

Gibberella zeae chitin synthase genes, *GzCHS5* and *GzCHS7*, are required for hyphal growth, perithecia formation, and pathogenicity

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Abstract *Gibberella zeae* causes Fusarium head blight of cereal crops, and sexual spores of the fungus play an important role as primary inocula. We isolated a restriction enzyme-mediated integration (REMI) transformant, ZH431, of *G. zeae* with defects in perithecia formation and virulence. Integration of the REMI vector resulted in disruption of *GzCHS7* gene, which encodes a putative class VII chitin synthase with high similarity to *Fusarium oxysporum* ChsVb. A second chitin synthase, *GzCHS5*, is adjacently located in a head-to-head configuration with *GzCHS7*, and its deduced protein sequence showed similarity with a class V chitin synthase in *F. oxysporum*. Neither $\Delta GzChs5$ nor $\Delta GzChs7$ mutants produced perithecia or caused disease on barley heads. Microscopic observation

revealed that both mutants formed balloon-shaped hyphae and intrahyphal hyphae and that cell wall rigidity of the mutants was weaker than that of the wild-type strain. Transcription profiles of *GzCHS5* and *GzCHS7* were not altered in $\Delta GzChs7$ and $\Delta GzChs5$, respectively, suggesting that transcription regulations of the genes are independent of each other. Our results demonstrate that *GzCHS5* and *GzCHS7* are indispensable for perithecia formation and pathogenicity as well as normal septa formation and hyphal growth in *G. zeae*.

Keywords Balloon-shaped hyphae · Cell wall rigidity · *Fusarium graminearum* · Intrahyphal hyphae · Septa formation

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Introduction

Gibberella zeae (anamorph: *Fusarium graminearum*) is a filamentous ascomycete fungus that causes Fusarium head blight (FHB) of cereal crops and Fusarium ear and stalk rot of maize in many regions of the world (Leslie and Summerell 2006). The fungus produces mycotoxins such as trichothecene and zearalenone that pose serious threats to human and animal health (Desjardins 2006).

Sexual reproduction in *G. zeae* is an important factor for survival under field conditions and helps maintain a genotypically diverse population structure. Sexual spores (ascospores) of *G. zeae* overwinter within a fruiting body (perithecium) on plant debris, and may serve as the primary inoculum for initiating disease epidemics (Sutton 1982; Trail et al. 2002). The osmotic pressure generated by concentrating potassium ions and chloride ions within the asci triggers forcible discharge of ascospores and initiates the transfer of ascospores from plant debris to cereal flowers

(Trail et al. 2005). Thus, studies of sexual developments in *G. zeae* have gained attraction because certain genes responsible for sexual reproduction in *G. zeae* are closely related to disease development (Han et al. 2007; Hou et al. 2002; Lee et al. 2009; Shim et al. 2006; Urban et al. 2003; Yu et al. 2008).

Fungal cell walls are the outermost layer of fungal cells against adverse environmental conditions. Chitin, a β -1,4-linked polysaccharide of *N*-acetylglucosamine, is a key structural component of the fungal cell wall (Bartnicki-Garcia 1968). Chitin also cross-links other cell wall components and plays an important role in mycelial growth and polarity maintenance in filamentous fungi (Takeshita et al. 2002).

Fungal chitin synthases have been divided into at least seven classes based on the structural properties (Mandel et al. 2006). Myosin motor-like chitin synthases, which are unique to filamentous fungi, are important for the maintenance of cell wall integrity and host infection (Liu et al. 2004; Madrid et al. 2003; Weber et al. 2006). These class V and class VII chitin synthases are characterized by the presence of two conserved domains: an N-terminal myosin motor-like domain (MMD) and a C-terminal chitin synthase domain (Martín-Urdíroz et al. 2008; Nino-Vega et al. 2004; Takeshita et al. 2006). The characteristics differentiating these two classes of chitin synthases are the length of MMD and the presence of ATP-binding motifs, i.e., P-loop, Switch I, and Switch II motifs (Nino-Vega et al. 2004; Takeshita et al. 2006).

One of our strategies to isolate genes associated with sexual development and virulence in *G. zeae* is the use of random insertional mutagenesis. We have generated over 20,000 mutants from a representative strain of *G. zeae*, GZ03643, by restriction enzyme-mediated integration (REMI) strategy (Han et al. 2004). REMI has been used successfully to identify novel genes associated with virulence not only in *G. zeae* but also in other filamentous fungi (Dufresne et al. 2000; Han et al. 2004; Inoue et al. 2002; Lu et al. 1994). During our REMI screen, we isolated a transformant (ZH431) with aberrant hyphal growth and perithecia formation, and significantly reduced virulence. We determined that REMI vector was inserted into a locus that encodes a chitin synthase (*GzCHS7*), and subsequently we learned that another chitin synthase gene (*GzCHS5*) is located adjacent to *GzCHS7* in a head-to-head configuration. The objective of this study was to functionally characterize the two chitin synthase genes, *GzCHS5* and *GzCHS7*. We demonstrate that these two chitin synthase genes, one class V and the other class VII, play an important role in sexual reproduction and pathogenesis in *G. zeae*. Furthermore, we show that *GzCHS5* and *GzCHS7* are required for normal hyphal tip growth and septum formation.

Materials and methods

Strains, culture condition, and plasmids

Gibberella zeae strain GZ03643, a zearalenone and deoxynivalenol producer, was obtained from Dr Robert L. Bowden (US Department of Agriculture, Manhattan, KS, USA) and was used as the wild-type strain. The mutant strain ZH431 was generated from GZ03643 by using REMI mutagenesis, as previously described (Han et al. 2004). Transgenic *G. zeae* strain, T39 Δ M1-3, which carries *mat1-1* deletion and thus self-sterile, was used as the female test strain for outcross analysis (Lee et al. 2003). For sexual crosses, carrot agar was used as previously described (Leslie and Summerell 2006). All strains were stored at -80°C in 25% glycerol. For DNA extraction, strains were grown in 30 ml of complete medium (CM) (Leslie and Summerell 2006) for 3 days at 25°C with shaking (150 rpm). For RNA extraction, fungi were grown in 50 ml CM at 25°C with shaking (150 rpm). To determine the effect of osmotic stabilizers, strains were grown on minimal medium supplemented with 1 M sorbitol, 1 M mannitol, 0.7 M NaCl, or 0.7 M KCl as osmotic stabilizer. Recombinant plasmids, pUCH1, and pII99 (Namiki et al. 2001) carrying the gene for resistance to hygromycin B (*hygB*) and geneticin (*gen*), respectively, were used as selection needs.

