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Functional Analysis of a Histidine Auxotrophic Mutation in *Gibberella zeae*

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A plant pathogenic fungus, *Gibberella zeae* (anamorph: *Fusarium graminearum*), not only generates economic losses by causing disease on cereal grains, but also leads to severe toxicosis in human and animals through the production of mycotoxins in infected plants. Here, we characterized a histidine auxotrophic mutant of *G zeae*, designated Z43R1092, which was generated using a restriction enzyme-mediated integration (REMI) procedure. The mutant exhibited pleiotropic phenotypic changes, including a reduction in mycelial growth and virulence and loss of sexual reproduction. Outcrossing analysis confirmed that the histidine auxotrophy is linked to the insertional vector in Z43R1092. Molecular analysis showed that the histidine requirement of Z43R1092 is caused by a disruption of an open reading frame, designated *GzHIS7*. The deduced product of *GzHIS7* encodes a putative enzyme with an N-terminal glutamine amidotransferase and a C-terminal cyclase domain, similar to the *Saccharomyces cerevisiae* HIS7 required for histidine biosynthesis. The subsequent gene deletion and complementation analyses confirmed the functions of *GzHIS7* in *G zeae*. This is the first report of the molecular characterization of histidine auxotrophy in *G zeae*, and our results demonstrate that correct histidine biosynthesis is essential for virulence, as well as sexual development, in *G zeae*. In addition, our results could provide a *G zeae* histidine auxotroph as a recipient strain for genetic transformation using this new selectable marker.

Keywords **:** *Gibberella zeae*, glutamine amidotransferase, histidine auxotrophy, REMI

Gibberella zeae (anamorph: *Fusarium graminearum*) has been recognized as an economically important plant pathogen since it was found to cause not only severe epidemics of cereal crop diseases (McMullen et al., 1997), but also serious mycotoxicoses in both humans and animals through the production of mycotoxins in infected crops (Marasas et al., 1984). To develop comprehensive control strategies for plant diseases caused by this homothallic, ascomycetous fungus, many researchers have attempted to identify genetic elements that govern the pathogenicity of *G zeae*. The recent release of a genome sequence assembly for *G zeae* has encouraged functional molecular approaches for the analysis of fungal traits important for disease development. To date, virulence factors such as mycotoxins, cyclic peptides, amino acids, and lipids have been shown to play critical roles in fungal pathogenesis and/or sexual development (Proctor et al., 1995; Lu et al., 2003; Han et al., 2004; Voigt et al., 2005; Oide et al., 2006; Kim et al., 2007). We first examined high-throughput functional genomics approaches using random insertional mutagenesis, i.e., restriction enzyme-mediated integration (REMI), in *G zeae* (Han et al., 2004). Several *G zeae* genes have already been identified using this strategy: genes found to be responsible for auxotrophic mutations have provided valuable information for understanding the biology of *G zeae*, both in terms of basic understanding and practical application (Han et al., 2004; Seong et al., 2005; Kim et al., 2007). Functional characterization of the auxotroph genes clearly demonstrated that fungal primary metabolism such as the methionine, arginine, or adenine biosynthetic pathways are directly connected to the expression of major traits for disease development in *G zeae*. In addition, a combination of an auxotrophic strain and the corresponding gene responsible for the mutation provides a new genetic complementation system for *G zeae* transformation, which is a useful alternative to methods using dominant selectable markers.

We identified and characterized a histidine auxotrophic mutant, designated Z43R1092, whose mutation is tagged to a putative glutamine amidotransferase gene (*GzHIS7*). This strain exhibited reduced virulence and no sexual reproduction. Targeted gene deletion and genetic complementation studies demonstrated that *GzHIS7* is directly responsible for histidine auxotrophy and other pleiotropic phenotypes in the Z43R1092 strain.

