens} has a relatively low homologous recombination frequency.} The predicted gene KDB17291.1 of \textit{U. virens} is orthologous to the yeast \textit{HOG1} and named \textit{UvHOG1} in this study. To generate the \textit{Uvhog1} deletion mutant, we first transformed the \textit{UvHOG1} gene replacement construct generated by the double-joint PCR approach\textsuperscript{26} into protoplasts of the wild-type strain UV-8b\textsuperscript{2}. After screening over 300 hygromycin-resistant transformants derived from at least five independent transformations, we failed to identify any \textit{Uvhog1} deletion mutant, indicating that the homologous recombination efficiency is relatively low in \textit{U. virens}, which is consistent with what has been observed in \textit{C. purpurea}\textsuperscript{14}.

Because \textit{Agrobacterium tumafaciens}-mediated transformation (ATMT) is known to increase the frequency of homologous recombination\textsuperscript{27}, we then cloned the \textit{UvHOG1} gene replacement construct generated by double-joint PCR into the binary vector pCAMBIA1300\textsuperscript{28}. The resulting vector was introduced into \textit{A. tumafaciens} and used to transform conidia of UV-8b. A total of 619 hygromycin-resistant ATMT transformants were screened by PCR with primers (see Supplementary Table S1 online) located in the deleted region. One putative knockout mutant M1 was identified (Fig. 1A) and further confirmed by Southern blot hybridization (Fig. 1B). In the wild type and ectopic transformant M2, a 3.5-kb \textit{BamHI} band was detected with an \textit{UvHOG1} fragment as the probe (Fig. 1B). The same probe had no hybridization signals in transformant M1. When hybridized with a fragment of the \textit{hph} gene, a 3.2-kb band of the expected size derived from the gene replacement event was detected in the \textit{Uvhog1} deletion mutant M1 (Fig. 1B). The wild type had no hybridization signals but ectopic transformant M2 had a 5.0-kb band (Fig. 1B). Therefore, the homologous recombination efficiency was as low as 0.16\% for the \textit{UvHOG1} gene in \textit{U. virens}.

\textbf{\textit{UvHOG1} is important for vegetative growth and conidiation.} Because the \textit{Uvhog1} mutant formed smaller colonies than the wild type (Fig. 1C), we assayed its growth rate on PDA, YT, and 5xYEG. On all the media tested, the \textit{Uvhog1} mutant was slightly reduced in growth rate (Table 1), indicating that its defects in vegetative growth was independent of nutrient conditions.

We also assayed the production of conidia in liquid YT cultures\textsuperscript{29}. Although it produced normal conidia, the \textit{Uvhog1} mutant was significantly reduced in conidiation in comparison with the wild type (Table 1). Whereas the wild-type strain produced \(8.2 \pm 1.0\times 10^5\) conidia/ml in 7-day-old cultures, the mutant produced less than \(1.8 \pm 1.0\times 10^5\) conidia/ml under the same conditions.

For complementation assays, the entire \textit{UvHOG1} gene (the coding region and its 1.5-kb promoter and 0.5-kb terminator sequences) was amplified from strain UV-8b and transformed into the \textit{Uvhog1} mutant. The resulting complemented transformant C1 was rescued in the defects of \textit{Uvhog1} mutant in hyphal growth and conidiation (Table 1). These results indicate that deletion of \textit{UvHOG1} is directly responsible for the mutant phenotypes and the \textit{Uvhog1} gene plays a role in hyphal growth and conidiation in \textit{U. virens}.

