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GIP2, a Putative Transcription Factor That Regulates the Aurofusarin Biosynthetic Gene Cluster in *Gibberella zeae*

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***Gibberella zeae* (anamorph: *Fusarium graminearum*) is an important pathogen of maize, wheat, and rice. Colonies of *G. zeae* produce yellow-to-tan mycelia with the white-to-carmine red margins. In this study, we focused on nine putative open reading frames (ORFs) closely linked to *PKS12* and *GIP1*, which are required for aurofusarin biosynthesis in *G. zeae*. Among them is an ORF designated *GIP2* (for G*ibberella zeae p*igment gene *2*), which encodes a putative protein of 398 amino acids that carries a $Zn(II)_2Cys_6$ binuclear cluster DNA-binding domain commonly found in transcription factors of yeasts and filamentous fungi. Targeted gene deletion and complementation analyses confirmed that *GIP2* is required for aurofusarin biosynthesis. Expression of *GIP2* in carrot medium correlated with aurofusarin production by *G. zeae* and was restricted to vegetative mycelia. Inactivation of the 10 contiguous genes in the Δ*GIP2* strain delineates an aurofusarin biosynthetic gene cluster. Overexpression of *GIP2* in both the Δ*GIP2* and the wild-type strains increases aurofusarin production and reduces mycelial growth. Thus, *GIP2* is a putative positive regulator of the aurofusarin biosynthetic gene cluster, and aurofusarin production is negatively correlated with vegetative growth by *G. zeae*.**

Fusarium head blight is caused by several species of *Fusarium*, including *Fusarium graminearum* (teleomorph: *Gibberella zeae*), *F. culmorum*, and *F. crookwellense*. These fungi are distributed worldwide and produce mycotoxins that cause economic losses in terms of crop and animal production. They also produce pigments that range from yellow to tan to carmine red (23). Two of the pigments produced by *G. zeae* and *F. culmorum* are naphthoquinones: aurofusarin and rubrofusarin (1, 8, 28). Aurofusarin is toxic to poultry and can reduce the nutritional quality of quail eggs (5, 6). Rubrofusarin is antimycobacterial, antiallergenic, and phytotoxic (7, 14, 15, 20).

We previously identified a type I polyketide synthase gene (*PKS12*) and a putative laccase gene (*GIP1*) that are required for aurofusarin biosynthesis in *G. zeae* (13). Thus, aurofusarin is synthesized in *G. zeae* in a manner similar to that used for other fungal polyketide pigments. *Agrobacterium*-mediated transformation of *F. pseudograminearum* also results in aurofusarin-deficient mutants, and targeted mutagenesis in *G. zeae* confirmed the function of *PKS12* (21). However, the role(s) of aurofusarin production in the physiology of *G. zeae* is not well understood. It may play a role in vegetative growth and zearalenone production in *G. zeae* based on the phenotype of aurofusarin-deficient mutants (21).

Genes involved in the biosynthesis of secondary metabolites are often clustered in filamentous fungi (11). For example, clusters for biosynthetic genes of trichothecenes (9), fumonisins (27), and gibberellins (31) have been identified in *Fusarium* species that produce these metabolites. These clusters include genes encoding metabolic enzymes, transcription factors, and transporters. Thus the genes encoding the protein products for aurofusarin biosynthesis might also be clustered. Previously, putative open reading frames (ORFs) were found near the *PKS12* gene in *G. zeae* (13, 21); one of them, which was similar to a fungal transcription factor, was involved in expression of *PKS12* (21). However, the role of this putative transcription factor for aurofusarin biosynthesis is unproven and the limits of the aurofusarin gene cluster are unknown.

Our objectives in this study were (i) to determine whether *GIP2* is required for aurofusarin biosynthesis, (ii) to identify the genes in the aurofusarin biosynthetic gene cluster, and (iii) to determine whether overproduction or underproduction of aurofusarin affects growth and colony morphology of *G. zeae*. The results from this study and further characterization of the aurofusarin biosynthetic genes will be used to determine the biological significance of aurofusarin in the growth and physiological activities of *G. zeae*.

MATERIALS AND METHODS

Strains and media. Strains SCKO4 and Z03643 were used as wild-type strains of *G. zeae* (13). SCKO4 is a lineage 6 strain that produces nivalenol and zearalenone. Z03643 is a lineage 7 strain that produces deoxynivalenol and zearalenone (24) and is more pigmented than SCKO4 (13). Fungal strains were stored as spore suspensions in 20% glycerol at −80°C. For inoculum and pigment production, the strains were grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) at 25°C. For DNA extraction, the strains were inoculated in 100 ml of complete medium in 250-ml Erlenmeyer flasks (4) and grown for 3 days at 25°C on a rotary shaker at 150 rpm. For RNA extraction and induction of sexual development, fungi were grown on carrot medium as previously described (16).

Nucleic acid manipulations and PCR primers. Fungal genomic DNA was extracted as described previously (12). *Escherichia coli* colonies carrying recombinant plasmids were screened by using a single-tube miniprep method (19). For fungal transformation, plasmids were purified from 5 ml of *E. coli* culture by

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TABLE 1. Primers used in this study

