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A Large Genomic Deletion in *Gibberella zeae* Causes a Defect in the Production of Two Polyketides but not in Sexual Development or Virulence

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Gibberella zeae (anamorph: Fusarium graminearum) is an important pathogen of cereal crops. This fungus produces a broad range of secondary metabolites, including polyketides such as aurofusarin (a red pigment) and zearalenone (an estrogenic mycotoxin), which are important mycological characteristics of this species. A screen of G zeae insertional mutants, generated using a restriction enzyme-mediated integration (REMI) procedure, led to the isolation of a mutant (Z43R606) that produced neither aurofusarin nor zearalenone yet showed normal female fertility and virulence on host plants. Outcrossing analysis confirmed that both the albino and zearalenone-deficient mutations are linked to the insertional vector in Z43R606. Molecular characterization of Z43R606 revealed a deletion of at least 220 kb of the genome at the vector insertion site, including the gene clusters required for the biosynthesis of aurofusarin and zearalenone, respectively. A re-creation of the insertional event of Z43R606 in the wild-type strain demonstrated that the 220-kb deletion is responsible for the phenotypic changes in Z43R606 and that a large region of genomic DNA can be efficiently deleted in G zeae by double homologous recombination. The results showed that 52 putative genes located in the deleted genomic region are not essential for phenotypes other than the production of both aurofusarin and zearalenone. This is the first report of the molecular characterization of a large genomic deletion in G zeae mediated by the REMI procedure.

Keywords : aurofusarin, gene deletion, *Gibberella zeae*, REMI, zearalenone

Gibberella zeae (anamorph: *Fusarium graminearum*), a homothallic ascomycetous fungus with a ubiquitous geographic distribution, is an important pathogen of cereal crops, causing head blight of small grains including wheat, barley, and rice (McMullen et al., 1997). This fungus produces mycotoxins such as trichothecenes and zearalenone on diseased crops and is a potential threat to both human and animal health (Marasas et al., 1984). Despite the economic importance of *G. zeae*, our understanding of the molecular mechanisms that control the major fungal traits involved in disease development is limited. The recent release of a genome sequence assembly for *G. zeae* allows genome-wide investigations of the molecular bases of most genetically determined processes in this species. In addition, high-throughput functional analyses of the entire *G. zeae* genome are beginning to be conducted. As first efforts toward functional genomics approaches, several strategies, such as random insertional mutagenesis and automatic gene deletion, have been suggested. The former, widely used in *G. zeae*, is termed restriction enzyme-mediated integration (REMI) (Han et al., 2004; Seong et al., 2005). Because it leads to a high frequency of successful introductions of random tagged mutations in the *G. zeae* genome, REMI mutagenesis is a powerful tool for investigating the genes involved in important *G. zeae* phenotypes. Several *G. zeae* genes have already been identified through REMI mutagenesis and have been shown to play essential roles in fungal pathogenesis and/or development. These genes encode a predicted NADH:ubiquinone oxidoreductase, a putative b-ZIP transcription factor, a transducin β-subunit-like protein, cystathionine β-lyase, *O*-acetyltransferase, and a polyketide synthase (Han et al., 2004; Kim et al., 2005a; Seong et al., 2005). We screened a collection of REMI transformants previously generated from the *G. zeae* Z03643 strain (Han et al., 2004) and identified a mutant designated Z43R606 that lacks the ability to produce two polyketides, aurofusarin (a red pigment) and zearalenone (an estrogenic mycotoxin). Characterization of Z43R606 revealed that the strain carries a unique vector insertion event that caused a large genomic deletion. Our findings provide new insight into the significance of the unexpected insertion event generated by REMI for functional genomics in *G. zeae*.