\textbf{Deletion of \textit{UvHOG1} results in defects in response to hyperosmotic stress.} Because the HOG pathway is well conserved in fungi for responses to hyperosmotic stress\textsuperscript{17,18}, we assayed the defects of the \textit{UvHog1} mutant in growth on YT medium with 0.5 M NaCl or 1 M sorbitol. In the presence of 0.5 M NaCl, the \textit{Uvhog1} mutant had no obvious growth after incubation for 14 days (Fig. 2A). Under the same conditions, the wild type was reduced in growth rate but still formed compact colonies (Fig. 2A; Table 2). We also assayed the effect of hyperosmotic stress on conidium germination. In the presence of 0.3 M NaCl, most of the wild-type conidia (72.1 ± 1.9\%) germinated after incubation in YTS medium for 16 h but only 3.2 ± 1.3\% mutant conidia germinated. Even after incubation for 24 h, only 5.0 ± 0.8\% conidia produced germ tubes. Moreover, germ tube growth was stunted by NaCl treatment in the \textit{Uvhog1} mutant (Fig. 2B; see Supplementary Table S2 online). Similar results were obtained with growth assays on medium with 1 M sorbitol. Treatments with 0.5 M NaCl or 1 M sorbitol resulted in over 99\% reduction in colonial growth in the \textit{Uvhog1} mutant (Table 2).

\textbf{\textit{UvHOG1} is also important for responses to cell wall and membrane stresses but not oxidative stress.} We also assayed the defects of the \textit{Uvhog1} mutant in response to oxidative, cell wall and membrane stresses on YT medium. In the presence of 0.07\% \textit{H2O2}, both the wild type and \textit{Uvhog1} mutant had a similar...
level of reduction in growth rate after incubation for 14 days (Fig. 2A; Table 2). On medium with 70 μg/ml Congo red, the growth rate was reduced by approximately 17% in the wild type but 50% in the *Uvhog1* mutant (Fig. 2A; Table 2). However, in the presence of 0.03% SDS (w/v), the *Uvhog1* mutant had no visible growth although the
Glycerol accumulation is also controlled by Uvhog1 but its contribution to adaptation to hyperosmotic stress under hyperosmotic conditions, and its accumulation is under the control of the wild-type strain UV-8b (Fig. 4). These results indicate that sorbitol is the main compatible solute in U. virens, their orthologs in Uvhog1, RNA samples were isolated from hyphae of the wild-type strain UV-8b and complemented transformant. Whereas rice shoots were green and began to form the first leaf by 5 days in the water treatment control, only whitish, short shoots were observed in samples treated with culture filtrates of strains UV-8b or wild-type strain UV-8b still produced compact colonies (Fig. 2A). After incubation in YTS medium with 0.03% SDS for 12 h, most of the wild type conidia (76.2 ± 1.2%) germinated but less than 2.5 ± 1.0% Uvhog1 mutant conidia produced germ tubes (see Supplementary Fig. S1 and Table S2 online). These results suggest that the Uvhog1 mutant also had increased sensitivity to Congo red and SDS. Therefore, the UvHOG1 pathway is involved in regulating responses to membrane and cell wall stresses, but not oxidative stress in U. virens.

Deletion of UvHOG1 affects the expression of UvATF1, UvSKN7, and UvAP1. The ATF1, SKN7, and API genes encode three transcription factors known to be involved in the regulation of stress-related genes in F. graminearum and other fungi. To determine whether deletion of UvHOG1 affects the expression of their orthologs in U. virens, RNA samples were isolated from hyphae of the wild-type strain UV-8b and Uvhog1 mutant that were harvested from 2-day-old regular YT cultures and cultures treated with 0.5 M NaCl, 0.03% SDS, or 0.07% H2O2. In the wild type, the expression of UvAPI, UvATF1, and UvSKN7 had no significant change when treated with NaCl. However, SDS treatment resulted in a 50% increase in the expression of UvAPI and UvSKN7, and H2O2 treatment caused an up-regulation of UvSKN7 expression (Fig. 3). In the Uvhog1 mutant, the expression of all these three genes were reduced over two-folds by treatment with 0.5 M NaCl. Nevertheless, the expression of UvAPI and UvSKN7 was up-regulated over two-folds in the presence of SDS (Fig. 3).

