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Functional Characterization of Genes Located at the Aurofusarin Biosynthesis Gene Cluster in *Gibberella zeae*

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Aurofusarin is a polyketide pigment produced by some *Fusarium* species. The *PKS12* and *GIP1* genes, which encode a putative type I polyketide synthase (PKS) and a fungal laccase, respectively, are known to be required for aurofusarin biosynthesis in *Gibberella zeae* (anamorph: *Fusarium graminearum*). The ten additional genes, which are located within a 30 kb region of *PKS12* and *GIP1* and regulated by a putative transcription factor (GIP2), organize the aurofusarin biosynthetic cluster. To determine if they are essential for aurofusarin production in *G zeae*, we have employed targeted gene deletion, complementation, and chemical analyses. *GIP7*, which encodes *O*-methyltransferase, is confirmed to be required for the conversion of norrubrofusarin to rubrofusarin, an intermediate of aurofusarin. *GIP1*-, *GIP3*-, and *GIP8*-deleted strains accumulated rubrofusarin, indicating those gene products are essential enzymes for the conversion of rubrofusarin to aurofusarin. Based on the phenotypic changes in the gene deletion strains examined, we propose a possible pathway for aurofusarin biosynthesis in *G zeae*. Our results would provide important information for better understanding of naphthoquinone biosynthesis in other filamentous fungi as well as the aurofusarin biosynthesis in *G zeae*.

Keywords : a gene cluster, aurofusarin biosynthesis, *Gibberella zeae*, polyketide

Fusarium species including *F. graminearum*, *F. culmorum*, and *F. crookwellense* cause *Fusarium* head blight (FHB) of cereal crops such as barley, rice, and wheat (McMullen et al., 1997), and *Fusarium* ear and stalk rot of maize (Kommedahl and Windels, 1981). FHB is a devastating plant disease in many regions of the world. These fungal species are important plant pathogens because they cause severe economic losses and produce mycotoxins that pose serious threats to human and animal health (Marasas et al., 1984). The fungi produce aerial mycelia that are yellow to tan with white to carmine red margins. Two pigments, rubrofusarin and aurofusarin, in the naphthoquinone group of polyketides have been firstly isolated from *F. culmorum* (Ashley et al., 1937; Gray et al., 1967; Tanaka et al., 1962). Aurofusarin causes a significant decrease in vitamins A and E, and fatty acids in egg yolk, and is toxic to poultry (Dvorska et al., 2001, 2004). Rubrofusarin has antimycobacterial and antiallergic activity and is phytotoxic to plants (Graham et al., 2004; Kimura et al., 1988; Kitanaka et al., 1998).

Many fungal polyketide pigments and other secondary metabolites have been intensively studied because of their biological importance (Keller and Hohn, 1997; Langfelder et al., 2003). These fungal metabolites contribute to virulence, protection against oxidative attack, and antifungal activity (Jahn et al., 2000; Langfelder et al., 1998; Linnemannstons et al., 2002). In many filamentous fungi, genes for the biosynthesis of polyketides are usually clustered in the fungal genomes (Keller and Hohn, 1997). These gene clusters encode not only polyketide synthase (PKS), but also other metabolic enzymes, transporters, and transcription factors. Gene clusters for the biosynthesis of trichothecenes (Hohn et al., 1995), zearalenones (Kim et al., 2005b), fumonisins (Seo et al., 2001), and gibberellins (Tudzynski et al., 1998) have been identified in *Fusarium* species.

We previously identified a type I PKS gene (*PKS12*), a putative laccase gene (*GIP1*), and a putative transcription factor (*GIP2*), all of which are essential for aurofusarin biosynthesis in *G zeae* (Kim et al., 2006). Using Northern analysis, we found that the nine additional genes surrounding *PKS12* are under the control of GIP2 (Kim et al., 2006). Maltz et al. (2005) also predicted the same aurofusarin gene cluster from reverse transcription (RT)-PCR analysis. Our objectives in this study were to determine if the additional

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genes in the gene cluster are required for aurofusarin production, and to propose a biosynthetic pathway for aurofusarin in *G. zeae*.

Materials and Methods

Strains and culture conditions. *Gibberella zeae* strain Z03643 was used as a wild-type strain. Z03643 is a lineage 7 strain that produces both zearalenone and deoxynivalenol (O'Donnell et al., 2000). Z03643 produces more red pigments than any other *G. zeae* strain (Kim et al., 2005a). Fungal strains were stored in 20% glycerol at –80°C and were reactivated on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA). For macroconidia formation, mycelial plugs of fungal strain were inoculated in CMC liquid medium (Han et al., 2004) at 25°C for 4-5 days with shaking at 150 rpm. For genomic DNA extraction, the fungus was grown in 50 ml of liquid complete medium (CM; Leslie and Summerell, 2006) in 250-ml Erlenmeyer flasks at 25°C for 3 days on a rotary shaking incubator at 150 rpm, and mycelia were harvested and lyophilized.