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Materials and Methods

Strains, media, and plasmid. *Gibberella zeae* strain Z03643, obtained from Dr. Robert L. Bowden (U.S. Department of Agriculture, Manhattan, KS, USA), was used as the wild-type strain. This strain was not only highly virulent on host plants, but also underwent self-fertile mating. The mutant strain, Z43R1092, was generated from Z03643 using REMI mutagenesis (Han et al., 2004). A *mat1-1*-deleted self-sterile strain, T39ΔM1-3, derived from the *G. zeae* strain Z03639, was used as the female test strain for outcrossing analysis (Lee et al., 2003). All strains were stored in 25% glycerol at –80°C and maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA). For the histidine auxotrophy test, fungal strains were inoculated into minimal medium (MM: 0.6% NaCl, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.15% KCl) or MM supplied with 0.25 mM histidine and grown at 25°C for 3-4 days (Han et al., 2004). For genomic DNA extraction, fungal strains were grown in 50 ml of complete medium (CM, Leslie and Summerell, 2006) at 25°C for 3 days, with shaking (150 rpm). For macrospore production, mycelial plugs of each strain were inoculated into CM liquid medium and grown as described earlier (Han et al., 2004). Carrot agar was used for sexual crosses (Leslie and Summerell, 2006). Recombinant *Escherichia coli* HB101 strain (Takara Korea Biomedical Inc., Seoul, Korea) was used for the propagation of plasmids recovered from the REMI mutant. Bacteria were grown on Luria-Bertani agar or in liquid medium supplemented with 75 μg/ml ampicillin. The REMI plasmid pIGPAPA, carrying a hygromycin-B resistance gene (*hygB*) (Horwitz et al., 1999) was used as a probe for *G. zeae* genomic DNA.

DNA manipulations, plasmid rescue, PCR primers, and sequencing. Fungal genomic DNA was isolated as previously described (Han et al., 2004). Plasmid DNA was purified from *E. coli* cultures using a plasmid DNA purification kit (NucleoGen Biotech, Siheung, Korea). Standard procedures were used for restriction endonuclease digestion, ligation, agarose gel electrophoresis, gel blotting, ^{32}P labeling of probes, and hybridization (Sambrook and Russell, 2001). A plasmid rescue procedure was used to recover the DNA regions flanking the vector insertion point in the Z43R1092 genome, as described previously (Yun et al., 1998). Primers were designed using the PrimerSelect program (DNAStar Inc., Madison, USA), synthesized by the Bioneer oligonucleotide synthesis facility (Bioneer Corporation, Chungwon, Korea), dissolved in 100 μM sterilized water, and stored at –20°C. Sequencing of the rescued plasmids was initiated with primers close to the *Kpn*I site on the pIGPAPA vector: pIGPAPA/P5 (5'-GGTCCCCCTCCCAATTCCTTTTC-3') and TSP3-2 (5'-GCTCCTCGCCCTTGCTCACCAT-3').

Double-joint (DJ) PCR. A fungal transforming construct for targeted gene deletion was made using a DJ-PCR method (Yu et al., 2004). DNA fragments corresponding to regions 5' (1.0 kb) and 3' (1.0 kb) of the *GzHIS7* gene were amplified from genomic DNA of Z03643 using the primer pairs His-5For (5'-ACCGCTGCCACATTCTTGACTTC-TACTCTA-3')/His-5Rev-tail (5'-ACCTCCACTAGTAGC-CAAGGGAAAACACCAACGAAGATGAAAT-3') and His-3For-tail (5'-TATGAAAATTCCGTCACCAGCGGC-CAAGTTCAAAATCCAATAAGA-3')/His-3Rev (5'-CGG-ATAAATAGTCACTTCGCTCACG-3'), respectively. A 2.1-kb fragment containing the *hygB* cassette was amplified from the plasmid DNA pBCATPH (Kim et al., 2007) using the primers Hyg5'for (5'-CTTGGCTGGAACAGCTAGTG-GAGGT-3') and Hyg3'rev (5'-GGCTGGTGACGGAATT-TTCATA-3'). The complementary sequences between the primers are underlined; these promote hybridization between the PCR products amplified by the primers. Three amplicons (the 5'-flanking region of *GzHIS7*, the *hygB* cassette, and the 3'-flanking region of *GzHIS7*) were mixed in a 1:2:1 molar ratio and used as the template for a second round of PCR without primers. A 4.1-kb fragment carrying the *hygB* cassette was amplified from the second PCR product using the nested primer pair His-5'nest (5'-TATT-CCAACCATCACCACTTTAG-3') and His-3'nest (5'-AACGGTTCCAGAGCCTTGACACTTC-3').