Nucleic acid manipulations, plasmid rescue, PCR primers, and sequencing

Fungal genomic DNA was extracted by using acetyltrimethyl ammonium bromide procedure (Leslie and Summerell 2006). Total RNA from *G. zeae* mycelia was prepared with TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) following the manufacturer's instructions. Plasmid DNA of recombinant *E. coli* strain was isolated using a NucleoGen plasmid purification kit (NucleoGen Biotech, Siheung, Korea). Restriction endonuclease digestion, gel electrophoresis, gel blotting, ligation, Southern, and northern hybridization with ^{32}P -labeled probes were performed following standard techniques (Sambrook and Russell 2001). PCR primers (Table 1) were suspended in sterile water to achieve 100 μM final concentration and stored at -20°C . DNA sequencing was performed at National Instrumentation Center for Environmental Management (Seoul National University, Seoul, Korea). The primers described by Han et al. (2004) were used to sequence the rescued plasmid from the mutant strain ZH431, and the sequence obtained was compared against the *Fusarium* Comparative Database at Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html) and the GenBank at National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) by BLAST algorithm.

Table 1 Primers used in this study

Primer	Sequence (5'–3')
CHS5-5f	CCTTTGGTTATAGCATCAACAA
CHS5-5r	ACCTCCACTAGCTCCAGCCAAGCATAACAGACAAGCCAACAAG
CHS5-3f	TATGAAAATTCCGTCACCAGCCACTGCATAAGAATCCATTGAA
CHS5-3r	GTACTACACCGAGATCACATGG
NCHS5-5	AGACGCGATTGAGACTTATTTTAGA
NCHS5-3	CACTCGTCTTCTGCGTCTACTCTA
SNCHS5-3	CTATCTATTAGACAGCCTGCGCACACCCT
CHS7-5f	CTGAGCGAGGGTAAGGGTGCAAGTAGACAA
CHS7-5r	TATGAAAATTCCGTCACCAGCCCCGAAAGAGGAACGACAGAC
CHS7-3f	ACCTCCACTAGCTCCAGCCAAGCATAACTTGTGGTGGGT
CHS7-3r	GCTACTATCGAGAACAGGT
NCHS7-5	GAATGAGATGGAAGTGCGGCAGAATGTAAT
NCHS7-3	CAAGTCCATCGGCAGAGAACAAACAAAG
CCHS7-5r	AGTCCGTGTGGGGCCCCACTGCATAAGAATCCATTGAA
CCHS7-3f	TTCATACCACACCTGCCACCATCCATCGAGGGCATTCACTT
ICL-f	GGGGCCCCACACGGACT
ICL-r	ATGGTGGGCAGGTGTGGTATGAA
Gen-f	CTTGGCTGGAGCTAGTGGAGGT
Gen-r	GGCTGGTGACGGAATTTTCATA

Vector constructions and fungal transformation

Gene deletion constructs were generated by double-joint PCR method (Yu et al. 2004). For targeted deletion of *GzCHS5* and *GzCHS7*, DNA fragments of 5' and 3' flanking regions of *GzCHS5* and *GzCHS7* were amplified from the wild-type GZ03643 genomic DNA using primer pairs CHS5-5f/CHS5-5r and CHS5-3f/CHS5-3r for *GzCHS5*, and CHS7-5f/CHS7-5r and CHS7-3f/CHS7-3r for *GzCHS7* (Fig. 1a, b). The primers used in this study were listed in Table 1. Simultaneously, a 1.6-kb fragment of geneticin resistance gene (*gen*) cassette was amplified from plasmid pII99 using the primers Gen-f and Gen-r. The 5' and 3' flanking regions of each gene were mixed with *gen* amplicon in a 1:2:1 molar ratio (5' fragment: *gen*: 3' fragment) and joined by PCR without any primers. Subsequently, the

6.0- and 3.5-kb PCR products harboring the *gen* marker fused to the flanking regions of *GzCHS5* and *GzCHS7* were amplified with primer pairs NCHS5-5/NCHS5-3 and NCHS7-5/NCHS7-3, respectively. For $\Delta GzChs5/7$ double mutant, the 3' flanking regions of *GzCHS5* and *GzCHS7* amplified above were fused with *gen* as described above (Fig. 1c). Nested primers SNCHS5-3 and NCHS7-3 were used to amplify the 3.6-kb amplicon carrying the *gen* marker fused to the 3' flanking regions of *GzCHS5* and *GzCHS7*, and the construct was transformed into GZ03643 to generate a double deletion of the *GzCHS5* and *GzCHS7* genes. We isolated two gene replacement mutant strains of $\Delta GzChs7$, three of $\Delta GzChs5$, and three of $\Delta GzChs5/7$ in this study.