Primer	Sequence[a] (5′ to 3′)	Position[b] (bp at contig) or description
G2-5′f	TTTGCGGGATGATATGACTGAG	123,112–123,091 at 1.116
G2-5′r	CTCCACTAGCTCCAGCCAAGC AAACGCAGCTAACAGAGGAGA	121,978–121,999 at 1.116
G2-3′f	TGAAAATTCCGTCACCAGCCTC TCGCGGCAGCTTGTAATCAT	120,415–120,394 at 1.116
G2-3′r	GCCATGCTAGCCCAACTCTCC	118,949–118,969 at 1.116
NG2-5′f	GCTTGCATCGGACCGAAGGAATGA	122,880–122,856 at 1.116
NG2-3′r	GCCCTGAGCTTGCAGCAGAGTGTCTT	119,164–119,189 at 1.116
P12-for	TATAGGGGATTGTGCTTC	136,498–136,481 at 1.116
P12-rev	AATACACAAACAGCCCTCTC	129,906–129,925 at 1.116
G1-for	AGGAGCCCTCCAAGTAAT	144,157–144,140 at 1.116
G1-rev	TCAACGCAGTCGATTGTA	141,752–141,769 at 1.116
G2-for	ACGACCTTCCTAAACTGCACCTATCA	122,182–122,157 at 1.116
G2-rev	GGATGCCTGCACCCACCTA	120,235–120,253 at 1.116
G3-for	GGCTTAACGGCTGAGGACCAAT	122,940–122,961 at 1.116
G3-rev	GCACGGCCGATCTCATCAAG	124,669–124,650 at 1.116
G4-for	GCATCACCATTCAATCCT	127,093–127,076 at 1.116
G4-rev	CCTTTTTATTGCATTGCA	125,277–125,294 at 1.116
G5-for	TCTCGTCAATACCAACCC	127,538–127,555 at 1.116
G5-rev	GCAGATTGCCTTCATTCT	129,844–129,827 at 1.116
G6-for	CGCCTCATAGTGATACCCAAGAAA	139,925–136,948 at 1.116
G6-rev	AAACGCGGATCTGCCTTCAT	137,264–137,245 at 1.116
G7-for	GAACACGGCATCCACTGTAAGAT	139,307–139,285 at 1.116
G7-rev	GCGATATCAGCGAGATCAAAAATA	137,647–137,670 at 1.116
G8-for	CACCCCGACCCGAAGAGC	139,452–139,469 at 1.116
G8-rev	CTCACAACAGTCAATCAGGAACCAC	141,641–141,617 at 1.116
G9-for	TCATCAATGTCAGCCAAG	144,377–144,394 at 1.116
G9-rev	CGAGTCGCACTGAGTATG	145,843–145,826 at 1.116
G10-for	GCTTGCCATCTCGAGTTTGAAT	148,254–148,233 at 1.116
G10-rev	CAGTGCGACTCGAATGAGGC	145,832–145,851 at 1.116
G2ov-p1	CATTACATACAGCCGTCAATA TGAGTTCCACAGACCCCCTTC	121,713–121,692 at 1.116
G2ov-p2	GTGTTGGTGCCGTTGCCC	119,150–119,167 at 1.116
G2ov-p3	TGTTCGTCGTCGGCTTCGTTC GCGGGGTCTACAATAAGC	123,273–123,293 at 1.116
G2ov-p4	GCACGGCCGATCTCATCAAG	124,669–124,650 at 1.116
bpro-5′	CGAAGCCGACGACGAACA	49,310–49,327 at 1.393
bpro-3′	ATTGACGGCTGTAGATGTAATG	51,455–51,434 at 1.393
NG2ov-5′f	ACCCAATACTATCGCCTGTCG	124,493–124,473 at 1.116
NG2ov-3′r	GGAACGCCTTGAAGAGAATGTC	119,613–119,634 at 1.116
nHygB-f	CTTGGCTGGAGCTAGTGGAGGT	For amplification of *hygB* cassette from pBCATPH
nHygB-r	GGCTGGTGACGGAATTTTCATA	For amplification of *hygB* cassette from pBCATPH

[a] Underlined sequences with the same patterns (e.g., light underline, heavy underline, double underline, dotted underline) are complementary and promote hybridization between the PCR products.

[b] The positions indicated as bp at contig are from the *F. graminearum* database at http://www.broad.mit.edu/annotation/fungi/fusarium/index.html.

using a plasmid purification kit (NucleoGen Biotech, Siheung, Korea). Total RNA was extracted from mycelia (0.1 to 0.2 g) or lawns of perithecia ground in liquid N_2 with 1 ml of TRIZOL reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. DNA and RNA gel blot hybridizations with ^{32}P-labeled probes were made as previously described (26). DNA fragments used as probes were amplified from genomic DNAs of Z03643 and SCKO4 with proper primer pairs (Table 1) as described previously (18). PCR primers were obtained from the Bioneer oligonucleotide synthesis facility (Bioneer Corporation, Chung won, Korea), resuspended at 100 μM in sterile water, and stored at −20°C.

Construction of transforming vectors. For PCR-based construction of fungal transforming vectors, a double-joint PCR method with appropriate primers (Table 1) was employed as previously described (33). To delete *GIP2*, DNA fragments corresponding to regions 5′ (1.1 kb) and 3′ (1.5 kb) of the *GIP2* ORF were amplified from genomic DNA of either Z03643 or SCKO4 with primer pair G2-5′f and G2-5′r and primer pair G2-3′f and G2-3′r, respectively. A 1.8-kb fragment containing the *hygB* gene under the control of the *Aspergillus nidulans trpC* promoter and terminator was amplified from the vector pBCATPH (34) with primers nHygB-f and nHygB-r. Three amplicons (5′-flanking region of *GIP2*, the *hygB* cassette, and the 3′-flanking region of *GIP2*) were mixed in a 1:2:1 ratio, and a second round of PCR was carried out. Using a new nested primer pair (NG2-5′f and NG2-3′r, which are nested in G2-5′f and G2-3′r, respectively) and the second PCR product as the template, a 4.4-kb fusion PCR product was amplified.

For the *GIP2* construct fused to a putative promoter of the *G. zeae* β-tubulin gene (GenBank accession no. AAP68979), a 2.6-kb region containing the *GIP2* ORF and its 3′ flank (1.3 kb) and a 1.4-kb 5′-flanking region of *GIP2* were amplified from Z03643 with primer pair G2ov-p1 and G2ov-p2 and primer pair G2ov-p3 and G2ov-p4, respectively. These amplicons and a 2.1-kb putative β-tubulin gene promoter amplified from Z03643 with primer pair bpro-5′ and bpro-3′ were fused each other in a PCR with a nested primer pair (NG2ov-5′f and NG2ov-3′r), resulting in a 5.5-kb fusion PCR product.