In comparison with the wild type, the expression of UvATF1, UvSKN7, and UvAPI was only slightly reduced in the Uvhog1 mutant (controls in Fig. 3). However, compared to the wild type, the expression of UvATF1 was reduced over 50% in the presence of NaCl and SDS but up-regulated approximately two-folds when treated with H2O2 in the Uvhog1 mutant (Fig. 3A). For UvSKN7, its expression in the mutant were significantly reduced when treated with NaCl but increased over two-folds in response to SDS (Fig. 3B). However, the wild type and Uvhog1 mutant had no obvious differences in the expression level of UvAPI when treated with SDS or H2O2 although the presence of 0.5 M NaCl down-regulated its expression more than 50% in the Uvhog1 mutant (Fig. 3C). These results indicate that deletion of UvHOG1 has different effects on these transcription factor genes in U. virens.

The accumulation of sorbitol and glycerol is not induced by NaCl treatment in the Uvhog1 mutant. Glycerol, sorbitol, and other neutral compatible solutes known to be accumulated in different fungi in response to hyperosmotic stress. To determine the compatible solutes accumulated in U. virens in response to hyperosmotic stress, we assayed metabolites in cultures treated with or without 1.0 M NaCl. In the wild type, sorbitol that has the retention time (RT) of 25.308 min was significantly induced by NaCl treatment (Fig. 4). Glycerol (RT = 6.031 min) was also slightly induced by NaCl treatment in UV-8b. However, the production of these two compatible solutes was not or barely detectable in hyphae of the Uvhog1 mutant treated with or without 1.0 M NaCl (Fig. 4). These results indicate that sorbitol is the main compatible solute in U. virens under hyperosmotic conditions, and its accumulation is under the control of the UvHOG1 MAP kinase pathway. Glycerol accumulation is also controlled by UvHOG1 but its contribution to adaptation to hyperosmotic stress may be not as significant as sorbitol.

Deletion of the Uvhog1 gene likely reduces the expression of UvUSTA gene. In F. graminearum, the Fghog1 mutant was significantly reduced in DON production. To assay the role of UvHOG1 in mycotoxin biosynthesis, we assayed the expression of the UvUSTA gene, a member of the gene cluster related to ustiloxin synthesis. In comparison with the wild type, the expression level of UvUSTA was reduced over 10-folds in the Uvhog1 mutant after incubation for 14 days in YT medium (see Supplementary Fig. S2 online). These results indicate that UvHOG1 may be involved in the regulation of UvUSTA expression in U. virens.

Culture filtrates of the Uvhog1 mutant is less inhibitory to rice seed germinating. To determine whether deletion of UvHOG1 affects the production of phytotoxins, we isolated the culture filtrates from YT cultures of 5-day-old wild-type strain UV-8b, 7-day-old Uvhog1 mutant M1, and 5-day-old complemented transformant C1 as described with minor modifications. The dry weights of vegetative hyphae were quantified to show that the wild type and Uvhog1 mutant strains had similar biomass in these YT cultures (see Supplementary Table S3 online). When assayed with rice seeds of cultivar YA-5A, culture filtrates of the wild type, Uvhog1 mutant, and complemented strains blocked root growth after incubation for 5 days at room temperature (Fig. 5). No visible root growth was observed in the rice seeds treated with culture filtrates of U. virens. Rice shoot growth also was significantly stunted in samples treated with culture filtrates of the wild type and complemented transformant. Whereas rice shoots were green and began to form the first leaf by 5 days in the water treatment control, only whitish, short shoots were observed in samples treated with culture filtrates of strains UV-8b or

| Strains  | Colony Diameter (mm) | PDA       | YT       | 5xYEG     | Conidiation (×10⁵/ml) |
|----------|----------------------|-----------|----------|-----------|----------------------|
| UV-8b    | 43.0 ± 0.1           | 48.0 ± 0.2 | 41.0 ± 0.1 | 8.2 ± 0.4  |
| M1       | 36.0 ± 0.3           | 38.0 ± 0.4 | 35.0 ± 0.2 | 1.8 ± 0.1  |
| C1       | 38.2 ± 0.4           | 48.0 ± 0.1 | 43.0 ± 0.1 | 8.5 ± 0.7  |