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Materials and Methods

Strains and media. *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. The REMI mutant strain Z43R606 was generated from Z03643 using REMI mutagenesis (Han et al., 2004). The *G. zeae* strain Tzp12-4, derived from Z03643, is a *pks12*-deleted strain that produces no aurofusarin (Kim et al., 2005a). T39ΔM2-1, derived from the *G. zeae* strain Z03639, is a *mat1-1*-deleted self-sterile strain that produces both zearalenone and aurofusarin (Lee et al., 2003). Fungal stock cultures stored at −80°C were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). To isolate genomic DNA, the fungal strains were grown in 50 ml of complete medium (CM, Correll et al., 1987) at 25°C for 3 days in a rotary shaker (150 rpm). For fungal sporulation, mycelial plugs of each strain were inoculated into CMC liquid medium and grown as described (Han et al., 2004). For zearalenone production, the strains were grown either in 25 ml of a starch-glutamate (SG) liquid medium (Kim et al., 2005b) or on rice grains (50 g) in 250-ml Erlenmeyer flasks. Carrot agar was used for sexual crosses (Klittich and Leslie, 1988). Recombinant *Escherichia coli* strains were grown on Luria-Bertani agar or in liquid medium supplemented with ampicillin.

DNA manipulations, PCR primers, and sequencing. Fungal genomic DNAs were isolated using standard procedures (Kerenyi et al., 1999). Plasmid DNAs for both fungal transformation and sequencing were 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, labeling of probes with ^{32}P, and hybridization (Sambrook and Russell, 2001). The DNA regions flanking the vector insertion point in the Z43R606 genome were recovered as described previously (Yun et al., 1998). The PCR primers used in this study were obtained from the Bioneer oligonucleotide synthesis facility (Bioneer Corporation, Chungwon, Korea), dissolved to 100 μM in sterilized water, and stored at −20°C. PCR reactions were performed as described (Kim et al., 2005a). Sequencing of the rescued plasmids was initiated with the specific primers pIGPAPA/P5 (5'-GGTCCCCCTCCCAATTCCTT-TTC-3') and TSP3-2 (5'-GCTCCTCGCCCTTGCTCACC-AT-3'), which matched the region close to the *Kpn*I site on the REMI vector pIGPAPA (Lee et al., 2003).

Fungal transformation, outcrossing, virulence tests, and chemical analysis. For fungal transformation, ~10 μg of the rescued plasmid pR606 linearized with *Bgl*II was added directly to fungal protoplasts along with polyethylene glycol. Further transformation steps were performed as described (Kim et al., 2006). For outcrossing (Lee et al., 2003; Kim et al., 2005a), a mycelial plug of the self-sterile *mat1-1* deletion strain (female) was placed on a carrot-agar plate and incubated at 25°C. After 7 days, a conidial suspension (10^5 conidia/ml) of a male *G. zeae* strain was applied to mycelia of the female strain. The plates were incubated for an additional 10-14 days under the same conditions. For virulence tests, macroconidia grown in CMC liquid for 5 days were suspended in sterile water at 10^6 spores/ml and sprayed onto heads of the barley cultivar 'SangRok' at the early anthesis stage. The inoculated plants were placed in a growth chamber for 2 days at 25°C and 100% relative humidity and then transferred into a greenhouse until disease symptoms appeared. To detect zearalenone, both the culture filtrate and rice cultures of the fungal strains were extracted and analyzed by high-performance liquid chromatography (HPLC) as described (Kim et al., 2005b).

Results

Phenotypes of the REMI mutant Z43R606. A REMI mutant of *G. zeae*, designated Z43R606, was initially selected from the insertional mutant collection generated by the REMI procedure (Han et al., 2004). Compared with its wild-type progenitor Z03643, the mutant Z43R606 exhibited several phenotypic differences. First, Z43R606 showed no pigmentation on PDA. Z03643 colonies usually began to produce yellow-to-tan mycelia with white-to-red margins on carrot agar by 6 to7 days after inoculation, whereas those of Z43R606 remained milky white even after four weeks (Fig. 1A). Second, the radial growth of Z43R606 on PDA was ~30% greater than that of Z03643. Third, Z43R606 produced neither zearalenone nor its derivative β-zearalenol during the entire cultivation period, either on the rice substrate or in SG liquid medium, a result that was confirmed by HPLC analysis (Fig. 1B). However, in other important phenotypes such as female fertility, conidiation, and virulence toward host plants, the characteristics of Z43R606 were similar to those of Z03643. The Z43R606 strain produced white aerial mycelia during the vegetative growth stage on carrot agar (up to 7 days after inoculation). After removal of the aerial mycelia for perithecial induction, Z43R606 formed abundant sexual fruiting bodies (perithecia) containing ascospores on the same plates within 2 weeks, as did Z03643 (Fig. 1A). Inoculation with a conidial suspension of Z43R606 on barley heads caused head-blight symptoms that began to appear as early as 3 days after inoculation and became obvious after 6 days, appearing typical and similar to those caused by Z03643 (Fig. 1C).