Fungal transformation. Following phenol extraction and ethanol precipitation, ~5 μg of the final PCR product alone, if it carried a selectable marker, was incorporated directly into fungal protoplasts or in combination with plasmid pSK660 which carries the geneticin resistance gene (*gen*) as a selectable marker as previously described (13).

RESULTS

Structural organization of a putative *PKS12* gene cluster. The 30 kb-region carrying *PKS12* and *GIP1*, required for au-

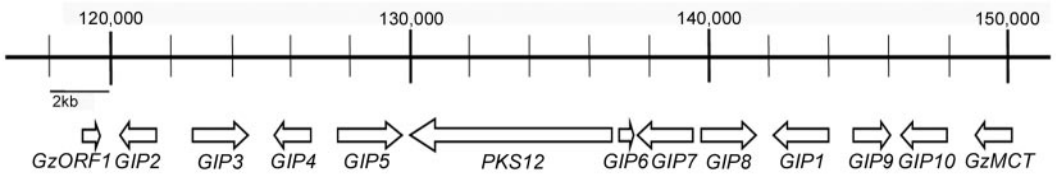

FIG. 1. Molecular organization of the putative aurofusarin biosynthesis cluster in *G. zeae*. This cluster was found in contig 1.116 from the *F. graminearum* genome databases. Numbers and arrows indicate possible ORFs and transcriptional directions, respectively. Nucleotide positions of the contig are indicated on the thick vertical bar.

rofusarin biosynthesis in *G. zeae*, contains several ORFs, including sequences that are similar to transcription factors, an efflux pump, and some metabolic enzymes (Fig. 1, Table 2). This putative *PKS12* gene cluster was arbitrarily defined, because there is no significant sequence structure outside of this region for proximity of a gene cluster. A 1.2-kb ORF designated *GIP2*, which contains no putative intron, exhibited the highest amino acid identity (21%) to the transcription factor AFLR required for sterigmatocystin or aflatoxin production in *Aspergillus* species (32). However, significant identity (53%) with AFLR was mostly due to the conserved $Zn(II)_2Cys_6$-type DNA binding motif that frequently occurs in other transcription factors of yeasts and filamentous fungi (3).

Targeted deletion of *GIP2*. We deleted *GIP2* from strains Z03643 and SCKO4. The entire ORF of *GIP2* was replaced with the fungal selectable marker *hygB* via double homologous recombination between a PCR fragment carrying both the 5′ and 3′ regions of the *GIP2* ORF fused to *hygB* and the corresponding genomic regions (Fig. 2A). Genomic DNAs of the Δ*GIP2* strains derived from Z03643 and SCKO4 carried a 13.1-kb band and a ~22.8-kb hybridizing band, when digested with KpnI, instead of the 2.5-kb and ~9.7-kb bands found in the wild-type strains, respectively, suggesting that the 1.2-kb *GIP2* ORF had been deleted and replaced with the *hygB* gene (Fig. 2B). The Δ*GIP2* strains derived from both strains could not produce aurofusarin when grown on PDA, but a transgenic strain carrying the transforming DNA at an ectopic position had red wild-type pigmentation (Fig. 2C). Radial growth on PDA of the Δ*GIP2* transgenic Z03643 strains increased ~30% relative to that of its wild-type progenitor. Mycelial growth of the transgenic Δ*GIP2* strain of SCKO4 was not significantly different from that of the untransformed parent (data not shown).

Complementation analyses. Intact copies of *GIP2* were introduced into a Δ*GIP2* mutant by cotransformation (Fig. 3A). Of 16 geneticin-resistant transformants with *GIP2*, four produced as much aurofusarin as Z03643 (Fig. 3B). One of these transformants has integrated the *GIP2* gene at its original genomic locus by homologous recombination (Fig. 3C). To determine whether the heterologous genes were expressed in these transformants, total RNA was extracted from colonies grown on carrot medium and hybridized with *GIP2*. In pigmented transformants carrying *GIP2*, *GIP2* transcripts were present at a level similar to that found in the wild-type strain (Fig. 3D).

Transcript analyses. To determine whether the nine genes closely linked to both *PKS12* and *GIP1* are associated with aurofusarin biosynthesis, we analyzed their expression patterns on carrot medium, which is conducive to aurofusarin production by *G. zeae* (13). Total RNA was extracted from the mycelia of both Z03643 and the transgenic Δ*GIP2* strain (Tzg2-1) grown for 3, 6, 9, 12, and 15 days. Transcripts of all 11 genes (*GIP2* to *GIP10*; Fig. 1) were detected beginning either 3 or 6 days after inoculation in Z03643, but none of the genes except *GIP3* and *GIP5* were expressed in Tzg2-1 during the entire incubation period (Fig. 4A). The expression pattern for most of the genes paralleled the time course of fungal pigmentation, but signal intensities differed between individual genes. The transcripts of *PKS12* and *GIP8* began to accumulate on day 6 and increased gradually until day 15. Transcripts of *GIP2*,

TABLE 2. Nucleotide sequence similarities of the genes located at the 30-kb region carrying *PKS12*