Nucleic acid manipulation. For DNA gel blot analysis, fungal genomic DNA was prepared using previously described methods (Han et al., 2004). Plasmid DNA was purified from 5 ml of *Escherichia coli* culture using a plasmid purification kit (NucleoGen Biotech, Siheung, Korea). The procedures for restriction endonuclease digestion, agarose gel electrophoresis, gel blotting, probe labeling with ^{32}P, and hybridization were performed as previously described (Sambrook and Russell, 2001). PCR primers were obtained from the Bioneer oligonucleotide synthesis facility (Bioneer Corporation, Chungwon, Korea), adjusted to 100 pM with sterile distilled water, and stored at –20°C. PCRs were performed as previously described (Kim et al., 2005a). Sequences of the *G. zeae* genome databases (http://www.broad.mit.edu/annotation/genome/fusarium_group/) were compared with those of other species using BLASTX (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov).

Construction of the transforming vector and fungal transformation. To construct the fungal transforming vector, a DNA fragment carrying a hygromycin resistance gene (*hygB*) flanked with 5' and 3' DNA of the targeted gene was amplified using double-joint PCR (DJ-PCR) as previously described (Kim et al., 2005a; Yu et al., 2004). A final PCR product harboring the *hygB* marker fused to the flanking regions of the ORF was directly added into fungal protoplasts for transformation, as previously described (Kim et al., 2005a). For complementation, an intact copy of the target ORF was amplified from Z03643 genomic DNA, and was incorporated into the fungal protoplast along with the plasmid DNA SK660 containing a geneticin resistance gene (*gen*) as a selectable marker. SK660 was obtained from Dr. Seogchan Kang (Department of plant pathology, Penn state university, USA). Fungal transformation methods were performed previously described (Kim et al., 2005a).

Pigment analysis by high-performance liquid chromatography (HPLC). To observe pigment production, the strains were grown on PDA for 2 weeks at 25°C in darkness, after which the cultures were dried and ground in a blender for pigment extraction. Aurofusarin was extracted from grinding PDA cultures of strains using chloroform and was analyzed using a HPLC (Waters 510 HPLC system, Waters, USA) equipped with a diode array detector and a 150 cm× 4.6 mm Luna C18 reverse-phase column (particle size 5 μm; Torrance, USA), as previously described (Kim et al., 2005a). The detection wavelength was 360 nm.

Virulence test. To test virulence toward host plants, the fungal strains were grown in CMC, and spores were collected by filtering through six layers of cheesecloth and centrifuging. The spores were adjusted to a concentration of 1×10^6 conidia/ml using a hemocytometer. The conidial suspension was sprayed onto the heads of a susceptible barley cultivar (SangRok) at the early anthesis stage. The inoculated plants were incubated in a growth chamber for 2 days at 25°C with 100% relative humidity and then transferred to a greenhouse for 7-9 days. Head blight symptoms appeared 1 week after inoculation.

Results

Molecular organization of the aurofusarin biosynthetic gene cluster. The clustered genes located around *PKS12* encode metabolic enzymes that are likely to be required for aurofusarin biosynthesis in *G. zeae* (Kim et al., 2006; Malz et al., 2005) (Fig. 1A). The PKS gene (*PKS12*), laccase gene (*GIP1*), and transcriptional regulator gene (*GIP2*) are essential for aurofusarin biosynthesis (Kim et al., 2005a). Transcripts of the genes located at the aurofusarin gene cluster were not detected in the *GIP2* deletion mutant (Kim et al., 2006). BLAST analyses of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) indicated the similarity of several other genes, for example *GIP4* and *GIP7*, to those that are found in the aflatoxin gene cluster of *Aspergillus parasiticus*. Sequence of *GIP4* has similarity to that of *aflT*, the major facilitator superfamily (MFS) transporter gene that is the transporter of aflatoxin in *A. flavus*. *GIP7* is similar to the *O*-methyltransferase gene of *G. fujikuroi* and to the co-activator gene *aflJ* in *A. flavus*. *GIP3* has similarity to FAD/FMN-containing dehydrogenases. *GIP5*