Table 1. Phenotypes of the Uvhog1 mutant in vegetative growth and conidiation. Colony diameter was measured with petri plate cultures (Φ 9 CM) after incubation for 14 days. Conidiation was assayed with 7-day-old YT cultures. Mean and standard deviation were calculated from three independent replicates. Different letters mark significant differences by ANOVA analysis (P = 0.05).
Figure 2. Growth and conidium germination of the Uvhog1 mutant in the presence of different stresses. (A) The wild-type strain UV-8b, Uvhog1 mutant, and complementary transformant were cultured on YT medium with or without 0.5 M NaCl, 0.07% H₂O₂, 0.03% SDS (w/v), and 70 μg/ml Congo red. Photographs were taken after incubation at 25 °C for 14 days. (B) Conidia of UV-8b and the Uvhog1 mutant were incubated in YTS with 0.3 M NaCl for 16, 20, or 24 h. Bar = 20 μm.
Table 2. Reduction in hyphal growth of the Uvhog1 mutant by different stresses. *Colony diameter was measured after incubation for 14 days on regular YT medium or YT with different chemicals. For each strain, the percentage of reduction in growth by different stresses was estimated in comparison with its growth on regular YT medium after deducting the diameter of the original inoculum (Φ 8 mm). Mean and standard deviation were calculated from three replicates. Different letters mark significant differences by ANOVA analysis (P = 0.05). **no visible growth.

| Strains          | 0.5 M NaCl | 1 M Sorbitol | 0.07% H₂O₂ | 0.03% SDS | 70μg/ml Congo red |
|------------------|------------|--------------|-------------|-----------|------------------|
| UV-8b (wild type)| 72.5 ± 0.3a| 76.0 ± 0.9b  | 71.9 ± 0.4c | 33.3 ± 0.2a | 16.7 ± 1.1a      |
| M1 (Uvhog1)      | 100% B     | 99.7 ± 0.0b  | 73.7 ± 0.2c | 100% B    | 44.7 ± 0.4b      |
| C1 (Uvhog1/UvHOG1)| 71.6 ± 0.6a| 71.9 ± 0.4c  | 69.8 ± 0.2a | 37.5 ± 0.2a | 20.8 ± 0.2a      |

Figure 3. Expression profiles of UvATF1, UvAP1, and UvSKN7. RNA samples were isolated from vegetative hyphae of UV-8b and the Uvhog1 mutant cultured in regular YT (CK) or YT with 0.5 M NaCl, 0.03% SDS, or 0.07% H₂O₂. The expression level of (A) UvATF1, (B) UvSKN7, and (C) UvAP1 in the wild type strain cultured in regular YT was arbitrarily set to 1. Mean and standard deviations were calculated with results from three independent replicates.
Figure 4. Metabolic profiles of the wild type and Uvhog1 mutant. Vegetative hyphae were harvested from two-day-old YT cultures of the wild type strain UV-8b and Uvhog1 mutant treated with or without 1.0 M NaCl for 1 h. Metabolites were extracted and analyzed by GC-MS. The X-axis represents the retention time (RT) in minutes. The Y-axis is the abundance of total ion current (TIC). The peaks with RT of 6.031 and 25.308 are glycerol and sorbitol, respectively. The peak with RT of 7.223 minutes is isocetane.

Figure 5. Assays for toxicity of U. virens culture filtrates with rice seeds. Seeds of rice cultivar YA-5A were incubated on filter papers soaked with blank control or filtrates of 5-day-old YT cultures of UV-8b, 7-day-old Uvhog1 mutant, and 5-day-old complementary strain. Shoot and root growth were examined after incubation at 25 °C for 5 days.
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Table 3. Inhibitory effects of culture filtrates of U. virens on rice seed germination. Root and shoot growth were measured after incubation of rice seeds on filter papers with culture filtrates for 5 days. Mean and standard deviation were calculated from three independent replicates with 50 seeds for each treatment. Different letters mark significant differences by ANOVA analysis (P < 0.05). N/D, not detectable. *Culture filtrates harvested from 5-day-old YT cultures. **Culture filtrates harvested from 7-day-old YT cultures.