Gene	Locus[a]	Similarity (accession no.)	Species	E value
PKS12	FG02324.1	Polyketide synthase (AF025541)	*A. fumigatus*	0
GIP1	FG02328.1	brown 2 (AF104823)	*A. fumigatus*	e−142
GIP2	FG02320.1	AFLR (AY618557)	*A. flavus*	8e−08
GIP3	FG02321.1	FAD/FMN-containing dehydrogenases (NZ_AAED01000004)	*Mesorhizobium* sp.	9e−29
GIP4	FG02322.1	DHA14-like major facilitator (AF238225)	*Botryotinia fuckeliana*	e−108
GIP5	FG02323.1	Putative transcriptional activator (NP_593170)	*Schizosaccharomyces pombe*	6e−14
GIP6	FG02325.1	Hypothetical protein	*A. nidulans*	3e−29
GIP7	FG02326.1	AFLJ (AY510453)	*A. flavus*	3e−24
GIP8	FG02327.1	Flavin-containing monooxygenase 5 (AAA67848)	*Cavia porcellus*	1e−44
GIP9	FG02329.1	Fasciclin I family protein, putative (BX649607)	*A. fumigatus*	8e−27
GIP10	FG02330.1	Ascorbate oxidase (AB010110)	*Acremonium* sp.	e−130
GzORF1	FG02319.1	Predicted protein (EAA69859)	*G. zeae*	2e−104
GzMCT	FG02331.1	Major superfacilitator superfamily monocarboxylate transporter, putative	*A. fumigatus*	6e−29

[a] The sources of each locus from the *F. graminearum* database can be found at http://www.broad.mit.edu/annotation/fungi/fusarium/index.html.

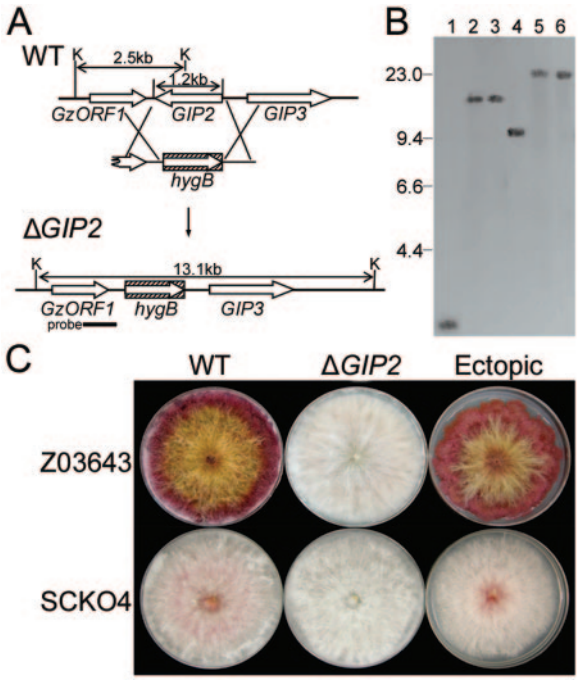

FIG. 2. Targeted deletion of *GIP2* from the genome of wild-type *G. zeae* strains Z03643 and SCKO4. (A) Deletion strategy. WT, genomic DNA of the wild-type strain Z03643; Δ*GIP2*, genomic DNA of the strain with *GIP2* deleted; K, KpnI; *hygB*, hygromycin B resistance gene. The probe used for blot hybridization, which is amplified from genomic DNA of Z03643 with primers G2-3′f and G2-3′r (Table 1), is indicated by a thick bar. (B) Gel blot of KpnI-digested genomic DNAs from Δ*GIP2* strains, hybridized with the probe. Lanes 1 and 4, Z03643 and SCKO4, respectively; lanes 2 and 3, the Δ*GIP2* strains of Z03643 (Tzg2-1 and Tzg2-2, respectively); lanes 5 and 6, the Δ*GIP2* strains of SCKO4 (Tsg2-1 and Tsg2-2, respectively). The sizes of λDNA standards (in kilobases) are indicated on the left of the blot. (C) Pigmentation of transformants of Z03643 and SCKO4. WT, wild-type strains; Δ*GIP2*, the *GIP2*-deleted strains Tzg2-1 and Tsg2-1; Ectopic, transformants carrying ectopic vector integrations (Tzg2-5 and Tsg2-8).

GIP4, *GIP5*, and *GIP7* were first detected on day 3, and the accumulation levels remained high through day 15. No transcripts of the putative ORFs (*GzORF1* and *GzMCT*) that were located 3′ of *GIP2* and 5′ of *GIP10*, respectively, were detected in either Z03643 or Tzg2-1 (data not shown). Thus, the coregulated set of 11 contiguous genes probably defines the aurofusarin biosynthetic gene cluster regulated by *GIP2*. No transcripts of *GIP2* or *PKS12* were detected in Z03643 during sexual development, i.e., during perithecial formation on carrot agar medium (Fig. 4B).

Overexpression of *GIP2*. We cotransformed the Δ*GIP2* mutant with a 5.5-kb DNA fragment (Fig. 5A) carrying the *GIP2* ORF fused to the β-tubulin promoter of *G. zeae* to assess the effects of GIP2 overexpression. Of 15 geneticin-resistant transformants derived from Tzg2-1, four were sensitive to hygromycin B, which suggested that the heterologous *GIP2* gene under the control of β-tubulin gene promoter was integrated into the original *GIP2* genomic locus. The presence of hybridizing bands (2.5 kb and 1.3 kb) of the expected size confirmed this type of integration (Fig. 5B). All four $hygB^S$ gen^R transformants were more pigmented than was Z03643 (Fig. 5C). These transformants began to produce red pigments on PDA 2 days after inoculation, while strain Z03643 did not begin to produce pigments until day 4. Mycelial growth of all of four transformants was severely retarded relative to that of Z03643. A similar pattern was observed when GIP2 was overproduced in the Δ*GIP2* SCKO4 strains, but the reduction in radial growth of these transformants was not as severe as was the reduction in the strains derived from Z03643 (Fig. 5C). The overexpressing transformants produced ~50% less fungal mass but three times more aurofusarin in potato broth at 3 days after inoculation compared with wild-type strain results (data not shown).