Table 1. Primers used in this study

Name	Sequence (5' to 3')	Position
P12-5f	GGGGGAGGCCATCTTTATCT	7494509–7494490 at supercontig 1
P12-5r	CTCCACTAGCTCCAGCCAAGGAGAGGTAATGAAGCACAAT	7493239–7493258 at supercontig 1
P12-3f	TGAAAATTCCGTCACCAGCCCTTTGTATTGCGCTTCGTGTT	7486557–7486537 at supercontig 1
P12-3r	CTAATATTCATCAGCTTTCACTCC	7485252–7485275 at supercontig 1
NP12-5f	GATATCGCGACTGACGTACTAACAGGTGG	7494422–7494394 at supercontig 1
NP12-3r	GCGACACGTATGGGCCAAAGAATG	7485320–7485343 at supercontig 1
G1-5f	CATGGCTGAACAGGAACTTG	7501971–7501952 at supercontig 1
G1-5r	CTCCACTAGCTCCAGCCAAGCGCTGTCAGCTTATTGCAGT	7500854–7500873 at supercontig 1
G1-3f	TGAAAATTCCGTCACCAGCCTGTTATCGTGCTTCCATTTG	7498578–7498559 at supercontig 1
G1-3r	GATGAACCGCTACACTCCTG	7497380–7497399 at supercontig 1
NG1-5f	CATTGGTTGGAGCAAAGA	7501920–7501903 at supercontig 1
NG1-3r	TACCTTTGACCTCAAGCC	7497449–7497466 at supercontig 1
G2-5f	TTTGCGGGATGATATGACTGAG	7479880–7479880 at supercontig 1
G2-5r	CTCCACTAGCTCCAGCCAAGCAAACGCAGCTAACAGAGGAGA	7478746–7478767 at supercontig 1
G2-3f	TGAAAATTCCGTCACCAGCCTCTCGCGGCAGCTTGTAATCAT	7477183–7477162 at supercontig 1
G2-3r	GCCATGCTAGCCCAACTCTCC	7475717–7475737 at supercontig 1
NG2-5f	GCTTGCATCGGACCGAAGGAATGA	7479647–7479624 at supercontig 1
NG2-3r	GCCCTGAGCTTGCAGCAGAGTGTCTT	7475932–7475957 at supercontig 1
G3-5f	ACCGCTGAGAAGGGCTGT	7478325–7478342 at supercontig 1
G3-5r	CTCCACTAGCTCCAGCCAAGGATAAGCTGGCCCTTTTGAG	7479752–7479733 at supercontig 1
G3-3f	TGAAAATTCCGTCACCAGCCATGAGATCGGCCGTGCTACA	7481422–7481441 at supercontig 1
G3-3r	GGCGTCGGCTGGTTCAA	7482623–7482607 at supercontig 1
NG3-5f	CCACGTCGCTCGCATCTTTGACAC	7478357–7478380 at supercontig 1
NG3-3r	CATGTCAGTCGGTGGCGGTCTTATCAC	7482579–7482553 at supercontig 1
G4-5f	GCCGTGTAGAATCTTGGTC	7484955–7484937 at supercontig 1
G4-5r	CTCCACTAGCTCCAGCCAAGGCTGCGAAAGAGAGGGTATT	7483809–7483828 at supercontig 1
G4-3f	TGAAAATTCCGTCACCAGCCAAACATGGTTCATTTCTGGG	7482091–7482072 at supercontig 1
G4-3r	ATCAGCAACCCTGTCAATGT	7480958–7480977 at supercontig 1
NG4-5f	GAGGATGGACATTGCTGA	7484935–7484918 at supercontig 1
NG4-3r	CGGCACATCAGTATCTCC	7480985–7481002 at supercontig 1
G5-5f	AGTAAAAGCTCCTCCGATCA	7483209–7483228 at supercontig 1
G5-5r	CTCCACTAGCTCCAGCCAAGTTGTTGTTGGGATAATTCGT	7484361–7484342 at supercontig 1
G5-3f	TGAAAATTCCGTCACCAGCCACGAAGCGCAATACAAAGTA	7486540–7486559 at supercontig 1
G5-3r	GGGTCTCGACGTTGAATCTA	7487770–7487751 at supercontig 1
NG5-5f	CACATACCCAGCACGTTT	7483275–7483292 at supercontig 1
NG5-3r	TGAACTGTCCCACTTTGG	7487741–7487724 at supercontig 1
G7-5f	TCAAACATGAAGAATGCGG	7497853–7497835 at supercontig 1
G7-5r	CTCCACTAGCTCCAGCCAAGGGCTGGCTAGTGATTGACTG	7495961–7495980 at supercontig 1
G7-3f	TGAAAATTCCGTCACCAGCCGGGGGAGGCCATCTTTAT	7494509–7494492 at supercontig 1
G7-3r	CTTGTTCGTCGTTCGGTG	7493454–7493471 at supercontig 1
NG7-5f	GCACCTGTGAAGCCTCTAGACTTTTGCC	7497746–7497721 at supercontig 1
NG7-3r	GCAGCAGTCTCTCGTCATTCGGGC	7493523–7493546 at supercontig 1
G8-5f	AATCACATGCTTGCTTACTGC	7494924–7494944 at supercontig 1
G8-5r	CTCCACTAGCTCCAGCCAAGAAATACGAGAAAGCTGGTTGAT	7496390–7496369 at supercontig 1
G8-3f	TGAAAATTCCGTCACCAGCCGAGGGTCACTGGTCATAGGAA	7498282–7498302 at supercontig 1
G8-3r	AGGGAGTGATAGTGTGGGTGTG	7499510–7499489 at supercontig 1
NG8-5f	GCGGAGGTGTGGATAAGCAGCGG	7495008–7495030 at supercontig 1
NG8-3r	CCACCAGCACGCAAGGCAGATG	7499404–7499383 at supercontig 1
G9-5f	TTTGATTAGCAATGCAGTGC	7500100–7500119 at supercontig 1