| Strains | Shoot growth (mm/5-day) | Root growth (mm/5-day) |
|---------|--------------------------|------------------------|
| Water   | 23.4 ± 6.4              | 10.9 ± 8.6             |
| UV-88°  | 11.5 ± 5.8              | N/D                    |
| MI°     | 19.4 ± 4.5              | N/D                    |
| CI°     | 12.6 ± 5.4              | N/D                    |

Discussion

Agrobacterium tumefaciens-mediated transformation (ATMT) has been widely used in various fungi, including U. virens42, and it has been reported to increase the frequency of homologous recombination23,26. In this study, we failed to isolate Uvhog1 deletion mutants with PEG-mediated protoplast transformation. Even with the ATMT approach, only one Uvhog1 mutant was identified among the 617 hygromycin-resistant transformants screened, which was less than 0.2% for the gene replacement frequency. In separate studies in generating knockout mutants of two other genes in U. virens, we had similar low homologous recombination frequency with the ATMT approach. Whereas the frequency of gene replacement was 1-2% in ATMT transformants of C. purpurea33,14, over 50% the ATMT transformants had targeted gene deletion in Aspergillus nidulans39. Our data suggest that homologous recombination may occur at a relatively lower frequency in U. virens. To introduce targeted mutations or deletion more efficiently in U. virens, it may be helpful to generate the UvKu70 or UvKu80 deletion mutant, which is known to increase in homologous recombination frequency in other fungi40,41. The other option is to use the CRISPR42 approach to generate targeted gene disruption mutants in U. virens.

The most conserved function of the Hog1 MAP kinase pathway in yeast and filamentous fungi is for regulating responses to hyperosmotic stress27,43. Therefore, it is not surprising that hyphal growth of the Uvhog1 mutant was significantly stunted on YT plate with 0.5 M NaCl or 1 M sorbitol. In response to hyperosmotic stress, fungal hyphae often accumulate compatible solutes, including glycerol, sorbitol, and proline35. The accumulation of sorbitol was observed in hyphae treated with 1.0 M NaCl in the wild type but not the Uvhog1 mutant. Although to a lesser degree, glycerol accumulation was slightly increased in response to NaCl treatment. Therefore, although glycerol may play a minor role, sorbitol is the major compatible solute accumulated by U. virens in response to hyperosmotic stress, which is regulated by the UvHOG1 pathway. In F. graminearum, glycerol, arabitol, mannitol, and sucrose are accumulated in response to hyperosmotic stress29. However, in Beauveria bassiana, erythritol and arabitol, but not glycerol and mannitol, are the major compatible solutes for adaptation to hyperosmotic stress44. Similarly, arabitol is a major compatible solute accumulated in M. oryzae under hyperosmotic stress conditions22. Differences in compatible solutes that are induced by NaCl treatment indicate that the HOG1 pathway may be involved in the regulation of diverse metabolic pathways in response to hyperosmotic stress in different fungi.

In filamentous fungi, it has been shown that the Hog1 MAP kinase pathway also plays roles in regulating responses to oxidative stress27. In A. fumigatus and B. cinerea, vegetative growth of the mutants blocked in this conserved MAPK pathway, such as the AskA and Abcsak1 mutants, were hypersensitive to H2O2 treatment21,24. Whereas the Fhog1 mutant was slightly increased in sensitivity to H2O2 than the wild type in F. graminearum20, the Uvhog1 mutant had no significant changes in sensitivity to H2O2 in comparison with the wild type in U. virens. Therefore, the UvHog1 pathway may not play a significant role in response to oxidative stress in the rice false smut fungus. However, the Uvhog1 mutant was also hypersensitive to cytoplasm membrane and cell wall stresses. Therefore, it is likely that the UvHog1 MAP kinase pathway also is involved in regulating responses to various environmental stresses in U. virens, which is similar to what has been observed in several other filamentous fungi20,24.