Fungal transformation, outcrossing, and virulence tests. For targeted gene deletion, about 5 mg of the DJ-PCR product was added directly to fungal protoplasts together with polyethylene glycol, as described previously (Kim et al., 2006). For complementation, the PCR product carrying an intact copy of *GzHIS7* was added to fungal protoplasts together with pII99 containing a geneticin resistance gene (*gen*) as a selectable marker (Kim et al., 2007). Both selfing and outcrossing of the *G. zeae* strains was performed as previously described (Lee et al., 2003). For virulence tests, macroconidia harvested from cultures grown on CMC liquid medium for 5 days were suspended in sterile water at 10^6 spores per ml and sprayed onto the heads of a susceptible barley cultivar, 'SangRok,' at the early anthesis stage. The plants were placed in a growth chamber for 2 days at 25°C and 100% relative humidity and then transferred to a greenhouse.

Results

Phenotypes of the REMI mutant Z43R1092. A REMI mutant of *G. zeae*, designated Z43R1092, was initially

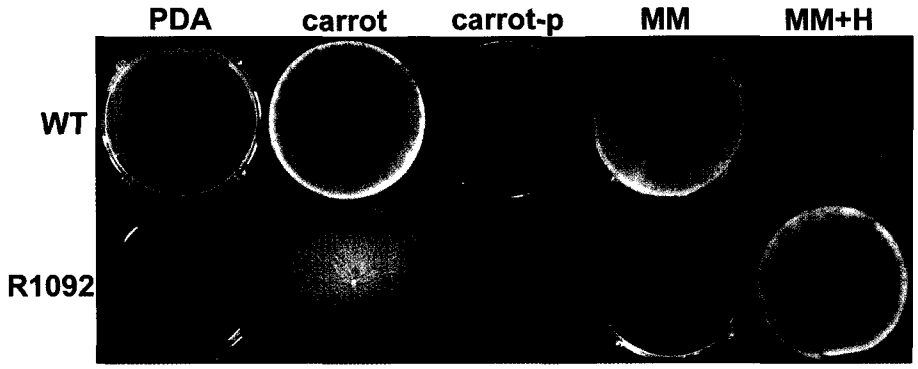

Fig. 1. Mycelial growth of wild-type strain Z03643 (WT) and the REMI mutant Z43R1092 (R1092) on potato dextrose agar (PDA), carrot agar for vegetative growth (carrot), carrot agar for perithecial formation (carrot-p), minimal medium (MM), or MM supplemented with 0.25 mM histidine (MM+H).

selected from the insertional mutant collection generated in the REMI procedure (Han et al., 2004). The mutant Z43R1092 differed from its wild-type progenitor Z03643 in several traits such as mycelial growth, pigmentation, sexual development, and virulence on host plants (Figs. 1-2). We observed a reduction of about 30% in the radial growth of Z43R1092, with fewer aerial mycelia and no red pigmentation, compared to that of Z03643 when grown on PDA or carrot agar; Z43R1092 did not grow on MM (Fig. 1). In addition, the wild-type strain began to form fertile perithecia 4 days after removing the aerial mycelia that had already grown on carrot agar for 1 week, whereas the mutant formed no perithecia, even after 6 weeks of incubation (Fig. 1). However, all the growth defects in Z43R1092 were restored to wild-type levels when 0.25 mM histidine was supplied exogenously, demonstrating that Z43R1092 is a histidine auxotroph. Inoculation of barley heads with a conidial suspension of Z43R1092 caused almost no disease symptoms, whereas Z03643 caused typical head blight symptoms that began to appear as early as 3 days after inoculation and became obvious after 6 days (Fig. 2). The ability of Z43R1092 to cause disease symptoms was also evident on young corn ears (Fig. 2).