Table 1. Continued

Name	Sequence (5' to 3')	Position
G9-5r	CTCCACTAGCTCCAGCCAAGTTTCGCCAATTATCTTCACA	7501199–7501180 at supercontig 1
G9-3f	TGAAAATTCCGTCACCAGCCTTAACAAATGTAGACCAGGC	7502531–7502550 at supercontig 1
G9-3r	ATCGAACTGTCCAATAGCAC	7503597–7503578 at supercontig 1
NG9-5f	CTCGGCAGCCATGTACTC	7500170–7500187 at supercontig 1
NG9-3r	TGGAAGGCTTATCGGAGA	7503554–7503537 at supercontig 1
G10-5f	TCGGCGCCGGTTTGATTATTAC	7506601–7506580 at supercontig 1
G10-5r	CTCCACTAGCTCCAGCCAAGCGTCGTCGTCGGTCAGCATTTA	7504904–7504925 at supercontig 1
G10-3f	TGAAAATTCCGTCACCAGCCGAGTATGATGGGACACGCTA	7502702–7502683 at supercontig 1
G10-3r	AGGCTCGCTTCACTGTTTA	7501456–7501474 at supercontig 1
NG10-5f	CCCGTACAATGGTTCAGCAGCAAACTG	7506482–7506456 at supercontig 1
NG10-3r	CAGCTGAGCGTCTCCCGTCAATGG	7501579–7501602 at supercontig 1
nHygB-f	CTTGGCTGGAGCTAGTGGAGGT	For amplification of *hyg* cassette from pBCATPH
nHygB-r	GGCTGGTGACGGAATTTTCATA	For amplification of *hyg* cassette from pBCATPH

[a]The sequences underlined with the same patterns are complementary each other for promotion of hybridization between the PCR products.

is similar to a putative fungal transcriptional activator, and has a fungus-specific transcription factor domain and a binuclear zinc cluster motif. *GIP8* is similar to a gene that encodes the flavin-containing monooxygenase of *Cavia porcellus*, the domestic guinea pig. GIP9 has similarity to a putative fasciclin I family protein in *A. fumigatus*. Finally, *GIP10* has similarity to the ascorbate oxidase gene of *Acremonium* sp. HI-25 (GenBank accession no. AB010110). RPS-BLAST analysis of GIP10 indicates that it is highly similar to a multicopper oxidase (pfam00394), as found by RPS-BLAST for GIP1.

Targeted deletion of genes in the aurofusarin gene cluster. To identify the roles of the additional genes in aurofusarin biosynthesis, we generated *G. zeae* deletion mutants of each gene from the wild-type strain Z03643. The entire ORF of each gene was replaced with the fungal selectable marker *hygB* via double homologous recombination between the genomic region of the target gene and a DJ-PCR product carrying both 5' and 3' regions of the target