For overexpression of *GzCHS7* gene in the $\Delta GzChs5/7$ double-deletion mutant, the entire *GzCHS7* gene including

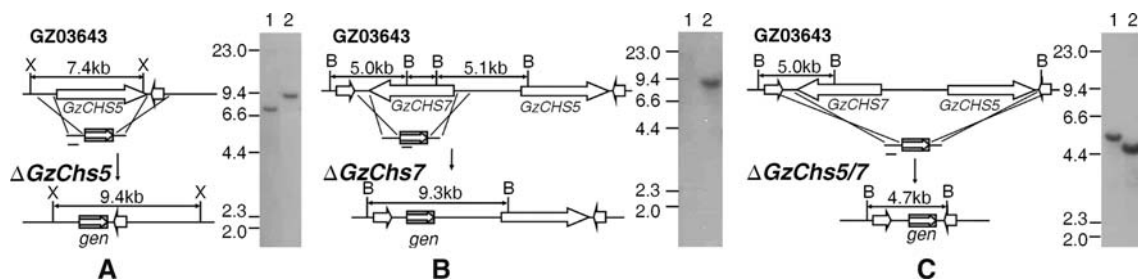


Fig. 1 Targeted gene deletion of *GzCHS5* (a), *GzCHS7* (b), and both *GzCHS5* and *GzCHS7* (c) in the wild-type *G. zeae* GZ03643 strain. Restriction enzymes used in this experiment are *Xba*I (X) and *Bgl*II (B). In Southern blots, lane 1 is GZ03643, and lane 2 is the mutant that

the target gene was replaced with geneticin-resistant gene (*gen*). The probe used for each hybridization is indicated by a bar. The sizes of the DNA standards (in kilobases) are indicated on the left of the blot

the native terminator was amplified, and the 3' flanking region of *GzCHS5* was amplified with primers CCHS7-3f and CHS7-3r/CHS5-3r and CCHS7-5f, respectively. The strong constitutive promoter of *Neurospora crassa* isocitrate lyase gene, ICL, was amplified from pIGPAPA (Horwitz et al. 1999) with primers ICL-f and ICL-r. These three amplicons were fused after purification with same nested primer pair used to construct the double-deletion construct by using a double-joint PCR method (Yu et al. 2004), and the final construct was transformed into the $\Delta GzChs5/7$ double-deletion mutant by co-transformation with pUCH1.

Sexual reproduction and virulence assays

Sexual crosses were performed as previously described (Lee et al. 2003). For self-fertilization, GZ03643 strain was inoculated on carrot agar and incubated at 25°C for 7 days (Leslie and Summerell 2006). Aerial mycelia were removed by scrubbing with 1 ml of sterile 2.5% Tween60[®] solution (v/v) using a glass rod. The plates were incubated for additional 10 days at 25°C under the mixture of fluorescent cool white and black lights with a 12-h photoperiod.

For outcrossing ZH431, a mycelia agar block of T39 Δ M1-3 was placed on a carrot agar plate and incubated at 25°C for 7 days. A conidial suspension (1×10^5 conidia ml⁻¹) of ZH431 was applied to the mycelia of T39 Δ M1-3 strain that was established on carrot agar as described above. The plates were incubated for an additional 10–14 days under the same conditions as the self-fertilized plates.

Fungal strains were incubated in CMC liquid medium (Capellini and Peterson 1965) at 25°C with shaking (150 rpm) for 5 days. Macroconidia of each isolate were grown in CMC and then harvested and re-suspended in sterile water (1×10^6 spores ml⁻¹). The spore suspension was sprayed on barley heads at early anthesis. Inoculated plants were incubated in a growth chamber at 25°C with 100% humidity for 2 days and then transferred to a greenhouse. FHB symptoms were observed 10 days after inoculation.

Microscopy

For scanning electron microscope (SEM), mycelial plugs from PDA plates were immersed at 4°C overnight in modified Karnovsky's fixation buffer (2% paraformaldehyde and 2% glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2) and washed with the same buffer three times each for 10 min. Post-fixation with 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) was performed at 4°C for 2 h, and the samples were washed twice with distilled water. The fixed samples were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, and 100%, and three times in

100% each for 10 min) and then transferred to 100% isoamyl acetate. The samples were dried with liquid carbon dioxide and then mounted on metal stubs. Samples were coated with gold and observed with a SEM JSM 5410LV (JEOL Ltd, Tokyo, Japan).

For transmission electron microscopy (TEM), the fixation protocol was the same as described for SEM. After post-fixation, the samples were en bloc stained with 0.5% uranyl acetate at 4°C for overnight, were dehydrated in a graded ethanol series. The samples were treated twice with 100% propylene oxide for 10 min and were embedded in Spurr's resin and sectioned using ultramicrotome MT-X (RMC, Tucson, AZ, USA). The specimens were stained with 2% uranyl acetate and Reynolds' lead citrate each for 7 min, and then observed with a TEM JEM-1010 (JEOL Ltd).

For atomic force microscopy (AFM), autoclaved cover glasses (each 18 mm \times 18 mm) with growing hyphae on the surface were collected from cultures growing on PDA plates. The samples were fixed to a steel disc (18 mm in diameter) with the hyphae upward using two-sided adhesive tape. The steel disc was magnetically mounted on a piezoscanner (maximum xy-scan range of 5 μ m) of an AFM (AutoProbe-CP; Park Scientific Instruments, Sunnyvale, CA, USA). Force modulation microscopy was used to acquire elastic images under ambient conditions by using V-shaped silicon cantilevers with a force constant of 0.26 N/m. The microscope was operated by applying forces of 10.2 nN to the specimen surface with optimized feedback parameters at a scan frequency of 1 Hz. Elastic images over $1 \times 1 \mu\text{m}^2$ scan areas were recorded from four hyphae on the cover glass of each strain. Based on the amplitude of cantilever modulation, the z-axis values of elastic images represent the hardness of the specimen surface (Li et al. 1998). The mean height (voltage in the z-axis) values of each elastic image were derived by using a software package (ProScan version 1.51b; Park Scientific Instruments) and processed to compare the arithmetic mean values of the four mean height values between strains.

Results

Genetic analysis of the REMI mutant ZH431

A total of 3,912 REMI transformants were screened for defects in perithecia formation and FHB virulence. We isolated one transformant, designated ZH431, that failed to produce perithecia on carrot agar. ZH431 and GZ03643 also differed dramatically in other phenotypes including hyphal growth, pigmentation, and virulence. The mutant grew slower and produced less aerial mycelia than did GZ03643 on both PDA and carrot agar. Also, ZH431

produced a yellow–orange pigment absent in GZ03643. ZH431 showed drastically reduced virulence; while the wild-type progenitor caused severe FHB symptoms 7 days after inoculation, ZH431 developed few small necrotic spots on spikelets of barley heads 10 days after inoculation.