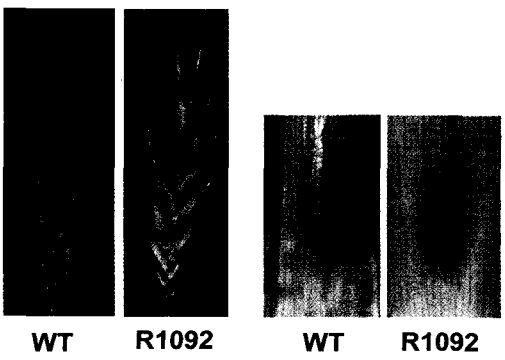

Fig. 2. Virulence of the wild-type strain (WT) and the REMI mutant Z43R1092 (R1092) on barley heads (left panel) and young corn ears (right panel).

Genetic analysis of Z43R1092. To determine whether the histidine auxotrophy and other phenotypic changes in Z43R1092 were tagged by the *hygB* gene on the REMI vector, Z43R1092 was outcrossed to the *G zeae mat1-1*-deleted strain, T39ΔM1-3 (the female parent), which is self-sterile and resistant to geneticin (gen^R). Random ascospores obtained from the outcross segregated in equal proportions into parental phenotypes for *hygB* and *gen* (data not shown). All of the $hygB^R$ progeny showed histidine auxotrophy, together with other pleiotropic changes found in Z43R1092, indicating that the mutation in Z43R1092 is linked to the insertion site of the *hygB* gene.

Molecular characterization of Z43R1092. A gel blot of Z43R1092 genomic DNA was hybridized with the entire REMI vector, pIGPAPA. In genomic DNA digested with *Kpn*I, which was the enzyme used for the linearization of pIGPAPA in the REMI procedure, the probe hybridized to a single ~5.9-kb fragment the same size as pIGPAPA (Fig. 3A). When the genomic DNA was digested with *Bgl*II, which has no recognition site in the vector, a single hybridizing band of ~17.0 kb appeared (Fig. 3A). These hybridization patterns, typical of a real REMI event, indicate that pIGPAPA was integrated at a *Kpn*I site in the Z43R1092 genome and that both *Kpn*I sites at the ends of the linearized pIGPAPA were retained during the REMI event. The 17.6-kb *Bgl*II fragment that appeared on the DNA gel blot was recovered using a plasmid rescue procedure and designated pZ43R1092. Nucleotide sequencing of pZ43R1092 revealed that it contains 4.7 kb of genomic DNA to the left side and 6.5 kb to the right side of the vector. BLAST searches of the *G zeae* genome database (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html) revealed that the immediate left and right flanking regions of the vector could be connected

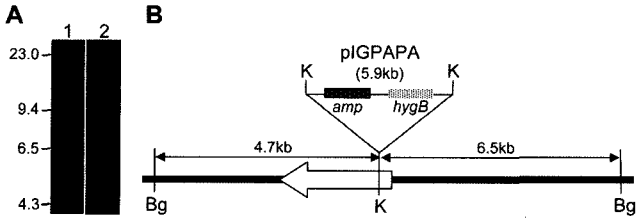

Fig. 3. Molecular characterization of the vector insertion event in the Z43R1092 genome. (A) Gel blot of Z43R1092 genomic DNA digested with *Kpn*I (lane 1) or *Bgl*II (lane 2), hybridized with pIGPAPA. The sizes of λ DNA standards (in kilobases) are indicated to the left of the blot. (B) Molecular structure of the vector insertion site in Z43R1092. The ORF within the vector insertion site is indicated by the open arrow. Restriction enzyme sites: K, *Kpn*I; Bg, *Bgl*II; *amp*, ampicillin resistance gene; *hygB*, hygromycin-B resistance gene.

