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Brief Communication

Engineering false smut resistance rice via host-induced gene silencing of two chitin synthase genes of *Ustilaginoidea virens*

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Rice false smut (RFS), caused by the pathogenic ascomycete fungus *Ustilaginoidea virens* (*U. virens*), is one of the severely devastating diseases worldwide (Sun *et al*., 2020; Zhang *et al*., 2014). *U. virens* infects rice flowers causing over 25% yield loss, also producing poisonous mycotoxins that threaten the health of humans and animals (Fan *et al*., 2016; Zhang *et al*., 2014). So far, rice varieties showing resistance to RFS are rarely mined and none resistant genes are genetically confirmed (Zhang *et al*., 2019). Host-induced gene silencing (HIGS) is a powerful strategy to control diseases, where small interference RNAs (siRNAs) are produced by plants to silence essential genes of pathogens. Application of HIGS to control fungal diseases is successfully reported in several crops (Hou and Ma, 2020). Here, we evaluated the potential of HIGS for engineering rice against RFS by silencing chitin synthase genes of *U. virens*.

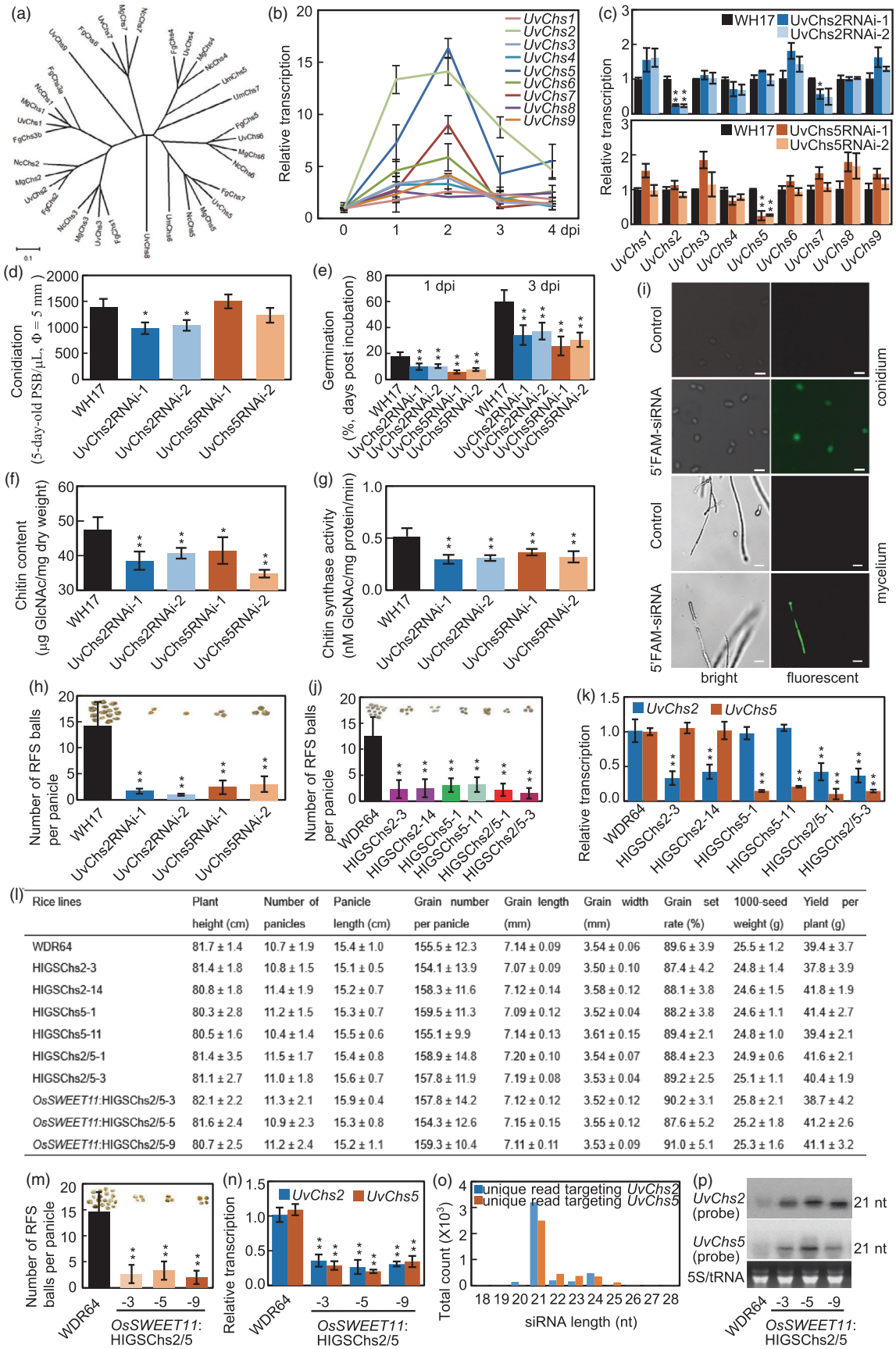
Chitin synthases are key enzymes controlling the formation of chitin, which is a major component of fungal cell wall and plays vital roles in hyphal growth, fungal morphogenesis and pathogenesis (Lenardon *et al*., 2010). The *U. virens* genome contains nine chitin synthase genes Uv8b_7958, Uv8b_3908, Uv8b_7948, Uv8b_4757, Uv8b_3222, Uv8b_3223, Uv8b_3210, Uv8b_7677 and Uv8b_1761 (Zhang *et al*., 2014), which are named as *UvChs1*-*UvChs9* (Figure 1a). These *UvChs* genes had differential expression patterns during the colonization of *U. virens* on rice spikelets. *UvChs2* and *UvChs5* were significantly activated, whereas others were slightly activated upon *U. virens* infection (Figure 1b), indicating that *UvChs2* and *UvChs5* had possibly major roles in the process of *U. virens* infection.