To determine if the phenotypic changes in ZH431 were caused by the insertion of REMI vector, we crossed ZH431 with the heterothallic strain, T39 Δ M1-3, carrying a *mat1-1* deletion (Lee et al. 2003). One hundred random ascospores from the outcross segregated into parental phenotypes at 53:47 that fits a 1:1 ratio ($\chi^2 = 0.36$), and all hygromycin-resistant progeny had the ZH431 phenotypes. This result suggested that the mutation in ZH431 occurred at a single locus and that this mutation was tightly linked to or resulted directly from the REMI vector insertion.

To confirm the insertion of REMI vector in ZH431, a Southern analysis was performed. ZH431 genomic DNA was digested with *Hind*III and *Bgl*II, separated by electrophoresis in an agarose gel, and transferred to a nylon membrane. We probed the blot with REMI vector pUCH1 (5.2 kb) and observed a single band, which indicated the integration of a single copy of pUCH1 into the ZH431 genome (data not shown).

Molecular characterization of disrupted loci in ZH431

We hybridized ³²P-labeled pUCH1 to the *Bgl*II-digested ZH431 genomic DNA blot and identified a single 7.2-kb band, which is expected to contain *G. zeae* genomic DNA flanking pUCH1. The DNA fragment, designated pZH431B, was rescued and subsequent sequencing revealed that the 5'-flanking region (0.5 kb) and the 3'-flanking region (1.6 kb) correspond to *G. zeae* gene FGSG_12039.3, which is 5,305-bp in length and encodes a 1,558-amino acid polypeptide interrupted by three putative introns. The vector insertion occurred at a *Hind*III site located 2,448-bp downstream of the FGSG_12039.3 translational start site. FGSG_12039.3 share a significant similarity (91%) with *ChsVb*, a *F. oxysporum* f. sp. *lycopersici* class VII chitin synthase (GenBank EF673037.1) that is required for septation (Martín-Urdíroz et al. 2008). The N-terminal MMD in FGSG_12039.3 is approximately 220 amino acids in length and lacks ATP-binding motifs, which are conserved in class VII chitin synthases such as *F. oxysporum* *ChsVb* and *Aspergillus nidulans* *CsmB* (Martín-Urdíroz et al. 2008; Nino-Vega et al. 2004). We identified a second gene 4,350-bp upstream of the FGSG_12039.3 translational start site, located in a head-to-head orientation (Fig. 1). This gene (FGSG_01964.3) encodes a 1,865-aa protein that shares 94% identity with a *F. oxysporum* class V chitin synthase gene (GenBank AF484941.1) (Madrid et al. 2003); the MMD is about 740 amino acids in length and the ATP-binding motifs are highly conserved. This

gene is similar to class V chitin synthases *F. oxysporum* *ChsV* and *A. nidulans* *CsmA*. Based on this similarity and the structural properties, we designated these two putative *G. zeae* chitin synthase genes FGSG_12039.3 and FGSG_01964.3 as *GzCHS7* and *GzCHS5*, respectively.

Targeted deletion of *GzCHS5* and *GzCHS7*

To further characterize the functions of *GzCHS5* and *GzCHS7* in *G. zeae*, we generated *GzCHS5*-deletion (Δ *GzChs5*), *GzCHS7*-deletion (Δ *GzChs7*), and double-deletion (Δ *GzChs5/7*) mutants from wild-type strain GZ03643. A 5.7-kb region containing *GzCHS5* and a 5.3-kb region containing *GzCHS7* were completely replaced with the 1.6-kb geneticin (*gen*) marker via double homologous recombination. Southern analyses confirmed these replacements in the *GzCHS5* and *GzCHS7* loci. *Xba*I-digested genomic DNA of Δ *GzChs5* mutants had a 9.4-kb band instead of the 7.4-kb wild-type band when a 5' PCR fragment of *GzCHS5* was used as a probe (Fig. 1a). *Bgl*II-digested genomic DNA of Δ *GzChs7* mutants contained a 9.3-kb band when the *gen* cassette was used as a probe, confirming that the *GzCHS7* gene was replaced with the marker (Fig. 1b). *Bgl*II-digested genomic DNA of Δ *GzChs5/7* strains had a single 4.7-kb hybridizing band instead of the 5.0-kb wild-type band, suggesting that the 15.3-kb region harboring *GzCHS5* and *GzCHS7* was deleted and replaced with the *gen* marker (Fig. 1c).

Phenotypes of Δ *GzChs5*, Δ *GzChs7*, and Δ *GzChs5/7* strains

All mutants showed phenotype similar to the REMI mutant ZH431. The mycelia growth rate of deletion mutants, Δ *GzChs5*, Δ *GzChs7*, and Δ *GzChs5/7*, was slower than that of GZ03643 on PDA (Fig. 2a). GZ03643 strain produced aerial mycelia when growing on PDA, but very little aerial mycelia was observed in Δ *GzChs5*, *GzChs7*, and Δ *GzChs5/7*. All mutants failed to produce perithecia on carrot agar and showed drastically reduced virulence on barley heads when compared to the wild type (Fig. 2b). Conidia production was also severely reduced in all mutants. When incubated for 7 days GZ03643 produced 2.3×10^6 conidia ml⁻¹ in CMC medium, however, Δ *GzChs5*, Δ *GzChs7*, and Δ *GzChs5/7* averaged 7.8×10^4 , 6.3×10^4 , and 6.0×10^4 conidia ml⁻¹, respectively, suggesting that *GzCHS5* and *GzCHS7* contribute to *G. zeae* conidia production. To determine whether *GzCHS5* and *GzCHS7* can mediate osmotic stability, Δ *GzChs5*, Δ *GzChs7*, and Δ *GzChs5/7* strains were grown on minimal media supplemented with 1.0 M sorbitol, 1.0 M mannitol, 0.7 M NaCl, or 0.7 M KCl as an osmotic stabilizer. Defective mycelial growth observed in Δ *GzChs5*, Δ *GzChs7*, and Δ *GzChs5/7* strains grown on minimal media was partially