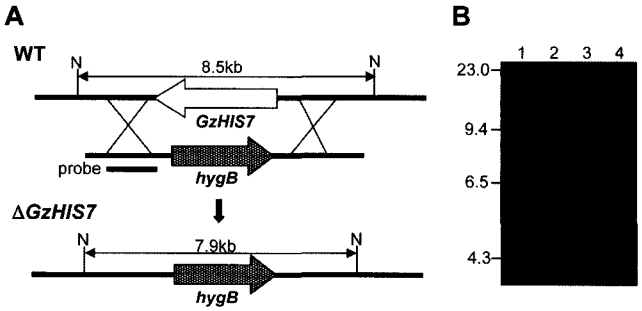

Fig. 4. Targeted deletion of *GzHIS7* from the genome of *G. zeae* wild-type strain Z03643. (A) Deletion strategy by double homologous recombination between the fusion PCR product and the corresponding genomic regions in Z03643. WT, genomic DNA of Z03643; Δ*GzHIS7*, genomic DNA of the *GzHIS7*-deleted strain; N, *Nhe*I; *hygB*, hygromycin-B resistance gene. The probe for gel blot hybridization is indicated by a bar. (B) Blot of *Nhe*I-digested genomic DNA of the Δ*GzHIS7* strains. Lane 1, Z03643; lanes 2-4, the Δ*GzHIS7* strains. The sizes of λ DNA standards (in kilobases) are indicated to the left of the blot.

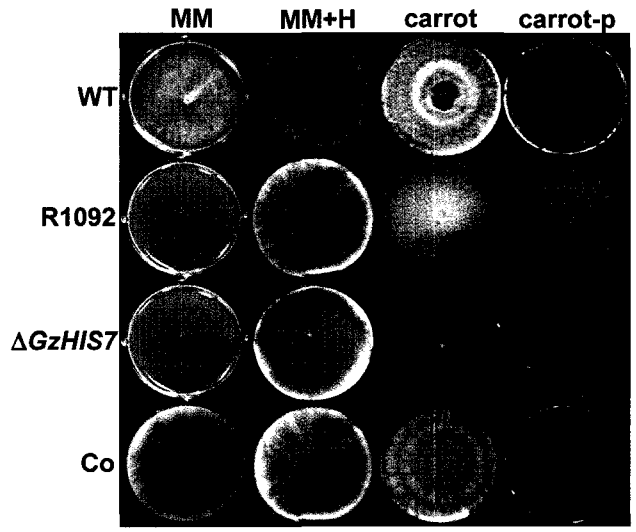

Fig. 5. Mycelial growth of *G. zeae* strains. WT, Z03643; R1092, Z43R1092; Δ*GzHIS7*, *GzHIS7*-deleted strain; Co, transgenic Δ*GzHIS7* strain carrying an intact copy of *GzHIS7*.

into a continuous open reading frame (ORF) of 549 amino acids, interrupted by one putative intron, which was annotated FG09984.1 and located at contig 1.415 in the fungal genome database. This ORF, designated *GzHIS7*, had a high sequence similarity to the *HIS7* gene (accession no. X69815) with 59.1% amino acid similarity; the *HIS7* gene codes for the *Saccharomyces cerevisiae* HIS7 protein, which is essential for histidine biosynthesis (Kuenzler et al., 1993). The vector insertion point in Z43R1092 was a *Kpn*I site located 404 bp downstream of the putative start codon of the *GzHIS7* ORF (Fig. 3B).

Sequence alignment analysis revealed that the N-terminal segment (amino acids 1 to 204) of the deduced GzHIS7 sequence showed significant homology (34.4%) to the *E. coli* glutamine amidotransferases (HisH: YP853139), whereas the C-terminal region (amino acids 230 to 549) was similar (30.6%) to the *E. coli* cyclase (HisF: P60664). Thus, the putative primary structure of GzHIS7 indicates that it is a bifunctional enzyme with an N-terminal glutamine amidotransferase and a C-terminal cyclase domain, as in the *S. cerevisiae* HIS7, catalyzing the fifth and sixth step of the histidine biosynthetic pathway in this yeast.