To verify the potential roles of *UvChs2* and *UvChs5* on chitin content and pathogenesis of *U. virens*, we selected a 598-bp partial *UvChs2* coding region (1192- to 1789-bp of its cDNA) and a 527-bp partial *UvChs5* coding region (2043- to 2569-bp of its cDNA), which were highly specific to *UvChs2* or *UvChs5* with no consecutive nucleotide sequences matching rice and human genomes, to generate RNAi constructs driven by the promoter of *U. virens β-actin* gene. The constructs were introduced into *U. virens* isolate WH17, each two transgenic *U. virens* strains designating as UvChs2RNAi and UvChs5 RNAi were selected for analysis. *UvChs2* and *UvChs5* had significantly reduced transcripts, while other *UvChs* genes had unchanged expressions in the transgenic *U. virens* strains compared with non-transgenic WH17 (Figure 1c), suggesting the RNAi constructs are able to effectively and specifically silence the target genes in *U. virens*. In comparison with WH17, conidiation was reduced in UvChs2RNAi but not in UvChs5RNAi *U. virens* (Figure 1d). Both UvChs2RNAi and UvChs5RNAi *U. virens* had significantly reduced conidium germination (Figure 1e), decreased chitin accumulation (Figure 1f) and reduced chitin synthase activity (Figure 1g). Additionally, we assessed their pathogenicity on rice spikelets by inoculation them on susceptible rice WDR64 panicles. The disease symptoms consisting of yellow smut balls were markedly compromised in panicles infected with UvChs2RNAi or UvChs5RNAi *U. virens* than WH17 (Figure 1h). Thus, *UvChs2* and *UvChs5* are essential for pathogenicity of *U. virens*.

To investigate that siRNA molecules from outside can enter into *U. virens* cells, commercial non- *U. virens* fluorescein (FAM)-labelled siRNAs (21 nt) were incubated with conidial spores of *U. virens* and assayed with a fluorescence microscope. The results showed that clearly observable fluorescence in the conidium and mycelium treated with FAM-siRNA (Figure 1i), indicating that exogenous siRNAs can actively migrate into *U. virens* cells.