The fused β-tubulin/GIP2 construct also was introduced into the wild-type Z03643 and SCKO4 strains. These transformants

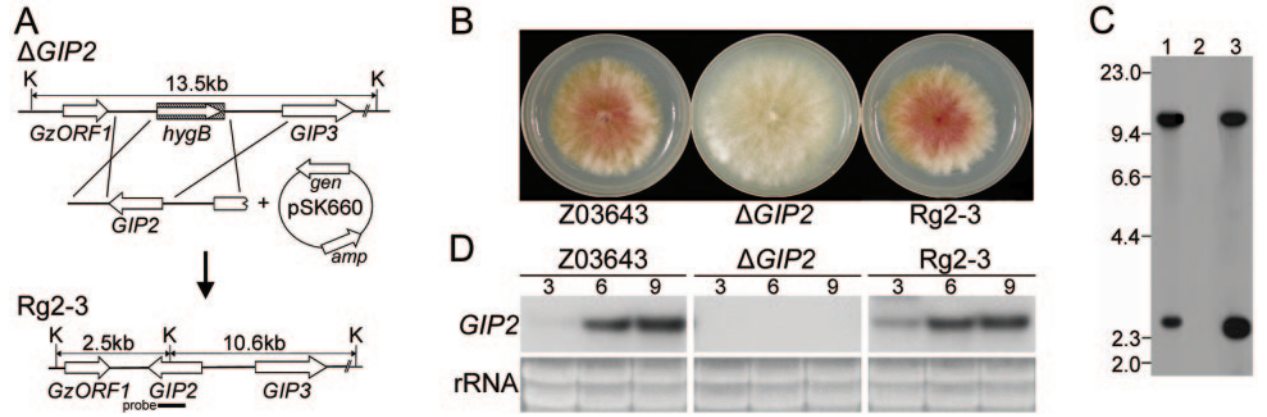

FIG. 3. Complementation of pigmentation in the *G. zeae* Δ*GIP2* strain. (A) Complementation strategy with *GIP2* from *G. zeae*. Δ*GIP2*, genomic DNA from the *GIP2*-deleted strain of Z03643 (Tzg2-1); Rg2-3, genomic DNA of the transgenic Tzg2-1 strain carrying *GIP2*; K, KpnI; *hygB*, hygromycin resistance gene; *gen* and *amp*, genes conferring resistance to geneticin and ampicillin, respectively. *GzORF1* and *GIP3* are indicated by open arrows. (B) Pigmentation of the transgenic strains examined by DNA gel blot analysis. Left, Z03643; middle, Tzg2-1; right, Rg2-3. (C) DNA gel blots of transformants derived from the Δ*GIP2* strain by using an intact copy of the *GIP2* ORF. Genomic DNAs of these transformants were digested with KpnI and hybridized with the probes indicated in panel A. The probe for hybridization with *GIP2* was amplified from genomic DNA of Z03643 with primers G2-for and G2-rev (Table 1). Lane 1, wild-type strain Z03643; lane 2, Δ*GIP2* recipient strain Tzg2-1; lane 3, transformants (Rg2-3) carrying *GIP2*. The sizes of λDNA standards (in kilobase) are indicated on the left of the blot. (D) RNA gel blot of strain Z03643 and the transgenic Δ*GIP2* strain probed with *GIP2*. Z03643 and Δ*GIP2*, total RNAs from wild-type strain and the Δ*GIP2* recipient strain Tzg2-1, respectively; Rg2-3, total RNAs from transgenic Tzg2-1 strains carrying *GIP2*. The probe and the incubation time are indicated on the left and above, respectively. The ethidium bromide-stained rRNAs are indicated as a loading control.

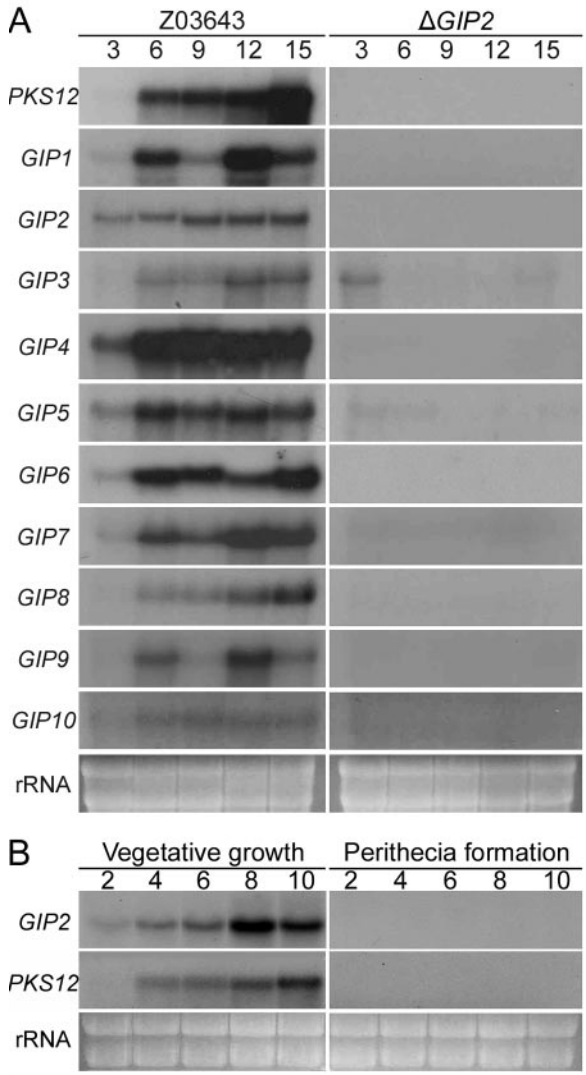

FIG. 4. Transcript analyses of the aurofusarin gene cluster. (A) RNA gel blots of Z03643 and its Δ*GIP2* strain Tzg2-1, probed with each cluster gene. Z03643 and Δ*GIP2*, total RNAs from strains Z03643 and Tzg2-1 grown on carrot medium, respectively. The probe and the incubation time are indicated on the left and above, respectively. The probes used for hybridization were amplified from genomic DNA of Z03643 by use of the following primer pairs: P12-for and P12-rev for *PKS12*, G1-for and G1-rev for *GIP1*, G2-for and G2-rev for *GIP2*, G3-for and G3-rev for *GIP3*, G4-for and G4-rev for *GIP4*, G5-for and G5-rev for *GIP5*, G6-for and G6-rev for *GIP6*, G7-for and G7-rev for *GIP7*, G8-for and G8-rev for *GIP8*, G9-for and G9-rev for *GIP9*, and G10-for and G10-rev for *GIP10* (Table 1). (B) RNA gel blots of Z03643 grown on carrot medium, probed with *GIP2* and *PKS12*, respectively. The incubation times for vegetative growth and perithecia induction are indicated above the blots. The ethidium bromide-stained rRNAs are indicated as a loading control.