Targeted deletion of *GzHIS7*. To confirm that the disruption of *GzHIS7* is a direct cause of the pleiotropic changes as well as the histidine auxotrophy in Z43R1092, the genomic copy of *GzHIS7* was deleted from the *G. zeae* Z03643 strain using a targeted gene-replacement strategy (Fig. 4). Insertion of the PCR product carrying a *hygB* gene cassette fused to the 5' (1.0 kb) and 3' (1.0 kb) regions flanking *GzHIS7* via a double crossover resulted in the deletion of *GzHIS7* (designated Δ*GzHIS7*) (Fig. 4A). *Nhe*I-digested genomic DNA of the Δ*GzHIS7* strains showed a single 7.9-kb hybridizing band instead of the 8.5-kb band hybridized in Z03643, confirming that the entire 1.7-kb *GzHIS7* ORF was deleted and replaced with *hygB*, as expected (Fig. 4B). All of the Δ*GzHIS7* strains exhibited the same pleiotropic changes as did the REMI mutant Z43R1092; these defects in the Δ*GzHIS7* strains were restored to the wild-type level when 0.25 mM histidine was supplied exogenously (Fig. 5). Other transformants carrying the fusion PCR product at an ectopic position were similar to Z03643 in all of the phenotypes examined (data not shown).

Genetic complementation of Δ*GzHIS7*. For further confirmation of the *GzHIS7* function in *G. zeae*, an intact copy of *GzHIS7* was introduced into the genome of a Δ*GzHIS7*

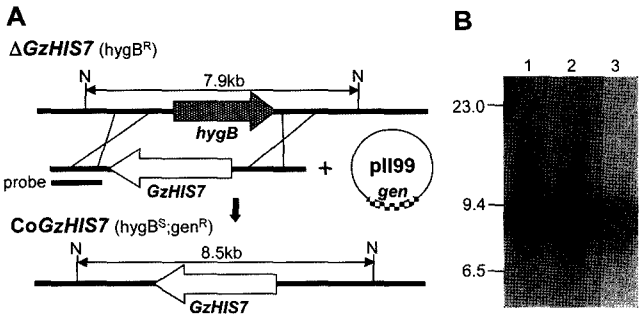

Fig. 6. Genetic complementation of the Δ*GzHIS7* strain. (A) Co-transformation strategy using circular pII99 DNA. Δ*GzHIS7*, genomic DNA of the *GzHIS7*-deleted recipient strain that is resistant to hygromycin-B (hygB^R); Co*GzHIS7*, genomic DNA of geneticin-resistant (gen^R) transformants; N, *Nhe*I; *hygB*, hygromycin-B resistance gene; *gen*, geneticin resistance gene. The probe for gel blot hybridization is indicated by a bar. (B) Gel blot of *Nhe*I-digested genomic DNA of the wild-type strain (lane 1), and the complemented transformants (lanes 2-3). The sizes of λ DNA standards (in kilobases) are indicated to the left of the blot.

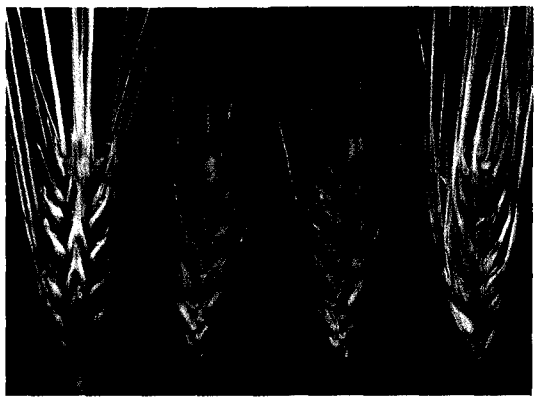

Fig. 7. Virulence of Z03643 (WT), Z43R1092 (R1092), a *GzHIS7*-deleted strain (Δ*GzHIS7*), and a transgenic Δ*GzHIS7* strain carrying *GzHIS7* (Co) on barley heads.