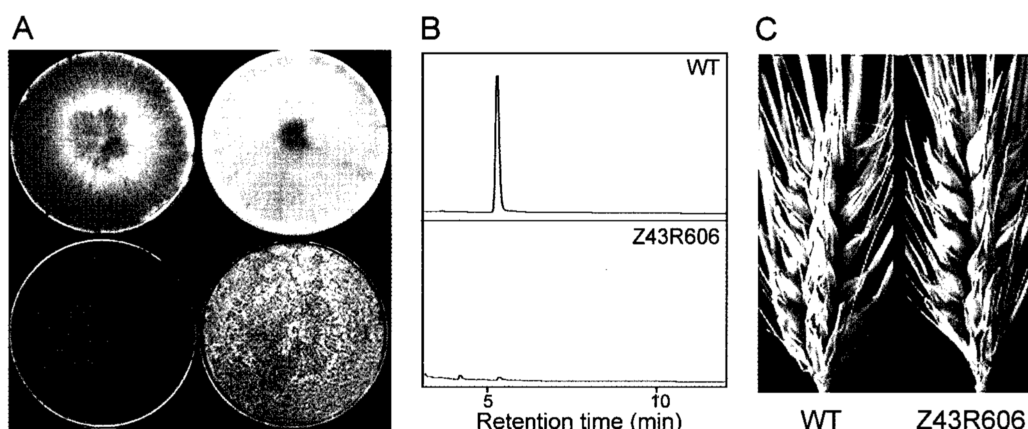

Fig. 1. Phenotypes of the REMI mutant Z43R606. (A) Pigmentation (top) and perithecia formation (bottom) of the wild-type progenitor Z03643 (left) and the mutant Z43R606 (right) on carrot agar. (B) HPLC chromatogram of rice culture extracts from Z03643 (WT) (1,000 times diluted) and Z43R606. The retention time of zearalenone was 5.4 min. (C) Head-blight symptoms on barley caused by Z03643 (left) or Z43R606 (right).

The ability of Z43R606 to cause disease symptoms was also evident on corn ears (data not shown).

Genetic analysis of the tagged mutation in Z43R606. To determine whether the phenotypic changes in Z43R606 were associated with the *hygB* gene derived from the REMI vector, a sexual outcross was performed using the *G. zeae mat1-1*-deleted strain T39ΔM1-3, which is self-sterile, resistant to geneticin (gen^R), produces zearalenone (zea^+), and has normal pigmentation (aur^+). Random ascospores obtained from the outcross between Z43R606 and T39ΔM1-3 segregated in equal proportions into parental phenotypes for *hygB* and *gen*, a result that was confirmed statistically ($\chi^2 = 3.143$; $0.05 < P < 0.1$) (Table 1). All of the $hygB^R$ progeny displayed the same $aur^-;zea^-$ phenotypes as Z43R606, and the other progeny were $aur^+;zea^+$, matching the other parent, indicating that the $aur^-;zea^-$ mutation in Z43R606 is linked to the insertion site of the *hygB* gene.

To determine whether the albino mutation in Z43R606 is genetically linked to the *PKS12* cluster required for aurofusarin production, Z43R606 was outcrossed to a *G. zeae pks12*-deleted strain (Kim et al., 2005a). In this outcross, a self-sterile progeny showing the Z43R606 phenotypes that was obtained from the outcross between Z43R606 and T39ΔM1-3 was used as the female parent. There was no recombination between *hygB* and *aur* in the progeny from this outcross and 100% of the progeny were albino, demonstrating that the albino mutation in Z43R606 maps at the aurofusarin biosynthesis gene cluster region in *G. zeae* (Table 1).