had unstable mycelial morphology and produced sectored colonies with differing morphologies. Seventeen transformants of Z03643 were highly pigmented and had slow growth. Fifteen of these 17 transformants frequently produced fast-growing sectors on PDA plates (Fig. 6A). When the mycelia of the sector were transferred to a fresh PDA plate, they grew as fast as the original wild-type strain but were much less pigmented; this phenotype remained stable for five successive transfers on PDA. When mycelia of the highly pigmented original transformants were transferred to fresh media, the resulting colonies continued to form sectors.

The highly pigmented transformants carried the heterologous *GIP2* gene, which was integrated into the genome by a single homologous recombination event between a circular PCR product and the corresponding genomic region (Fig. 6B). The hybridization patterns in transformant Ozg2-2-1 are consistent with this integration mechanism. The four expected hybridizing bands appeared when probed with *GIP2*, but only two of four bands were seen when probed with 3′ flanking region of *GIP2* (Fig. 6B and C). The presence of a ~3.0-kb band, instead of the expected 4.2-kb band, when probed with *GIP2* may have resulted from loss of terminal portions of the PCR product during the circularization through self-ligation. Genomic DNA of the mycelia from the fast-growing, less-pigmented sector had the same hybridization pattern as that of Z03643 when probed with *GIP2*, indicating that the sector no longer carried the heterologous *GIP2* sequence. These possible revertants could be generated by looping out and deleting the β-tubulin promoter from the chromosome through homologous recombination between adjacent two copies of *GIP2* (carrying heterologous and endogenous promoters, respectively) in the transformant Ozg2-2-1 (Fig. 6C). The same sectoring phenotype also was observed in the transformants of SCKO4 that carried the heterologous *GIP2* sequence (Fig. 6A).

Northern blot analysis confirmed overexpression of both *PKS12* and *GIP2* in transformants carrying the β-tubulin/GIP2 constructs. The transcripts of both genes were present at high levels from day 3 in the *GIP2*-overexpressing strains, but little or no transcript was detected in the wild-type strains (Fig. 7).

DISCUSSION

In filamentous fungi, genes responsible for the biosynthesis of secondary metabolites often are coordinately regulated and physically clustered in the genome. We previously described two aurofusarin biosynthetic genes, *PKS12* and *GIP1*, which encode an unreduced polyketide synthase and a putative laccase, respectively (13). In this report, we identify an additional gene, designated *GIP2*, that is closely linked to these genes and that is essential for aurofusarin biosynthesis in *G. zeae*. The requirement for all of these closely linked genes for aurofusarin biosynthesis suggests that aurofusarin is produced in *G. zeae* through the coordinated activities of the proteins encoded by the genes in the cluster, as is known for other fungal secondary metabolites (9, 11, 27, 31).

Eight additional genes were closely spaced within the 30-kb region carrying the three genes known to be involved in aurofusarin biosynthesis. Sequence similarity of the putative GIP2 protein to fungal transcription factors, especially those associated with a number of metabolic pathways (29), suggests that *GIP2* plays an important role in the transcription of the putative clustered genes. Malz et al. (21) reported that *GIP2* is important for regulating *PKS12* and other genes in the putative aurofusarin biosynthetic gene cluster. They provided reverse transcription-PCR data for altered expression of the genes in the *G. zeae* strains with insertional mutations in the promoter