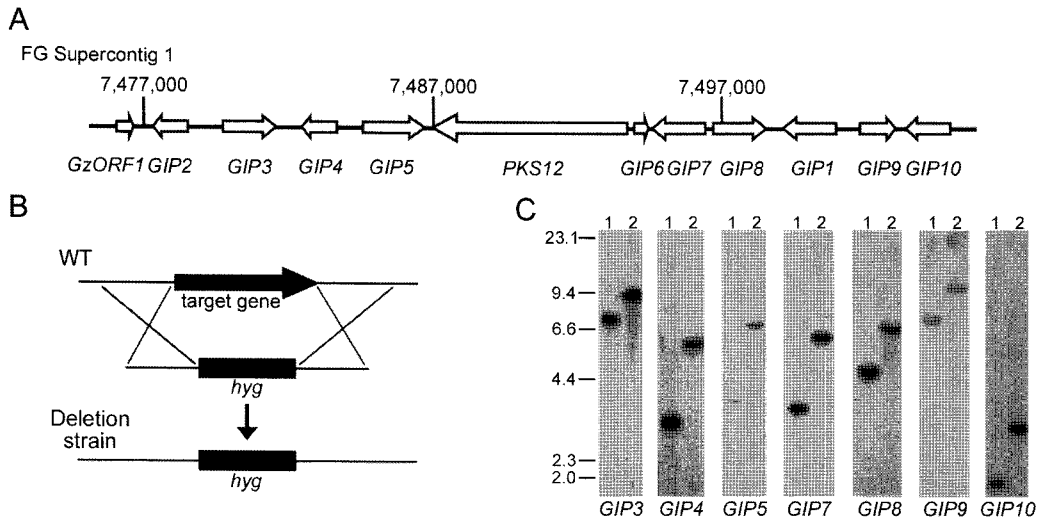

Fig. 1. Functional analysis of the aurofusarin biosynthetic gene cluster. (A) Molecular organization of the aurofusarin biosynthesis cluster in *G. zeae*. This cluster was found In supercontig 1 from the *F. graminearum* genome databases. Numbers and arrows indicate the ORFs and transcriptional direction, respectively. Nucleotide positions of the contig are indicated on the thick vertical bar. (B) Deletion strategy. WT, genomic DNA of wild-type strain Z03643; deletion strain, genomic DNA of the deleted strain; *hygB*, hygromycin B resistance gene. The restriction enzyme and probe used for hybridization are not indicated because the restriction enzymes and probes used varied with the genes. (C) Gel blots of genomic DNA from transgenic strains with deletions of aurofusarin biosynthetic genes, probed with the flanking region of each gene. In each blot: lane 1, wild-type strain Z03643; lane 2, the deleted strains of Z03643. The sizes of λ DNA standards (in kilobases) are indicated on the left of the blot. Each gene is indicated in the lower part of the blot.

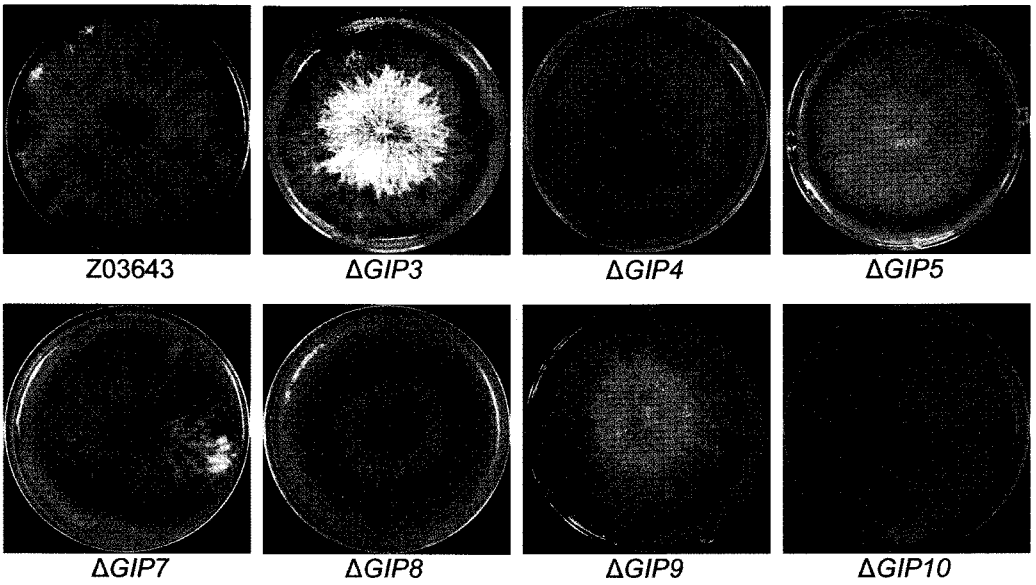

Fig. 2. Pigmentation of transgenic strains with gene deletion. Z03643, wild-type strain; Δ*GIP3*, Δ*GIP4*, Δ*GIP5*, Δ*GIP7*, Δ*GIP8*, Δ*GIP9*, and Δ*GIP10*; deletion strains of *GIP3* (Tzg3-2), *GIP4* (Tzg4-7), *GIP5* (Tzg5-1), *GIP7* (Tzg7-10), *GIP8* (Tzg8-3), *GIP9* (Tzg9-2), and *GIP10* (Tzg10-5), respectively.

gene with the *hygB* cassette (Fig. 1B). After protoplast transformation, hygromycin-resistant transformants were selected and verified by Southern analyses using the appropriate restriction enzyme for each gene (Fig. 1C). For example, the deletion of the 1.6-kb *GIP3* gene (Δ*GIP3*) was confirmed by the presence of a single 9.2-kb hybridizing band in a blot of *Sal*I-digested genomic DNA of the Δ*GIP3* strains, rather than the 7.2-kb band that hybridized to the probe in the wild-type strain. Using the same procedure, all selected mutants of the other six genes showed a single expected hybridizing band, indicating that only one copy of the *hygB* gene had replaced each target gene at the mutant genome (Fig. 1C). For verifying deletion of *GIP6*, approximately 40 transformants were selected and analyzed for the gene deletion by Southern analyses, but there were no *GIP6* deletion mutants.