When cultured on regular PDA, YT, and 5 × YEG media, the Uvhog1 mutant was reduced in growth rate and produced compact colonies, suggesting a role of UvHOG1 in hyphal growth under normal culture conditions regardless of stresses. Although this MAP kinase is dispensable for growth in fungi such as M. oryzae and B. bassiana22,44, deletion of its ortholog in F. graminearum, B. cinerea, A. fumigatus, and Metarhizium acridum affected hyphal growth20,21,24,45. In addition, condiation was reduced in the Uvhog1 mutant, which is similar to what has been observed in mutants defective in this MAP kinase pathway in B. cinerea, F. graminearum and B. bassiana20,21,44. The HOG MAP kinase pathway appears to have conserved roles in vegetative growth and condiation in a subset of filamentous ascomycetes.

Orthologs of ATF1, SKN7 and API transcription factors are known to play different roles in response to various stresses, fungicides resistance, and pathogenicity in different fungi33,46–48. For examples, the Moatf1 mutant has increased sensitivity to H2O246 but deletion of BcaTF1 has no effect on sensitivity to oxidative and hyperosmotic stresses in B. cinerea46. In Cryptococcus neoformans, the Δskn7 mutant is normal in sensitivity to H2O2 but
hypersensitive to NaCl47. Nevertheless, the ΔMrskn7 mutant is not sensitive to both of oxidative and hyperosmotic stresses in Metarhizium robertsii48. In F. graminearum, the Fgatf1, Fgap1, and Fgskn7 mutants all have increased sensitivity to oxidative stress but only FgATF1 and FgSKN7 are important for responses to hyperosmotic stress49. Although the exact functions of UvATF1, UvSKN7, and UvAPI2 are not clear, we found that their expression levels were all reduced over two-folds in the Uvhog1 mutant when treated with 0.5 NaCl, indicating that UvHOG1 may be functionally related to these transcription factors in response to hyperosmotic stress. However, deletion of UvHOG1 had different effects on their expression in response to SDS or H2O2 treatment. Only UvATF1 was significantly reduced in the Uvhog1 mutant in the presence of SDS. In fact, SDS treatment increased the expression of UvSKN7 and UvAPI2 in the mutant. Therefore, UvHOG1 may regulate response to SDS treatment via UvATF1. Interestingly, under oxidative stress, UvSKN7 expression was reduced in the Uvhog1 mutant but increased in the wild type although the changes in its expression level were less than 2-folds. It is likely that UvHOG1 is involved in the up-regulation of UvSKN7 expression in response to oxidative stress in the wild type. Down regulation of UvSKN7 in the Uvhog1 deletion mutant may be related to possible self-regulation of UvSKN7 in response to H2O2 treatment in U. virens.

Ustiloxins are mycotoxins produced by U. virens in false smut balls on rice plants. They can interfere cytoskeleton functions by inhibiting microtubule assembly and induce abnormal swelling of rice seedling roots55. The involvement of the HOG pathway in secondary metabolism has been reported in fungi such as F. graminearum, in which the FgHog1 mutant was reduced in DON production52. In U. virens, the UvUSTA gene encodes the precursor of ustiloxins56 and it had reduced expression level in the Uvhog1 mutant. However, in comparison with the U. virens α-tubulin gene as the internal reference control, the expression level of UvUSTA was low in both the wide type and mutant strains, which may be related to the fact that we failed to detect the production of ustiloxins in both the wild type and mutant strains in repeated attempts. Unfortunately, conditions to stimulate ustiloxin production in vitro remain to be developed and optimized in U. virens. Although the difference in UvUSTA expression between the wild type and mutant was significant in repeated experiments under the experimental conditions used in this study, to determine the regulatory role of the UvHog1 MAPK pathway in ustiloxin biosynthesis, it is necessary to assay the expression level of UvUSTA under better culture conditions that induce ustiloxin production in the future.