Next, these two RNAi constructs, and a chimeric RNAi construct that simultaneously silence *UvChs2* and *UvChs5*, driven by 35S promoter, were introduced into WDR64 by *Agrobacterium*-mediated transformation to generate transgenic plants (HIGSChs2, HIGSChs5 or HIGSChs2/5). After inoculation with *U. virens* WH17, WDR64 had about 13 RFS balls per panicle, whereas these transgenic lines showed dramatically attenuated disease symptoms with 2–4 RFS balls per panicle (Figure 1j). Consistent with enhanced resistance, transcripts of *UvChs2* and *UvChs5* were markedly reduced in the transgenic rice than in WDR64 (Figure 1k), suggesting RFS resistance in these transgenic lines was caused by *in planta*-derived silencing of the target genes. Notably, these transgenic lines maintained agronomic traits that were indistinguishable from those of wild type (Figure 1l).

Additionally, we transferred the chimeric *UvChs2* and *UvChs5* RNAi cassette driven by the promoter of *OsSWEET11* which is highly expressed in spikelet and dramatically activated by *U. virens* infection (Chu *et al*., 2006; Fan *et al*., 2020), into WDR64 to generate *OsSWEET11*:HIGSChs2/5 transgenic lines.



Rice lines	Plant height (cm)	Number of panicles	Panicle length (cm)	Grain number per panicle	Grain length (mm)	Grain width (mm)	Grain set rate (%)	1000-seed weight (g)	Yield per plant (g)
WDR64	81.7 ± 1.4	10.7 ± 1.9	15.4 ± 1.0	155.5 ± 12.3	7.14 ± 0.09	3.54 ± 0.06	89.6 ± 3.9	25.5 ± 1.2	39.4 ± 3.7
HIGSChs2-3	81.4 ± 1.8	10.8 ± 1.5	15.1 ± 0.5	154.1 ± 13.9	7.07 ± 0.09	3.50 ± 0.10	87.4 ± 4.2	24.8 ± 1.4	37.8 ± 3.9
HIGSChs2-14	80.8 ± 1.8	11.4 ± 1.9	15.2 ± 0.7	158.3 ± 11.6	7.12 ± 0.14	3.58 ± 0.12	88.1 ± 3.8	24.6 ± 1.5	41.8 ± 1.9
HIGSChs5-1	80.3 ± 2.8	11.2 ± 1.5	15.3 ± 0.7	159.5 ± 11.3	7.09 ± 0.12	3.52 ± 0.04	88.2 ± 3.8	24.6 ± 1.1	41.4 ± 2.7
HIGSChs5-11	80.5 ± 1.6	10.4 ± 1.4	15.5 ± 0.6	155.1 ± 9.9	7.14 ± 0.13	3.61 ± 0.15	89.4 ± 2.1	24.8 ± 1.0	39.4 ± 2.1
HIGSChs2/5-1	81.4 ± 3.5	11.5 ± 1.7	15.4 ± 0.8	158.9 ± 14.8	7.20 ± 0.10	3.54 ± 0.07	88.4 ± 2.3	24.9 ± 0.6	41.6 ± 2.1
HIGSChs2/5-3	81.1 ± 2.7	11.0 ± 1.8	15.6 ± 0.7	157.8 ± 11.9	7.19 ± 0.08	3.53 ± 0.04	89.2 ± 2.5	25.1 ± 1.1	40.4 ± 1.9
OsSWEET11:HIGSChs2/5-3	82.1 ± 2.2	11.3 ± 2.1	15.9 ± 0.4	157.8 ± 14.2	7.12 ± 0.12	3.52 ± 0.12	90.2 ± 3.1	25.8 ± 2.1	38.7 ± 4.2
OsSWEET11:HIGSChs2/5-5	81.6 ± 2.4	10.9 ± 2.3	15.3 ± 0.8	154.3 ± 12.6	7.15 ± 0.15	3.55 ± 0.12	87.6 ± 5.2	25.2 ± 1.8	41.2 ± 2.6
OsSWEET11:HIGSChs2/5-9	80.7 ± 2.5	11.2 ± 2.4	15.2 ± 1.1	159.3 ± 10.4	7.11 ± 0.11	3.53 ± 0.09	91.0 ± 5.1	25.3 ± 1.6	41.1 ± 3.2