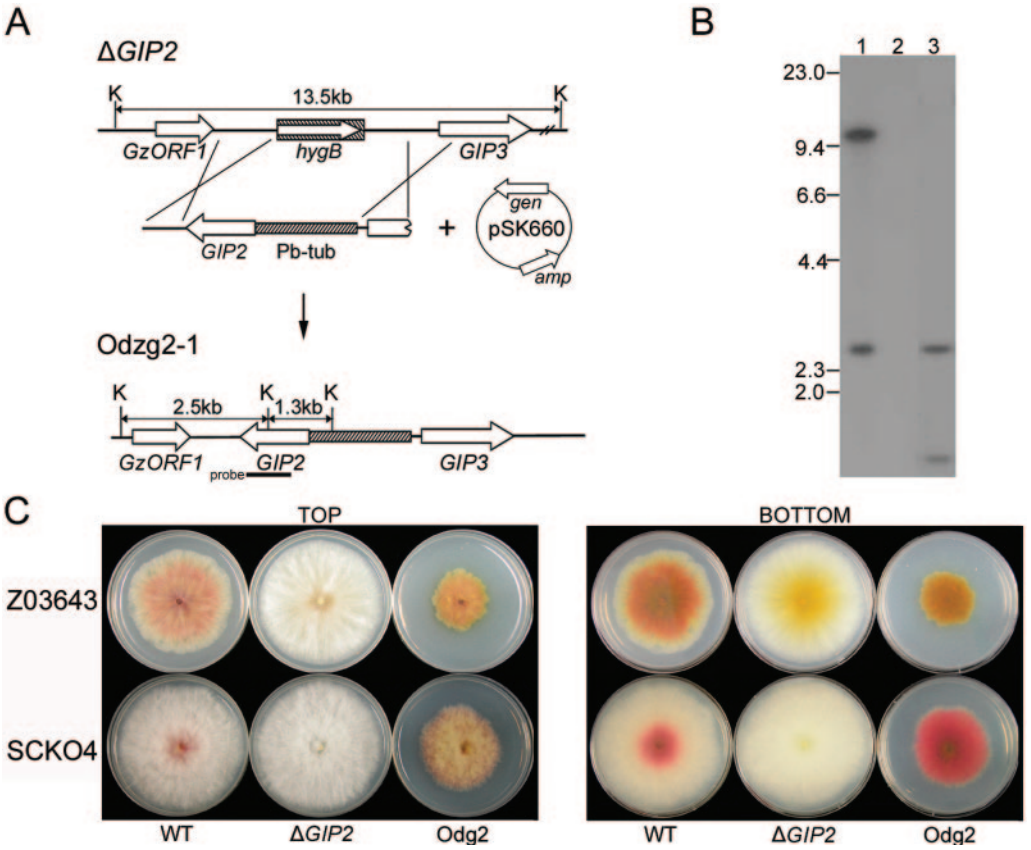

FIG. 5. Overexpression of *GIP2* in *G. zeae* Δ*GIP2* strains. (A) Overexpression strategy. Δ*GIP2*, genomic DNA of Tzg2-1, the Δ*GIP2* strain derived from Z03643; Odzg2-1, genomic DNA of Odzg2-1, the transgenic Tzg2-1 strain carrying the *GIP2* gene under control of the *G. zeae* β-tubulin gene promoter; pSK660, a vector used for cotransformation; K, KpnI; Pb-tub, promoter region of the β-tubulin gene from Z03643; *hygB*, hygromycin resistance gene; *gen* and *amp*, genes conferring resistance to geneticin and ampicillin, respectively. The probe used for blot hybridization, which is amplified from genomic DNA of Z03643 with primers G2-for and G2-rev (Table 1), is indicated by a thick bar. *GzORF1* and *GIP3* are indicated by open arrows. (B) DNA gel blot of transformants derived from Δ*GIP2* strains, hybridized with *GIP2*. Lane 1, Z03643; lanes 2, Tzg2-1, the Δ*GIP2* recipient strains derived from Z03643; lanes 3, Odzg2-1, a transformant derived from Tzg2-1. The sizes of λDNA standards (in kilobases) are indicated on the left of the blot. (C) Pigmentation of transgenic strains. The upper and lower plates in each panel indicate the strains derived from Z03643 and SCKO4, respectively. WT, wild-type strains; Δ*GIP2*, the transgenic strains with *GIP2* deleted (Tzg2-1 and Tsg2-2 derived from Z03643 and SCKO4, respectively); Odg2, the *GIP2*-overexpressing transformants from the Δ*GIP2* strains (Odzg2-1 and Odsg2–2 derived from Tzg2-1 and Tsg2-2, respectively).

region of *GIP2*. In this study we generated RNA gel blots with Δ*GIP2* and *GIP2*-overexpressing *G. zeae* strains to conclusively define the genes in the aurofusarin biosynthetic gene cluster in *G. zeae* and to show that *GIP2* was required for transcription of the clustered genes.

Coexpression of the same set of genes (11 contiguous genes) in the wild type and their lack of expression in the Δ*GIP2* strain under conditions favorable for pigmentation suggest that the gene cluster, coordinately induced by *GIP2*, is associated with aurofusarin biosynthesis in *G. zeae*. In addition, the high and early accumulation of *PKS12* transcripts in the overexpressing *G. zeae* strain is consistent with the hypothesis that *GIP2* is a transcriptional activator. Additional work remains to be done for the other eight genes in the aurofusarin biosynthetic cluster. We have not shown that GIP2 binds to specific sequences in the promoter regions of the aurofusarin genes. Neither has the role of two other putative transcription factors (*GIP5* and *GIP7*) been determined. Furthermore, it is possible that *GIP2* may regulate genes other than those involved exclusively in aurofusarin biosynthesis, as has been shown for a regulatory gene *TRI10* in trichothecene biosynthesis in *F. sporotrichioides* (25).

Aurofusarin production can significantly affect the growth of *G. zeae*, especially the mycelial growth. The faster growth rate of the Δ*GIP2* mutants of Z03643 suggests that aurofusarin accumulation may adversely affect mycelial growth of *G. zeae*. A similar result was obtained with the albino, Δ*PKS12* mutants from *G. zeae* strain PH-1 (21). These results are consistent with previous reports that highly pigmented *G. zeae* isolates infected with double-stranded RNAs also have reduced growth (2). However, this phenotypic change is neither simple nor universal, since Δ*GIP2* mutants from SCKO4 did not grow at a rate obviously different from that of the SCKO4 wild type. The different effects of aurofusarin deficiency on mycelial growth may be attributed to genetic differences between these three *G. zeae* strains. SCKO4, a lineage 6 strain obtained from barley in Korea (13), is less pigmented than are Z03643 and PH-1, which belong to lineage 7 and were collected in the