The HOG MAPK pathway varies significantly among different fungal pathogens for pathogenicity. In M. oryzae, this pathway is dispensable for plant infection22. However, it is essential for virulence in several plant, insect, and human pathogenic fungi, including M. graminicola, B. cinerea, B. bassiana, M. acridum, C. neoformans, and Candida albicans59,41,44,45,51,52. To determine whether UvHOG1 is important for virulence, we attempted several times to inoculate plants of rice cultivar YA-5A with conidia isolated from the wild-type strain UV-8b, the Uvhog1 mutant, and complemented transformant Cl. Unfortunately, we failed to observe in any of the plants that were inoculated. Because infection assays with the rice false smut fungus are known to be unreliable, we assayed the toxicity of culture filtrates on rice seed germination. Interestingly, culture filtrates of both the wild type and Uvhog1 mutant blocked root growth but only reduced rice shoot growth, suggesting that root growth is more sensitive to the inhibitory compounds present in U. virens culture filtrates than shoot growth. However, culture filtrates of the mutant were less inhibitory to shoot growth than those of the wild type and complemented transformant Cl. It is possible that UvHOG1 plays a role in regulating the production of inhibitory compounds or toxic secondary metabolites in U. virens cultures. It will be important to identify the exact chemical compounds in culture filtrates that are responsible for the inhibitory effects on rice shoot growth. One possibility is that ustiloxins are responsible for the inhibition of rice root and shoot growth by culture filtrates. However, we failed to detect ustiloxins in culture filtrates in repeated tries. Therefore, it is more likely that U. virens produces other secondary metabolites that are inhibitory or toxic to rice.

Methods

Strains and culture conditions. The wild-type strain UV-8b2 and all the transformants of U. virens generated in this study were routinely cultured on potato dextrose agar (PDA), YT (0.1% yeast extract, 0.1% tryptone, and 1% glucose)29, or 5xYE (0.5% yeast extract, 0.5% pepton, and 1% glucose) plates at 25°C. To test sensitivity against different stresses, vegetative growth was assayed after incubation at 25°C for 14 days on regular YT plates and YT with 0.5 M NaCl, 1 M sorbitol, 0.07% H2O2, 0.03% SDS (w/v), or 70 μg/ml Congo red. Conidiation was assayed with 7-day-old liquid YT cultures. Freshly harvested conidia were resuspended to 1 × 106 conidia/ml in YTS (0.1% yeast extract, 0.1% tryptone, and 1% sucrose) with or without 0.3 M NaCl, or 0.03% SDS and assayed for germination after incubation for 12, 16, 18, 20 and 24 h.

Generation of the UvHOG1 gene replacement construct. The 1.07-kb upstream and 1.01-kb downstream flanking sequences of UvHOG1 were amplified with primer pairs 1F/2R and 3F/4R (see Supplementary Table S1 online), respectively. The resulting PCR products were connected to the hygromycin phosphotransferase (hph) fragments amplified with primers HYGF/HYGR by double-joint PCR29. The resulting PCR product was then cloned between the EcoRI and PstI sites on pCDW22 as pCDW23. Plasmid pCDW22 was constructed by digestion of pCAMBIA1300 (CAMBIA, Canberra, Australia) with Xhol and EcoRI, and ligated with a 0.1-kb LacZ fragment amplified with primers LacZF/LacZR (see Supplementary Table S1 online) to eliminate the hph resistance marker on the vector backbone. Plasmid pCDW23 was transformed into A. tumefaciens strain AGL-1 by electroporation.

Transformation of U. virens. ATMT transformation of the wild-type strain UV-8b was performed as described3. For PEG-mediated transformation, conidia were harvested from 10-day-old YT cultures of the wild-type strain UV-8b by filtration through Miracloth53. A total of 106 conidia were inoculated into 100 ml YT medium and incubated for two days at 25°C. Hyphae were harvested by filtration with Miracloth, washed with
1.2 M KCl, and resuspended in driselase solution (15 mg/ml driselase in 1.2 M KCl). After digestion at 30°C for 3 h with gentle shaking (60 rpm), hyphal fragments were removed by filtration and protoplasts were collected by centrifugation for 10 min at 5000 rpm. After washing twice with STC solution (20% sucrose, 50 mM Tris-HCl, 50 mM CaCl₂), protoplasts were resuspended in STC to the final concentration of 10⁶ per milliliter. Plasmid DNA (5 μg) was mixed with 200 μl protoplasts and incubated for 25 min before mixing with 1 ml PTC solution (40% PEG8000 in STC). After incubation for 25 min, the transformation mixture was mixed with 8 ml TB3 liquid medium (3 g yeast extract, 3 g casamino acids, 20% sucrose, 1 liter H₂O) and shaken at 60 rpm overnight at 25°C. After mixing with 50 ml TB3 agar, an aliquot of 12 ml transformation cultures was plated out and overlaid with 15 ml of TB3 with 180 μg/ml of hygromycin B (Calbiochem, La Jolla, CA) for transformant selection.