Fig. 2 Growth of wild type and deletion mutants on PDA (a) and virulence on barley heads (b). Photographs were taken 5 and 10 days after inoculation on PDA and barley heads, respectively. GZ03643, wild-type *G. zeae* strain; $\Delta GzChs5$, *GzCHS5*-deleted mutant; $\Delta GzChs7$, *GzCHS7*-deleted mutant; $\Delta GzChs5/7$, double-deletion mutant of *GzCHS5* and *GzCHS7*

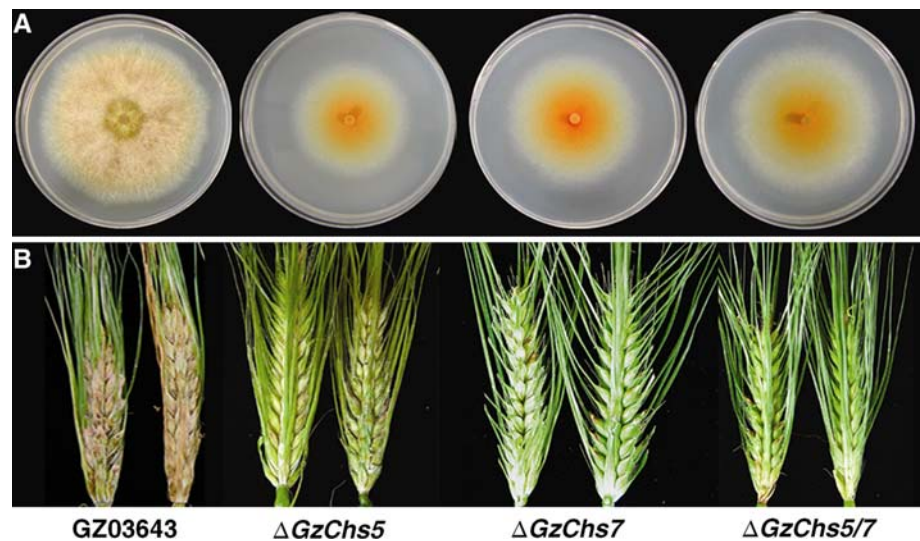
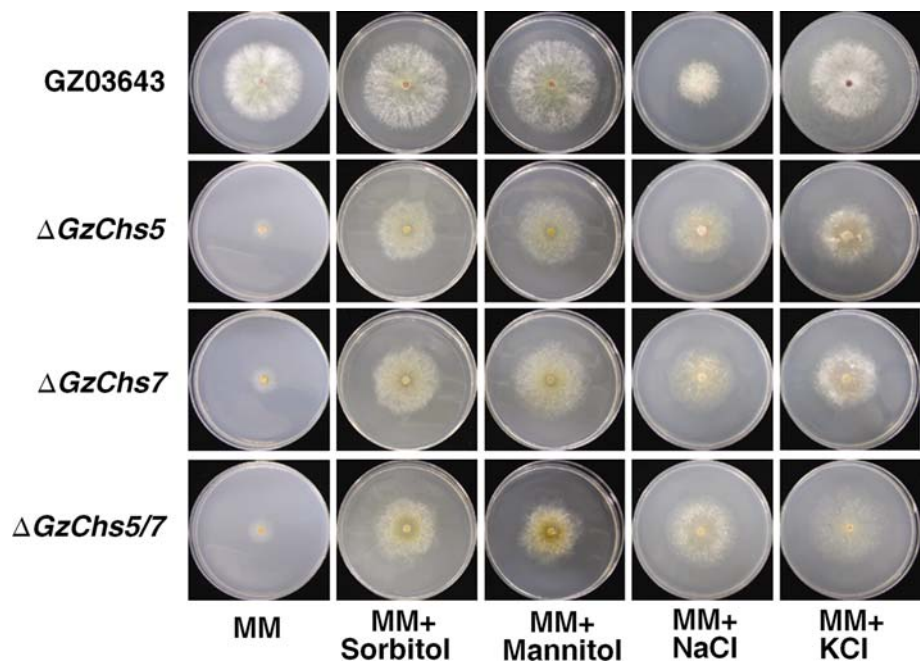


Fig. 3 Growth on minimal medium (MM) supplemented with 1 M sorbitol, 1 M mannitol, 0.7 M NaCl, or 0.7 M KCl as an osmotic stabilizer



restored by the addition of osmotic stabilizers (Fig. 3). In order to examine the effects of mutations on cell wall rigidity, we analyzed the wild type and mutants with an AFM. The cell walls of all three mutants were less rigid than those of the wild-type strain, with the $\Delta GzChs5$ cell wall being more rigid than that of $\Delta GzChs7$ strain (Fig. 4).

Microscopic observation of $\Delta GzChs5$, $\Delta GzChs7$, and $\Delta GzChs5/7$ strains

We investigated whether chitin synthase mutations had deleterious effect on *G. zeae* hyphae and conidia development. Under the light microscope, hyphae of three mutants appeared swollen when grown on PDA. Further analyses by

SEM asserted that mutant hyphae were heavily swollen in contrast to the wild-type strain (Fig. 5). Swollen balloon-shaped hyphae, as well as occasional crumpled balloon shapes, also were observed in the mutant hyphae (Fig. 5). TEM revealed typical hyphae enclosed with an electron-translucent cell wall in the wild-type strain (Fig. 6a). While intrahyphal hyphae can be found in the older mycelia of the wild-type strain (Fig. 6b), they were quite often found in the balloon-shaped hyphae of mutants (Fig. 6c, d). Moreover, one hypha often contained more than one intrahyphal hypha in the mutants (Fig. 6d). Swelling was evident in the mutant hyphae except for the septal region where normal hyphal thickness is likely maintained (Fig. 6e). At a higher magnification, we observed the septal pores in the mutant hyphae plugged by a woronin body-like structure (Fig. 6f).