Phenotypes of the deletion mutants. In mutants grown on PDA, the pigmentation pattern depended on the genes deleted (Fig. 2). The Δ*GIP3* and Δ*GIP9* strains did not produce aurofusarin, but had a yellowish pigment that was a little darker than that of the Δ*GIP1* strain. The mycelia of the Δ*GIP4* strain produced yellowish pigment with pinkish margin that was a little lighter than that of the wild-type strain. The Δ*GIP7* strains seemed to produce yellowish pigment for 3-4 days. However, after 1 week, the mycelia of the Δ*GIP7* strains turned new yellowish-green that were never found in the wild type or other mutants. The undersurface of this mutant was darker green than the aerial mycelia. Finally, the Δ*GIP8* mutants produced reddish-orange mycelia. The Δ*GIP5* and Δ*GIP10* strains showed no difference in pigmentation from the wild type. The deletion of each gene of the aurofusarin biosynthesis cluster, i.e., *GIP3*, *GIP4*, *GIP5*, *GIP7*, *GIP8*, *GIP9*, and *GIP10*, had no effect on conidiation or the mycelial growth rate on PDA. In addition, all of these mutants showed no difference in virulence on barley compared to the wild-type strain (data not shown).

Aurofusarin analysis by HPLC. After 7 days on PDA plates, the wild-type Z03643 strain produced both aurofusarin and rubrofusarin, as detected by HPLC (Fig. 3A). The peaks were identified by comparing with those of authentic standards. Norrubrofusarin, aurofusarin, and rubrofusarin were detected at approximately 14, 16, and 19 min, respectively. The Δ*GIP3* strain produced a small amount of rubrofusarin (approximately 3% of that of the wild type). The Δ*GIP7* strain did not produce aurofusarin or rubrofusarin, but rather a new green pigment (detected at 14 min) that was not detected in the wild-type strain. We confirmed that compound as norrubrofusarin using liquid chromatography-mass spectrometry (LC-MS). Only rubrofusarin was detected in the Δ*GIP8* strain producing an orange-yellow pigment on PDA. The Δ*GIP9* strain, the yellow pigment producer, had a peak with the same retention time as rubrofusarin. Both aurofusarin and rubrofusarin were detected in Δ*GIP4*, Δ*GIP5*, and Δ*GIP10* strains at similar levels as in the wild-type strain.