Molecular characterization of the vector insertion site in Z43R606. To characterize the vector insertion event in Z43R606, a gel blot of Z43R606 genomic DNA was hybridized with the entire REMI vector, pIGPAPA. In genomic DNA digested with *Kpn*I, the enzyme used for linearization of the vector in the REMI procedure, the probe hybridized to a single ~5.9-kb fragment, the size of pIGPAPA (Fig. 2A). Digestion of the genomic DNA with *Bgl*II, which has no recognition site in the vector, resulted in a single hybridizing band of ~16.0 kb (Fig. 2A). These hybridization patterns indicate that the vector integrated at a *Kpn*I site in the Z43R606 genome, and both *Kpn*I sites at the ends of the linearized vector were retained during the procedure. The 16.5-kb *Bgl*II fragment that appeared on the DNA gel blot was recovered using a plasmid rescue

Table 1. Segregation of genetic markers in outcrosses of Z43R606[a]

Cross	Number of progeny of each phenotype				
	$hygB^R;gen^R$	$hygB^R;gen^S$	$hygB^S;gen^R$	$hygB^S;gen^S$	Total
Z43R606 × T39ΔM1-3[b]	25	18	25	16	84
PZ43R606[c] × Tzp12-4[d]	79	58	0	0	137

[a] $hygB^R;gen^S;aur^-;zea^-$
[b] A *G. zeae mat1-1*-deleted, $hygB^S;gen^R;aur^+;zea^+$ strain of Z03639 (Lee et al., 2003).
[c] A *mat1-1*-deleted, $hygB^R;gen^R;aur^-;zea^-$ progeny obtained from an outcross between Z43R606 and T39ΔM1-3.
[d] A *pks12*-deleted $hygB^R;gen^S;aur^-;zea^+$ strain of Z03643 (Kim et al., 2005a).

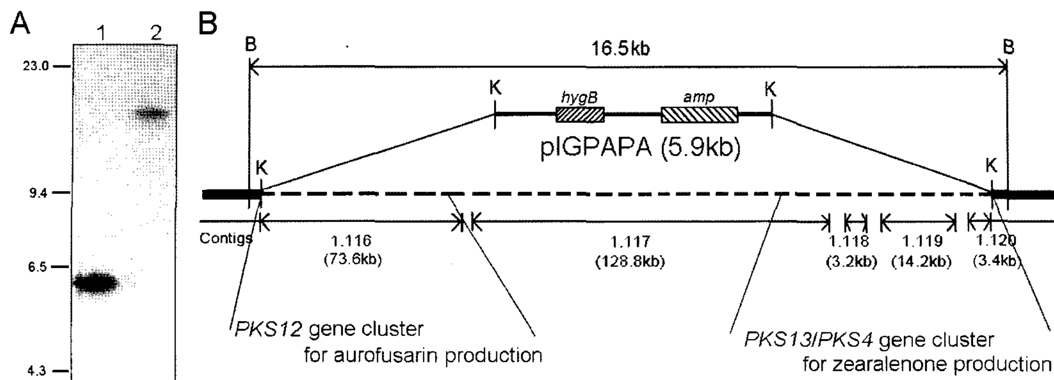

Fig. 2. Molecular characterization of the vector insertion event in the Z43R606 genome. (A) A gel blot of Z43R606 genomic DNA digested with *Kpn*I (lane 1) or *Bgl*II (lane 2) was hybridized with pIGPAPA. The sizes of λDNA standards (in kilobases) are indicated to the left of the blot. (B) Molecular structure of the vector insertion site in Z43R606. The fungal genome and the deleted genomic region are indicated by thick and dashed lines, respectively. The numbers and sizes of contigs from the *G. zeae* genome databases are shown below the corresponding genomic regions. Restriction enzyme sites: K, *Kpn*I; B, *Bgl*II.