**Complementation of the Uvhog1 mutant.** The entire UvHOG1 gene containing the coding region and its 1.5-kb promoter and 0.5-kb terminator sequence was amplified with primers CUvHOG1/F and CUvHOG1/R (see Supplementary Table S1 online), digested with EcoRI and PstI, and cloned into the vector pCBDW-GEN. The resulting construct, pCBDW-GEN-UvHOG1, was transformed into A. tumefaciens strain AGL-1 by electroporation. ATMT transformation of the Uvhog1 mutant M1 was performed as described. The resulting Uvhog1/ UvHOG1 transformants were confirmed by PCR.

**qRT-PCR assays.** Vegetative hyphae harvested from two-day-old YT cultures (started with 1 × 10⁶ conidia/ml) were further incubated in regular YT or YT with 0.5 M NaCl, 0.07% H₂O₂, or 0.03% SDS (w/v) for 30 min. RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with the Fermentas 1st CDNA synthesis kit (Hanover, MD). Primers used for qRT-PCR assays were listed in Supplementary Table S1. Relative expression levels of UvAPI, UvATF1, and UvSKN7 were calculated by the 2⁻ΔΔCT method with the U. virens α-tubulin gene as the endogenous reference. Data from three biological replicates were used to calculate the mean and standard deviation.

**Assays for compatible solutes.** Freshly harvested conidia were resuspended to 1 × 10⁶ conidia/ml in YT with sucrose replacing glucose and incubated for 2 days at 25°C. Vegetative hyphae were then harvested and divided into two halves. One half was incubated in regular YT and the other half was incubated in YT with 1.0 M NaCl at 25°C for 1 h. Hyphae were then harvested, rinsed with distilled water, ground in liquid nitrogen, and dried for 24 h in a freeze dryer. Six milligrams of ground hyphae were transferred into a 4 ml auto sampler vial, suspended in 2 ml methanol, and incubated overnight at 25°C. After centrifugation at 4,000 rpm for 10 min, 100 μl of the supernatant was transferred to a conical vial and dried under a gentle nitrogen stream. The content was then re-suspended in 100 μl of 1 M HCl, incubated at 50°C for 1 h, dried under a nitrogen stream as described. The solid extract was re-suspended in 100 μl of TMSI:TMCS (100:1), incubated at 37°C for 1 h, then mixed with 300 μl of isooctane and 300 μl water. After the aqueous and organic layers were completely separated, the top organic phase was analyzed with a GCMS-QP2010 (Shimadzu, Japan) in the scan mode (scanning from m/z 40 to 650). Helium was used as the carrier gas at a constant flow rate of 1 ml/min through an Rxi-5 ms (30 m × 0.25 μm) capillary column (Restek, Bellefonte, USA) with the injector temperature of 260°C and split ratio of 1:5.

**Toxicity assays with culture filtrates.** Hyphae were collected by filtration through two layers of Miracloth (Calbiochem, La Jolla CA, USA) from 5-day-old YT cultures of the wild type and complemented transformant C1 and 7-day-old liquid YT culture of the Uvhog1 mutant and measured for dry weights after dehydration in a freezer dryer for 18 h. Culture filtrates were then centrifuged at 7,000 rpm for 5 min to collect the supernatants. Seeds of rice cultivar YA-5A were incubated on filter papers soaked with the resulting culture filtrates at 25°C under 14 h light/10 h dark. Shoot and root growth were measured after incubation for 5 days.

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**Author Contributions**
D.Z., Y.W. and Y.H. performed the experiments and data analysis. J.X. was involved in experimental designs and manuscript preparation. C.W. designed the experiments, participated in data analysis, and wrote the manuscript. All authors read and approved the final manuscript.

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