Figure 1 Generation of strong resistance to RFS via HIGS-mediated silencing two chitin synthase genes. (a) Phylogenetic tree of fungal chitin synthase. (b) Transcription patterns of nine *UvChs* genes after *U. virens* infection. dpi, days post infection. (c) Transcripts of *UvChs* genes in UvChs2RNAi and UvChs5RNAi *U. virens* strains. (d–h) Comparison of number of conidiation (d), conidiation germination (e), chitin content (f), chitin synthase activity (g) and number of RFS balls per panicle (h) between non-transgenic WH17 and UvChs2RNAi or UvChs5RNAi *U. virens* strains. (i) Microscopic assay of siRNAs migrating into *U. virens* cells. (j) Comparison of number of RFS balls per panicle between wild-type WDR64 and HIGSChs2, HIGSChs5 or HIGSChs2/5 transgenic lines. (k) Transcripts of *UvChs2* and *UvChs5* in spikelets of HIGSChs2, HIGSChs5 or HIGSChs2/5 transgenic lines after *U. virens* infection. (l) Agronomic traits of wild-type WDR64 and HIGSChs2, HIGSChs5, HIGSChs2/5 or *OsSWEET11*:HIGSChs2/5 transgenic lines. (m) Comparison of number of RFS balls per panicle between wild-type WDR64 and *OsSWEET11*:HIGSChs2/5 transgenic lines. (n) Transcripts of *UvChs2* and *UvChs5* in spikelets of *OsSWEET11*:HIGSChs2/5 transgenic lines after *U. virens* infection. (o) Length distribution and abundance of siRNAs targeting *UvChs2* and *UvChs5* in *OsSWEET11*:HIGSChs2/5 transgenic lines. (p) Accumulation of siRNAs in *OsSWEET11*:HIGSChs2/5 lines by polyacrylamide gel electrophoresis-northern assay. Data represent mean ± SD. Asterisks indicate significant difference determined by two-tailed Student's *t*-test (**$P < 0.01$ or *$P < 0.05$).

Similarly, the lines displayed significantly lower transcripts of *UvChs2* and *UvChs5* and dramatically compromised disease symptoms than in WDR64 after *U. virens* infection (Figure 1m, n), whereas unchanged agronomic traits (Figure 1l). To confirm that silencing of *UvChs2* and *UvChs5* in infected *U. virens* was mediated by homologous siRNAs generated in transgenic rice spikelets, small RNA sequencing was carried out to identify siRNAs specific to the RNAi cassette in *OsSWEET11*:HIGSChs2/5 transgenic lines. The sequencing data showed that the unique small RNA reads mapping to *UvChs2* and *UvChs5* genes were 3.25% and 2.07% of the total small RNAs detected respectively. The lengths of siRNAs mapped to *UvChs2* and *UvChs5* were distributed between 18 and 28 bp, wherein 21 bp was the most abundant (Figure 1o). An ideal accumulation of 21-nt siRNA in *OsSWEET11*:HIGSChs2/5 transgenic lines but not in wild type was observed by polyacrylamide gel electrophoresis-northern assay (Figure 1p). These data indicate that the RNAi constructs were successfully processed into specific siRNA molecules in transgenic rice, and these siRNAs were translocated to fungal cells upon *U. virens* infection, thereby suppressing the expressions of *UvChs2* and *UvChs5* to attenuate pathogenicity of *U. virens*.

In summary, our results suggest that HIGS targeting chitin synthase genes involved in chitin synthesis and pathogenicity of *U. virens* is effective and can be used as an alternative strategy for developing RFS resistant rice.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

X.L., R.H. and M.Y. conceived the project. X.L., R.H., J.L., G.X. and M.Y. performed the research and analysed the data. M.Y. wrote the manuscript.

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