procedure and designated pR606. Nucleotide sequencing of pR606, initiated with primers that correspond to regions closer to the *Kpn*I site in pR606, revealed that pR606 contains 7.8 kb of genomic DNA 5' of the vector and 1.7 kb 3' of the vector. However, BLAST searches of the *G. zeae* genome databases (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html) revealed that the immediate 5' and 3' flanking regions of the vector could not be connected as a continuous genomic region. The 5' end of the vector in pR606 was identified as a *Kpn*I site 0.4 kb upstream of the *GzORF1* gene (located at contig 1.116 in the *G. zeae* genome databases), a member of the aurofusarin biosynthesis gene cluster, whereas the 3' end of the vector in pR606 is a *Kpn*I site 1.2 kb downstream of the *ZEB2* gene, a member of the zearalenone gene cluster at contig 1.120 (Fig. 2B). This vector insertion event indicates that a deletion of more than 220 kb of the genome spanning five contigs (from 1.116 to 1.120) occurred at the vector insertion site in Z43R606. BLAST searches of the MIPS *Fusarium graminearum* genome database (http://mips.gsf.de/projects/fungi/Fgraminearum.html) indicated that at least 52 putative genes are located in the deleted genomic region, including the gene clusters required for the biosynthesis of aurofusarin and zearalenone (Table 2).

Re-creation of the vector insertion event of Z43R606. To confirm that both the albino and zearalenone-deficiency mutations of Z43R606 result from the large genomic

Table 2. Sequence similarities of putative genes located within the ~220-kb DNA region that was deleted in Z43R606

Locus[a]	Contig	Gene[b]	Similarity (accession no.)	Species[c]	E value
FG02319.1	1.116	*GzORF1*	Predicted protein (EAA69859)	*G .zeae*	2E-104
FG02320.1	1.116	*GIP2*	AFLR (AY618557)	*G. zeae*	1.15E-270
FG02321.1	1.116	*GIP3*	FAD/FMN-containing dehydrogenases (NZ_AAED01000004)	*A. flavus*	8E-08
FG02322.1	1.116	*GIP4*	DHA14-like major facilitator (AF238225)	*B. fuckeliana*	E-108
FG02323.1	1.116	*GIP5*	Putative transcriptional activator (NP_593170)	*Sz. pombe*	6E-14
FG02324.1	1.116	*PKS12*	Polyketide synthase (AF025541)	*A. fumigatus*	0
FG02325.1	1.116	*GIP6*	Hypothetical protein	*A. nidulans*	3E-29
FG02326.1	1.116	*GIP7*	AFLJ (AY510453)	*A. flavus*	3E-24
FG02327.1	1.116	*GIP8*	Flavin-containing monooxygenase 5 (AAA67848)	*Cavia porcellus*	1E-44
FG02328.1	1.116	*GIP1*	Brown 2 (AF104823)	*A. fumigatus*	E-142
FG02329.1	1.116	*GIP9*	Fasciclin I family protein, putative (BX649607)	*A. fumigatus*	8E-27
FG02330.1	1.116	*GIP10*	Ascorbate oxidase (AB010110)	*Acremonium* sp.	E-130
FG02331.1	1.116	*GzMCT*	Major superfacilitator superfamily (MFS) monocarboxylate transporter, putative	*A. fumigatus*	6E-29
FG02332.1	1.116	–	Oxidoreductase, aldo/keto reductase family protein	*Oryza sativa*	8e-24
FG02338.1	1.116	–	Beta-Ig-H3/fasciclin (YP_400623)		4e-06