strain. A 4.0-kb DNA fragment, which included the entire *GzHIS7* ORF, was amplified from the genomic DNA of Z03643 using the primer pair His-5For and His-3Rev and directly added into protoplasts of the Δ*GzHIS7* strain, along with pII99 carrying *gen* as a fungal selectable marker (Fig. 6A). Three of 24 gen^R transformants were no longer resistant to hygromycin-B and were able to grow normally on MM plates, as could the Z03643 strain. The complemented strains from the Δ*GzHIS7* mutant were just as female-fertile and virulent as the wild-type Z03643 strain (Figs. 5&7). DNA gel blot analysis revealed that the *GzHIS7* gene was integrated at its original genomic locus of the recipient Δ*GzHIS7* strain by homologous recombination, leading to the loss of *hygB* in the complements (Fig. 6B). Prototropic transformants could also be selected on MM when the *GzHIS7* PCR product alone was added into protoplasts of the Δ*GzHIS7* strain (data not shown).

Discussion

The histidine biosynthetic pathway comprises an invariable series of 11 enzymatic steps that are conserved in all microorganisms and plants investigated thus far (Alifano et al., 1996; Stepansky and Leustek, 2006). However, most studies using histidine auxotrophy have focused on uncovering the histidine biosynthetic pathway itself or on more fundamental mechanisms in biology such as the operon for enzyme regulation (Stepansky and Leustek, 2006). There are few reports on how the histidine auxotroph affects some major phenotypes in filamentous fungi (Sweigard et al., 1998; Busch et al., 2001). Thus, we present the first characterization of a histidine auxotrophic mutation caused by the disruption of *GzHIS7* in *G. zeae*. The REMI mutant and Δ*GzHIS7* strains presented here are the first histidine auxotrophs available for assessing the role of histidine metabolism in the traits important for disease development in *G. zeae*. As in other *G. zeae* auxotrophs or in those from other plant pathogenic fungi, histidine auxotrophy is confirmed to affect the basic physiology of *G. zeae*, leading to pleiotropic changes such as altered vegetative growth and pigmentation, female sterility, and reduced virulence. This raises the question of whether blocking these basic physiological steps is a histidine-specific effect or a general, indirective effect due to histidine deficiency in *G. zeae*. From studies with microorganisms, it is known that histidine biosynthesis is integrated into multiple metabolic pathways, e.g., purine, folic acid, tryptophan, and methionine metabolism (Alifano et al., 1996; Stepansky and Leustek, 2006). Because several *G. zeae* REMI mutants that impair these metabolic pathways, e.g., auxotrophs for methionine, arginine, or purine, are already known to cause the same phenotypic changes as the histidine auxotrophs presented here (Han et al., 2004; Kim et al., 2007), it is plausible that the effect of histidine auxotrophy on fungal virulence and sexual development in *G. zeae* may be indirect, when considering all the metabolic interconnections (Busch et al., 2001). In this respect, the reduced virulence of a histidine auxotroph of *Magnaporthe grisea*, which was generated by the disruption of *pth3* encoding a putative imidazole glycerol phosphate dehydratase (Sweigard et al., 1998), could be comparable to the case of the *G. zeae* Δ*GzHIS7* strains. However, because histidine auxotrophy led to impaired embryo development in *Arabidopsis* (DeFraia and Leustek, 2004), and blockage of sexual fruiting body formation in *A. nidulans* (Busch et al., 2001) and *G. zeae*, this may indicate a more specific connection between histidine biosynthesis and sexual reproduction in

fungi and higher plants. In addition to the significance of the gene functional analysis, the availability of the *ΔGzHIS7* strain and an intact copy of *GzHIS7* could be useful for the development of a new transformation system using genetic complementation in *G. zeae*. In summary, we have clearly demonstrated that the disruption of the *GzHIS7* gene encoding a putative glutamine amidotransferase, an essential enzyme for histidine biosynthesis, is responsible for histidine auxotrophy, which subsequently leads to reduced virulence and the loss of sexual development in *G. zeae*.

Acknowledgements

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