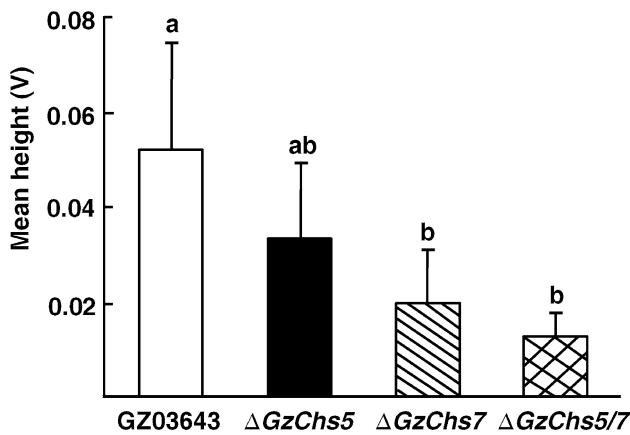


Fig. 4 Comparison of cell wall rigidity of hyphae measured by atomic force microscopy. The y-axis represents mean height (voltage in unit) of elastic images from each fungal specimen by force modulation microscopy. Higher voltages indicate increased hardness. Error bar indicates standard deviation of four biological replicates, and the significance among strains is labeled with *a* and *b*, which are significantly different according to Tukey’s test ($P < 0.05$), on the error bar

Overexpression of GzCHS7 in ΔGzchs5/7

Our original REMI mutation was in *GzCHS7* gene, and we were interested in testing whether the *GzCHS7* overexpression can overcome the double mutant phenotype. The *GzCHS7*-coding region was amplified from GZ03643

strain, fused to a constitutive *N. crassa* ICL promoter, and subsequently transformed into Δ*Gzchs5/7* strain (Fig. 7a). Two out of ten hygromycin-resistant transformants had the construct integrated into the *GzCHS7*-deletion locus by homologous recombination, which was confirmed by Southern analysis (Fig. 7b). However, none of the transformants carrying the *ICLp::GzCHS7* construct could be clearly distinguished morphologically from the Δ*GzChs5/7* mutant (Fig. 7c). In *Cchs7* strain, the transformant with *ICLp::GzCHS7*, *GzCHS7* was expressed at a higher level than that in the wild type, but *GzCHS5* expression was not detected (Fig. 8).

Northern analyses of *GzCHS5* and *GzCHS7* during hyphal growth

GzCHS5 and *GzCHS7* genes are positioned in a head-to-head configuration, which suggests that two genes share a common promoter region. We investigated whether the expression of *GzCHS5* or *GzCHS7* was altered in Δ*GzChs5*, Δ*GzChs7*, and Δ*GzChs5/7* strains. The overall expression levels of *GzCHS5* and *GzCHS7* in Δ*GzChs7* and Δ*GzChs5*, respectively, were similar to those of the corresponding genes in the wild type (Fig. 8). However, while both genes exhibited gradual increase in expression in the wild type, we did notice that *GzCHS5* and *GzCHS7* expressions peaked on day 4 in the mutant strains.

Fig. 5 Scanning electron micrographs of hyphae. The mutants were mainly characterized by balloon-shaped hyphae. Scale bars represent 10 μm

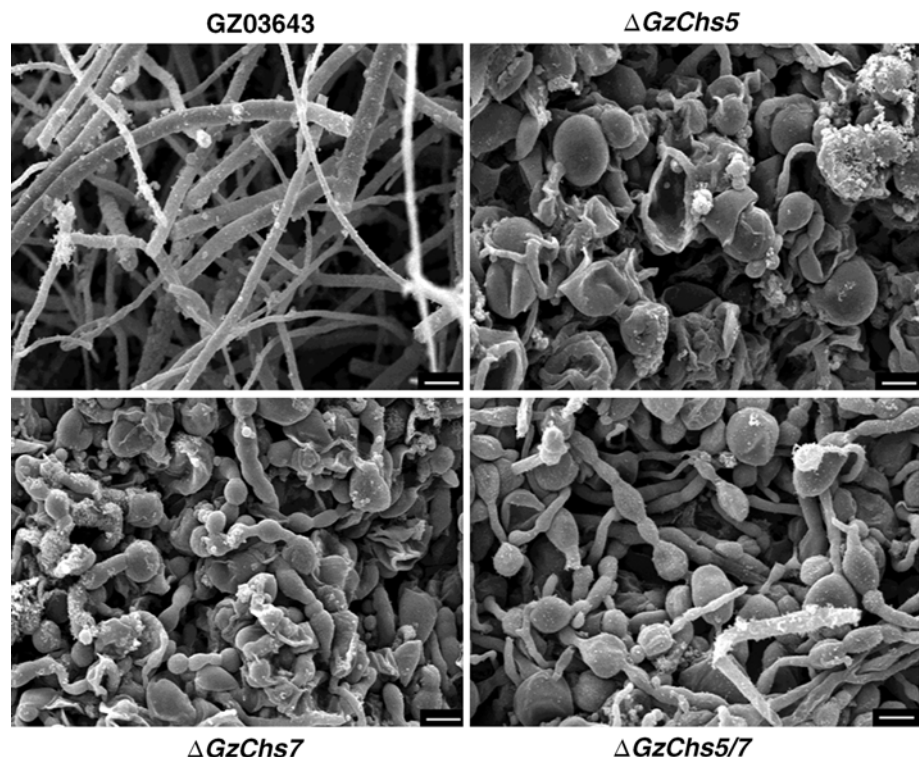


Fig. 6 Transmission electron micrographs of hyphae. **a** Hyphae of the wild-type strain GZ03643. **b** Intrahyphal hyphae (arrow) in old mycelia of GZ03643. **c** Intrahyphal hyphae (arrow) in a balloon-shaped hypha of $\Delta GzChs7$. **d** Three intrahyphal hyphae in mycelia of $\Delta GzChs7$. **e** Hyphae of $\Delta GzChs7$. **f** Woronin body-like organelle (arrowhead) plugging a septal pore in $\Delta GzChs7$. (Scale bars in **a**, **c** and **e** represent 3.0 μm , in **b** and **f** 1.0 μm , and in **d** 2.0 μm)