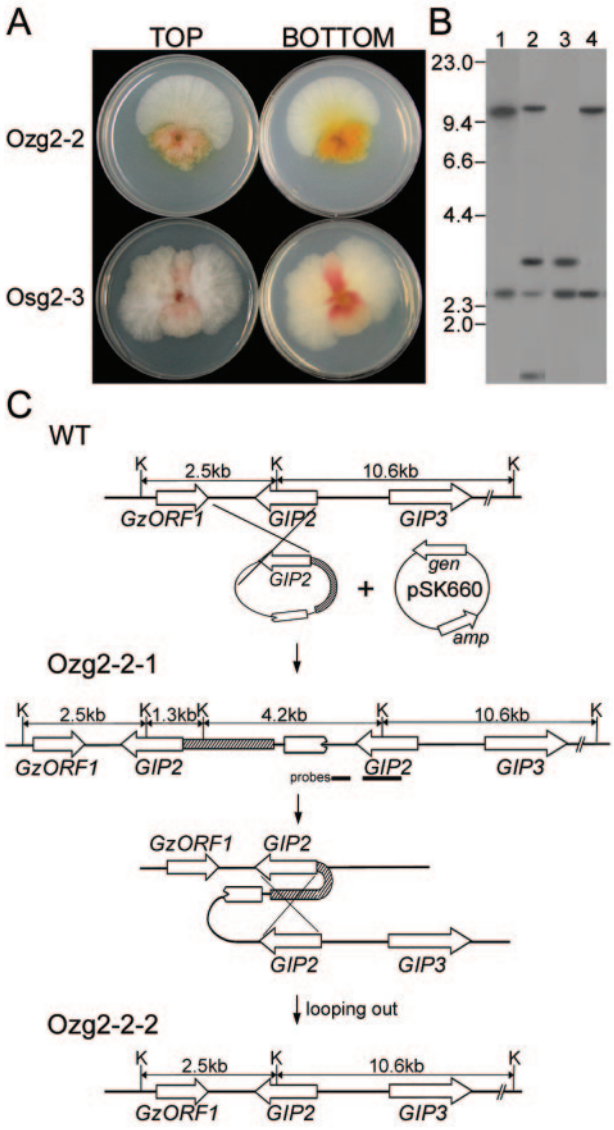

FIG. 6. Overexpression of *GIP2* in wild-type *G. zeae* strains Z03643 and SCKO4. (A) Pigmentation of transgenic strains. The upper and lower plates in each panel indicate transgenic strains carrying the heterologous *GIP2*, Ozg2-2, and Osg2-3 derived from Z03643 and SCKO4, respectively. (B) DNA gel blot of transformants derived from wild-type strains. Lane 1, Z03643; lanes 2 and 3, Ozg2-2-1 probed with *GIP2* and a 3′ flanking region of *GIP2*, respectively; lane 4, Ozg2-2-2, probed with *GIP2*. The sizes of λDNA standards (in kilobases) are indicated on the left of the blot. (C) Overexpression strategy. WT, genomic DNA of Z03643; Ozg2-2-1, genomic DNA from mycelia of the highly pigmented original transformant Ozg2-2; Ozg2-2-2, genomic DNA from the wild-type sector from Ozg2-2; pSK660, a vector used for cotransformation; K, KpnI; Pb-tub, promoter region of the β-tubulin gene from Z03643; *gen* and *amp*, genes conferring resistance to geneticin and ampicillin, respectively. The probes of *GIP2* and the 3′ flank of *GIP2*, which are amplified from genomic DNA of Z03643 with primer pairs G2-for and G2-rev and G2-3′f and G2-3′r, respectively (Table 1), are indicated by thick bars. *GzORF1* and *GIP3* are indicated by open arrows.

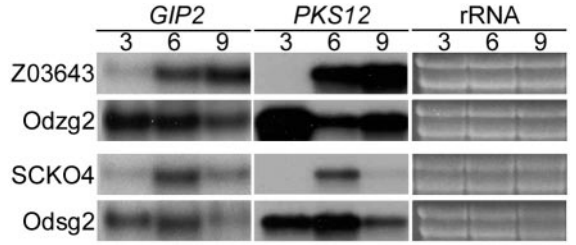

FIG. 7. RNA gel blots of the *GIP2*-overexpressing transformants derived from the Δ*GIP2* strains, probed with *GIP2* and *PKS12*, respectively. Z03643 and SCKO4, total RNAs from the wild-type strains; Odzg2-1 and Odsg2–2, total RNAs from the transgenic strains overexpressing *GIP2*, derived from the Δ*GIP2* strains of Z03643 and SCKO4, respectively. The probe and the incubation time are indicated above each blot. The ethidium bromide-stained rRNAs are indicated as a loading control.

United States (13, 24). The aurofusarin deficiency may have little effect on mycelial growth of SCKO4, because this strain has already adapted to growth with less pigmentation. To clearly evaluate the effect of aurofusarin accumulation on fungal growth, we generated aurofusarin-overproducing strains through the constitutive expression of heterologous *GIP2* sequences in both nonpigmented Δ*GIP2* mutants and pigmented wild-type strains. Severe growth reduction resulted in the highly pigmented transformants derived from both types of strains, which is consistent with the hypothesis that aurofusarin biosynthesis should be reduced or inhibited for normal early vegetative growth by *G. zeae*.

The effect of aurofusarin overproduction on mycelial growth also depended on the wild-type strain from which the *GIP2*-overexpressing mutants were derived. Mycelial growth of Δ*GIP2* mutants from Z03643 was more increased than was that of the Δ*GIP2* mutants from SCKO4, suggesting that proper aurofusarin biosynthesis is more important for hyphal growth of Z03643 than it is for SCKO4. The frequent revertants arising in cultures of aurofusarin-overproducing mutants also suggests that early accumulation of aurofusarin can alter the fungus's metabolism and favor genomic rearrangements that reduce the physiological stress. We do not know why the revertants of *GIP2*-overexpressing transformants derived from wild-type strains were less pigmented than their wild-type progenitors.

Aurofusarin biosynthesis may be associated with a particular developmental stage of *G. zeae*, as are other developmentally-regulated fungal pigments (10, 17, 22, 30). Neither *GIP2* nor *PKS12* is transcribed during sexual development, which is consistent with the hypothesis that aurofusarin biosynthesis is coordinated with mycelial growth, especially as the hyphae mature or age. Further analysis is needed of the regulatory mechanism(s), including those mediated by GIP2 that act on the aurofusarin gene cluster in response to environmental changes. Also, the possible utilization of aurofusarin by *G. zeae* for other purposes, e.g., for survival, for protection against adverse environmental conditions, or as an antimicrobial agent, also should be more carefully evaluated.

In conclusion, through functional studies of the *GIP2* gene, which encodes a putative transcription factor, we identified the members of the aurofusarin biosynthetic gene cluster, whose products appear to have a role in the biosynthesis of the compound. We also determined that this pigment may have a significant impact on colony growth rate and morphology, although the mechanism(s) by which these unanticipated mor-

phological changes are mediated remains to be elucidated. Thus, aurofusarin has a previously unanticipated biological role that is worthy of further investigation.

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