Genetic complementation. To verify that the phenotypes

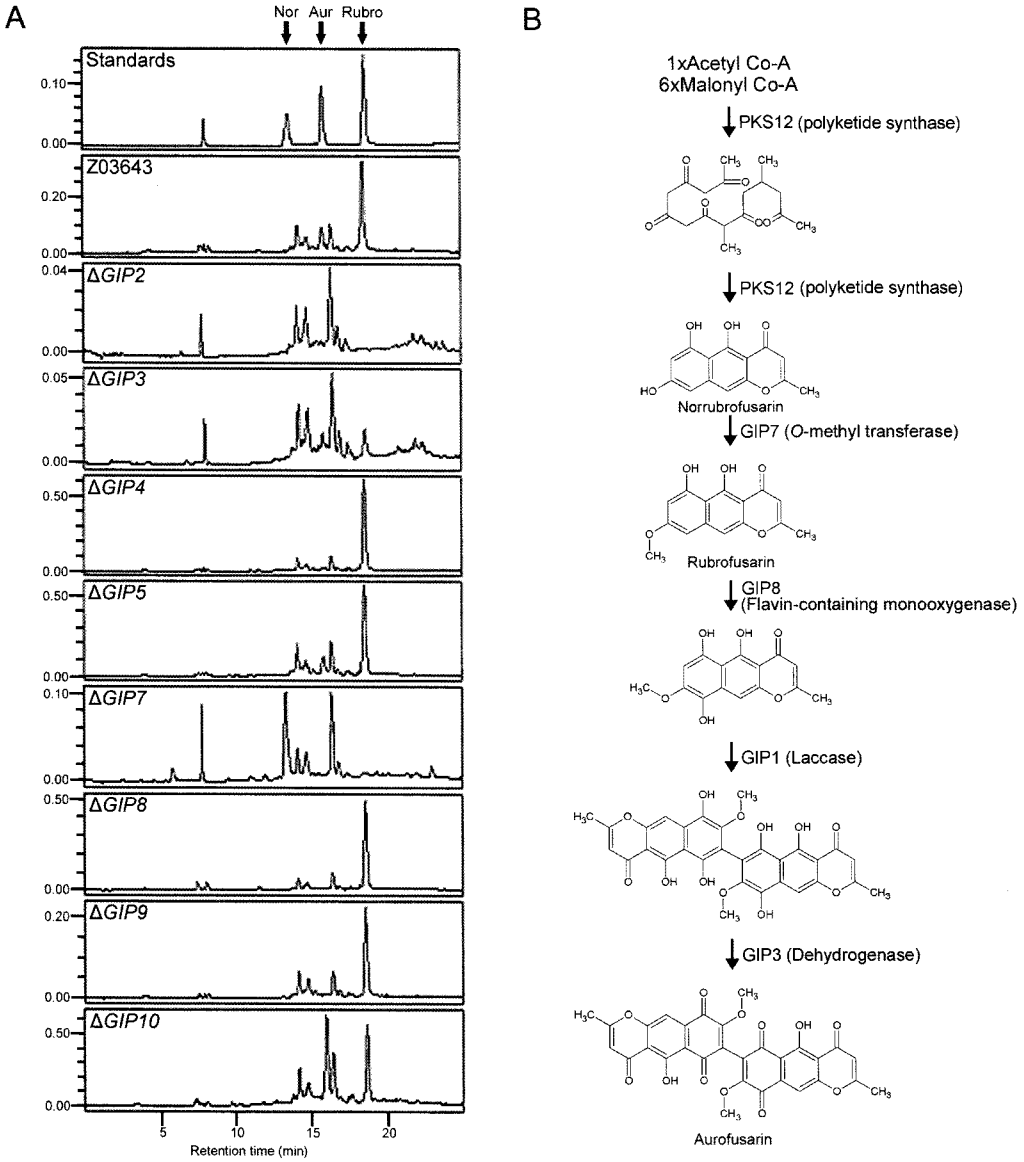

Fig. 3. HPLC analyses and predicted aurofusarin biosynthesis pathway. (A) HPLC chromatograms of pigments in wild-type *G. zeae* and transgenic strains with the deletion of aurofusarin biosynthetic genes. Nor, norrubrofusarin; Aur, aurofusarin; Rubro, rubrofusarin. (B) The predicted pathway from acetyl CoA to aurofusarin in aurofusarin biosynthesis. The hydroxyl group of primary product produced by *PKS12*, norrubrofusarin, is converted to a methyl group by *GIP7* resulting rubrofusarin. After oxidation by *GIP8* and dimerization of monomers by *GIP1*, aurofusarin is synthesized as a result of the dehydrogenation by *GIP3*. The order of reactions by *GIP1*, *GIP3*, and *GIP8* is not clear.

of the deletion strains were caused by the targeted deletion of the specific genes, the deletion strains of each gene were transformed with intact copies of the genes, including ~1 kb upstream and downstream of the coding region, using a co-transformation procedure. Among more than 25 geneticin-resistant transformants from each case, aurofusarin-producing transformants were examined using Southern hybridization. All of the pigmented transformants carrying an intact copy of the gene showed the phenotypes similar to that of Z03643 on PDA. HPLC analysis confirmed that these transformants produced aurofusarin at similar levels to the wild type (data not shown).

Discussion

Many filamentous fungi use the polyketide biosynthesis pathway to produce pigments, e.g., green conidial pigments (Langfelder et al., 1998), bikaverin (Linnemannstons et al., 2002), and melanin (Langfelder et al., 2003). Aurofusarin, a dimeric naphthoquinone metabolite, is a pigment that is produced by polyketide biosynthesis in some *Fusarium* species (Kim et al., 2005a; Malz et al., 2005). Many

researchers have intensively studied the functional characterization of the gene clusters involved in secondary metabolite biosynthesis after the release of the whole genome sequences for several fungi, including *G. zeae* (Frandsen et al., 2006; Kim et al., 2005b; Malz et al., 2005). Previously, we identified three genes involved in aurofusarin biosynthesis: *PKS12*, *GIP1*, and *GIP2*, which encode an unreduced fungal PKS, a putative laccase, and a putative transcription factor, respectively (Kim et al., 2006). Northern and RT-PCR analyses identified a 30-kb region including the three genes as a gene cluster for aurofusarun production in *G. zeae* (Kim et al., 2006; Malz et al., 2005).

The organization of the aurofusarin gene cluster is similar to that of other fungal polyketide biosynthetic gene clusters. The *GIP2*, *GIP4*, and *GIP7* genes show high similarity with *aflR*, *aflT*, and *aflJ*, respectively, which are involved in aflatoxin biosynthesis in *A. parasiticus*. GIP2 contains a zinc cluster motif that is found in aflR and other fungal transcriptional activators associated with several metabolic pathways (Kim et al., 2006; Malz et al., 2005). GIP4 is similar to aflT, a MFS transporter in the aflatoxin gene cluster. Although many MFS transporters like TRI12 are required for the secretion of fungal toxins, *aflT* does not have a significant role in aflatoxin secretion (Chang et al., 2004). The Δ*GIP4* strains showed little difference from the wild-type strain on PDA (Fig. 2) with similar pigment production, suggesting that the gene product of *GIP4* is not essential for aurofusarin biosynthesis, as shown for *aflT* in *A. parasiticus*. GIP7 exhibits a 29% amino acid identity to aflJ, which is a coactivator in aflatoxin biosynthesis. The *aflJ* deletion strains of *A. parasiticus* significantly decreased transcript levels of the aflatoxin biosynthetic genes (Chang, 2003). LC-MS analysis confirmed that the Δ*GIP7* strain accumulated the yellowish-green pigment, norrubrofusarin lacking one methyl group in rubrofusarin. This result suggests that norrubrofusarin is the precursor of rubrofusarin. *GIP7* also showed a higher similarity to the gene encoding *O*-methyltransferase than *aflJ*. Frandsen et al. (2006) found that GIP7 has an *O*-methyltransferase family 2 domain, which is shared with OmtA that encodes *O*-methyltransferase involved in aflatoxin biosynthesis. They also found no difference in the expression of aurofusarin biosynthetic genes between the *GIP7* (*aurJ*)-deleted strain and the wild type. These results confirmed that the function of *GIP7* is an *O*-methyltransferase, rather than a coactivator, in aurofusarin biosynthesis. GIP5 and GIP2 have a specific fungal transcription domain. Frandsen et al. (2006) demonstrated an increase in the rubrofusarin to aurofusarin ratio in a Δ*GIP5* (*aurR2*) strain compared to the wild type. They suggested that GIP5 could act as a co-regulator of GIP2 and fine-tune pigment production levels. However, the RT-PCR results showed no effects on the expression of aurofusarin biosynthetic genes in the cluster. To determine whether GIP5 also regulates the aurofusarin biosynthetic gene cluster, transcript analyses were performed in the wild-type and Δ*GIP5* strains. The expression levels of aurofusarin biosynthetic genes did not differ between the Δ*GIP5* and wild-type strains (data not shown), suggesting that GIP5 does not act as a transcription activator in aurofusarin biosynthesis even though GIP5 has a fungal transcription factor domain.