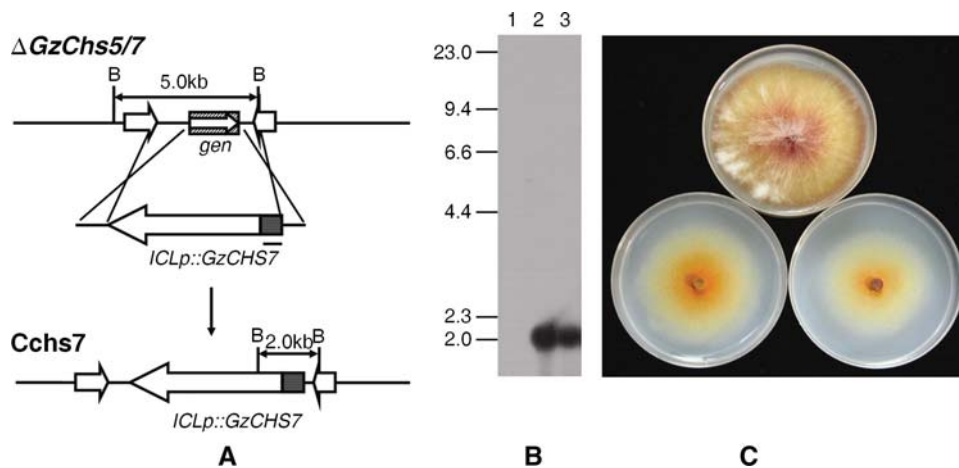
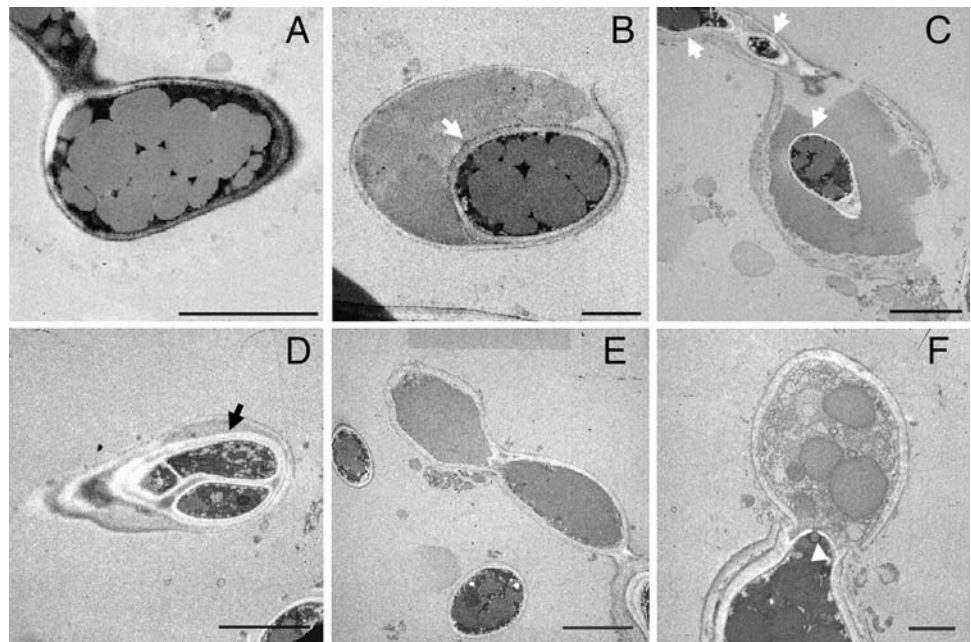


Fig. 7 Overexpression of GzCHS7. **a** Schematic depiction of overexpression construct and homologous recombination strategy to overexpress GzCHS7 in the double mutant $\Delta GzChs5/7$. $GzCHS7$ ORF was fused to the ICL promoter, and the fused construct was transformed into $\Delta GzChs5/7$ to generate $Cchs7$ mutants. Restriction enzyme *Bgl*III site is indicated with a bar. **b** Southern hybridization to confirm the integra-

tion of the fused construct. Lane 1 the wild-type *G. zeae* GZ03643, lanes 2 and 3 independent $Cchs7$ mutants. The sizes of the DNA standards (in kilobases) are indicated on the left of the blot. **c** Growth of $Cchs7$ mutants on PDA. The plate on top is the wild-type GZ03643, the plate on bottom left is $Cchs7$ mutant, and the plate on bottom right is $\Delta GzChs5/7$

Discussion

Chitin comprises 10–20% of filamentous fungal dry weight, and the chitin synthases play critical roles in hyphal development and fungal pathogenicity (Madrid et al. 2003; Martín-Urdíroz et al. 2008; Takeshita et al. 2006; Werner et al. 2007). Fungal chitin synthases have been divided into seven classes based on the structural properties (Mandel et al. 2006). In *A. nidulans*, six different genes designated *chsA*, *chsB*, *chsC*, *chsD*, *csmA*, and *csmB* correspond to classes II, III, I, IV, V, and VI, respectively (Culp et al.

2000; Horiuchi et al. 1999; Motoyama et al. 1994, 1996; Takeshita et al. 2006). In the tomato pathogen *F. oxysporum* f. sp. *lycopersici*, five chitin synthases, designated *CHS1*, *CHS2*, *CHS3*, *CHSV*, and *CHSVb*, have been isolated and characterized (Martín-Urdíroz et al. 2004, 2008). The *F. graminearum* genome database (<http://www.broad.mit.edu/annotation/genome/>) recognizes eight chitin synthases (FGSG_01272.3, FGSG_01949.3, FGSG_01964.3, FGSG_02483.3, FGSG_03418.3, FGSG_10116.3, FGSG_10327.3, and FGSG_12039.3). The predicted amino acid sequence of these genes showed the presence of conserved

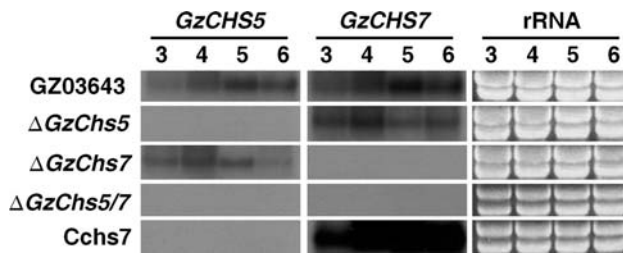


Fig. 8 Transcription profiles of *GzCHS5* and *GzCHS7* on complete media. Numbers above the blot indicate incubation days. Ethidium bromide-stained rRNA is shown as a loading control

chitin synthase 2 (CS2) domain. Furthermore, phylogenetic analysis using the CS2 domain indicated that all eight genes can be assigned to one of the chitin synthase classes (data not shown), suggesting that *G. zeae* chitin synthases are also highly conserved.