Based on predicted functions of the gene products in the cluster and the results from HPLC of deletion strains, we propose an aurofusarin biosynthetic pathway (Fig. 3B). It is notable that the Δ*PKS12* strain did not produce pigments. We propose that the pathway is initiated by the condensation of one acetyl-CoA and six malonyl-CoA and cyclization by PKS12. The Δ*GIP7* strain did not produce aurofusarin or rubrofusarin, but rather norrubrofusarin, a precursor of rubrofusarin. Thus, GIP7 catalyzes a primary reaction that modifies the PKS12 product to aurofusarin. GIP7 acts as an *O*-methyltransferase to convert the hydroxyl group of norrubrofusarin into a methyl group, converting norrubrofusarin to rubrofusarin. The Δ*GIP1*, Δ*GIP3*, and Δ*GIP8* strains all produced rubrofusarin. After oxidation by GIP8, GIP1 is probably involved in the dimerization of two monomers. Laccase catalyzes both the oxidation of hydroquinones and the dimerization of monomers (Claus, 2004; Marshall et al., 2000). In aurofusarin biosynthesis, the gene product of *GIP1* encoding fungal laccase acts in the latter case. GIP3 has a dehydrogenase domain that may catalyze the removal of a hydrogen atom from the dimeric structure. Aurofusarin is synthesized as a result of the dehydrogenation of the dimeric structure by GIP3. Frandsen et al. (2006) also used a HPLC analysis to suggest a similar aurofusarin biosynthetic pathway. GIP10 is similar to a multicopper oxidase regulated by GIP2, but the Δ*GIP10* strain did not show any difference from the wild type. The function of *GIP6* could not be determined since none of the Δ*GIP6* strains was generated in this study.

Pigments are known to be important for fungal survival, longevity, and virulence (Langfelder et al., 1998), and the protection of conidia against oxidative attack (Jahn et al., 2000). Some pigments also have antiprotozoan and antifungal activity (Linnemannstons et al., 2002). The aurofusarin-deficient transformants generated by Δ*PKS12* produce more zearalenone than does the wild type (Jung et al., 2006; Malz et al., 2005), whereas aurofusarin-overproducing strains produce no zearalenone, suggesting that aurofusarin negatively regulates zearalenone biosynthesis, although the underlying mechanism has not been elucidated. However, aurofusarin biosynthesis was not linked to fungal pathogenicity. Medentsev et al. (2005) showed that pigment biosynthesis in *Fusarium* sp., including aurofusarin biosynthesis, is affected by the pH of the culture

medium. Aurofusarin production is associated with a decrease in growth rate in extreme conditions, e.g., nitrogen or phosphorus deficiency, oxidative stress, or inhibition of respiration. Malz et al. (2005) demonstrated that the growth rate and conidia formation of aurofusarin-deficient mutants were higher than those of the wild type under pigment-inducing conditions. Our previous works also showed that aurofusarin production can significantly affect the growth of *G. zeae*, especially its mycelial growth (Kim et al., 2006; Lee et al., 2006). The aurofusarin-overproducing strains that had early accumulation of aurofusarin showed severe growth reduction. We propose that different effects of aurofusarin deficiency on mycelial growth might be attributed to genetic differences among *G. zeae* strains. It is possible that the early accumulation of aurofusarin can alter fungal metabolism against physiological stress.

In conclusion, through functional studies of the genes located at the aurofusarin biosynthetic gene cluster, we proposed a role for each gene product in the aurofusarin biosynthesis pathway in *G. zeae*. Our results will contribute to the understanding of naphthoquinone biosynthesis in other filamentous fungi as well as in *G. zeae*. However, further investigations should be required to determine the mechanism(s) underlying the aurofusarin-related phenotypes in which other fungal pigments may have a role.

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