Chitin synthases are known to directly associate with hyphal growth and asexual development in filamentous fungi. In *A. nidulans* *chsA*, *chsC*, and *chsD* play a role in conidiation (Ichinomiya et al. 2005; Motoyama et al. 1996). Deletion of *chsB* in particular resulted in hyper branching of hyphae, enlarged tips, and disorganized lateral cell walls. Disruption of *csmA*, the first class V chitin synthase to be identified, resulted in morphological abnormalities such as balloon-like hyphal tips, sparse conidiophores, and abnormal septa formation (Horiuchi et al. 1999). The follow-up study on *A. nidulans* *csmB*, a class VII chitin synthase gene, was determined to have a role in hyphal tip and septa formation (Takeshita et al. 2006). Significantly, in several plant pathogenic fungi, class V and VII chitin synthases have important roles in pathogenicity. *F. oxysporum* *CHSV* and *CHSVb*, class V and class VII chitin synthases, respectively, were directly linked to pathogenicity (Madrid et al. 2003; Martín-Urdíroz et al. 2008). In maize anthracnose fungus *Collectotrichum graminicola*, *ChsV* is essential for appressorium formation thus directly impacting host penetration. Appressoria of *ChsV* deletion mutant were severely distorted and the appressorial cell wall appeared to be disintegrated (Werner et al. 2007).

To date, only *FgCHS1*, a class I chitin synthase gene, has been cloned, but the report was limited to gene sequence analysis and its transcription profile during fungal development (Li et al. 2003). However, our understanding of chitin synthases in *G. zeae*, particularly the functional roles in development and virulence, is limited. In this study, we characterized the functional roles of *GzCHS5* and *GzCHS7* by using gene-deletion mutants. Similar to other filamentous fungi, mutants were viable but showed a number of abnormal hyphal morphologies. Both $\Delta GzChs5$ and $\Delta GzChs7$ mutants had swollen hyphal tips and reduced hyphal growth without apparent aerial hyphae. TEM revealed that intrahyphal hyphae were formed frequently in

the mutants. Similar intrahyphal hyphae were observed in *A. nidulans* *csmA* and *csmB* mutants, suggesting that septum formation and hyphal development were uncoordinated in these mutants (Horiuchi et al. 1999; Takeshita et al. 2006). In some cases, we observed a woronin body-like organelle plugging a septum pore in the swollen mycelia of mutants (Fig. 6f). Woronin bodies are dense-core vesicles that are localized in the close vicinity of septal pores, and when hyphae are damaged, these organelles plug the septal pores rapidly to prevent cytoplasmic content leakage (Asiegbu et al. 2004). While further characterization is needed to unambiguously determine whether these organelles are indeed woronin bodies, we can hypothesize that a class V or class VII chitin synthase deficiency lead to the formation of deviant woronin body or that abnormal phenotypes caused by gene mutations induce the movement of woronin body to septal pores. These observations also suggest that $\Delta GzChs5$ and $\Delta GzChs7$ mutants are under stress, perhaps osmotic, due to the weakened or irregular hyphal tip development, and therefore exhibit aberrant phenotypes such as intrahyphal hyphae and woronin body-plugged septal pores.

When we compared the $\Delta GzChs5$ and $\Delta GzChs7$ mutant phenotypes with other filamentous fungi, we recognized one unique feature in *G. zeae* that was not observed in other fungi. Previous studies indicated fungal chitin synthases are essential for cell wall synthesis and maintenance, conidiation, hyphal tip growth, and septum formation. In *G. zeae*, mutations in *GzCHS5* and *GzCHS7* impacted the ability to produce perithecia. The function of those genes is directly related to the production of female fruiting bodies, perithecia, since the mutants completely lost their self-fertility while maintaining their male fertility. Perithecia consist of several layers of cells (Trail and Common 2000), and in particular this structure may require higher chitin content than do vegetative growth or asexual development. Therefore, it is reasonable to believe that the defect in fertility in the mutants is due to the defect of chitin in perithecia formation rather than defects in fertilization or post-fertilization.

The class V and VII chitin synthases in *G. zeae* are important for proper hyphal development and perhaps specialization. In *A. nidulans*, *CsmA* mutants form abnormal conidiophores and balloon hyphae. Significantly, while chitin synthase genes are redundant in filamentous fungi, functional role of genes from these two classes are not complementary in *G. zeae*. Similar to what we have observed in this study, it is likely that expression of certain class of chitin synthase may not be influenced when either or both *GzCHS5* and *GzCHS7* are deleted in *G. zeae*.

We showed that mutation in *GzCHS5* and *GzCHS7* caused *G. zeae* to be avirulent. In order to establish successful infection, fungal pathogens must overcome highly effective,

constitutive physical and chemical host barriers, and employ a range of different infection strategies. A number of important steps in the infection process are common to fungal pathogens, including adhesion to the surface of the plant, penetration of the plant surface, and acquisition of nutrients from the plant cells (Hardham 2001). After successful penetration, hyphae of pathogenic fungi colonize host cells mechanically by expansion of the growing hyphal tips. Therefore, it is reasonable to hypothesize that opportune fungal cell wall biogenesis and hyphae development are critical for virulence. However, testing this hypothesis has been difficult in *G. zeae* due to the lack of mutant strains and other tools. Our study may provide a new insight into the complex mechanisms associated with early host–*G. zeae* interactions. In addition, chitin synthases characterized in this study may provide opportunities to develop a new chemical for controlling FHB.

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