Table 2. Continued

Locus[a]	Contig	Gene[b]	Similarity (accession no.)	Species[c]	E value
FG02339.1	1.116	–	Beta-fructofuranosidase (AAW46258)	*Cr. neoformans*	6e-155
FG02342.1	1.116	–	Cutinase (CAA46582)	*D. rabiei*	2e-57
FG02343.1	1.116	–	Trichothecene efflux pump (AAK33071)	*F. sporotrichioides*	1e-33
FG02346.1	1.116	–	C-14 sterol reductase (XP_956111)	*N. crassa*	1e-160
FG02347.1	1.116	–	Homogentisate 1,2-dioxygenase (XP_750969)	*A. fumigatus*	0.0
FG02349.1	1.117	–	Tyrosyl-tRNA synthetase (XP_720447)	*C. albicans*	5e-91
FG02350.1	1.117	–	Cellobiose dehydrogenase (XP_747382)	*A. fumigatus*	1e-32
FG02351.1	1.117	–	Cellobiose dehydrogenase (XP_749254)	*A. fumigatus*	1e-25
FG02352.1	1.117	–	Class V chitinase (XP_754890)	*A. fumigatus*	6e-117
FG02356.1	1.117	–	Cellulose-binding GDSL lipase/acylhydrolase (XP_749187)	*A. fumigatus*	7e-83
FG02356.1	1.117	–	Aldehyde dehydrogenase (XP_746469)	*A. fumigatus*	2e-148
FG02357.1	1.117	–	N amino acid permease (XP_748333)	*A. fumigatus*	3e-117
FG02358.1	1.117	–	Probable alpha-glucoside transport protein (CAD21259)	*N. crassa*	0.0
FG02360.1	1.117	–	Extracellular GDSL-like lipase/acylhydrolase (XP_749219)	*A. fumigatus*	9e-76
FG02364.1	1.117	–	Putative pod-specific dehydrogenase SAC25 (AAW46549)	*Cr. neoformans*	1e-18
FG02366.1	1.117	–	Monooxygenase, *verA* (AAS90100)	*A. flavus*	3e-63
FG02367.1	1.117	–	Cytochrome P450 monoxygenase (CAI59266)	*Cl. purpurea*	9e-30
FG02368.1	1.117	–	Cytochrome P450 monoxygenase (CAI59266)	*Cl. purpurea*	9e-30
FG02369.1	1.117	–	Cytochrome P450 monoxygenase (CAI59266)	*Cl. purpurea*	9e-30
FG02370.1	1.117	–	Cercosporin toxin biosynthesis protein (ABC79591)	*Ce. nicotianae*	2e-04
FG02371.1	1.117	–	Cytochrome P450 monooxygenase (AAS66021)	*A. parasiticus*	3e-34
FG02376.1	1.117	–	Glucokinase GlkA (XP_747854)	*A. fumigatus*	3.9
FG02379.1	1.117	–	Nitroalkane oxidase (AAL57485)	*F. oxysporum*	1e-116
FG02382.1	1.117	–	MFS transporter (XP_754701)	*A. fumigatus*	6e-149
FG02383.1	1.117	–	Adenosine deaminase (NP_595058)	*Sz. pombe*	3e-73
FG02384.1	1.117	–	C_6 zinc cluster transcription factor (XP_754652)	*A. fumigatus*	2e-08
FG02386.1	1.117	–	Pectate lyase (ABF50862)	*A. nidulans*	6e-76
FG02387.1	1.117	–	Flavin-containing monooxygenase (XP_748309)	*A. fumigatus*	5e-62
FG02392.1	1.117	*GzALD*	Aldehyde dehydrogenase (XP_751026)	*A. fumigatus*	3e-102
FG02393.1	1.117	*GzHET*	Heterokaryon incompatibility protein (AAF18153)	*N. crassa*	7e-25
FG02394.1	1.117	*GzNPS*	Non-ribosomal peptide synthetase (CAI38799)	*H. lixii*	0.0
FGd118-10	1.117	*GzKAT*	Voltage-gated potassium channel beta-2 subunit (AAW45573)	*Cr. neoformans*	4e-68
FG02395.1	1.118-1.119	*PKS13*	Polyketide synthase (AAR90251)	*B. fuckeliana*	0.0
FG02396.1	1.119	*PKS4*	Polyketide synthase (AAR90244)	*B. fuckeliana*	0.0
FG02397.1	1.119-1.120	*ZEB1*	Isoamyl alcohol oxidase (XP_749868)	*A. fumigatus*	2e-69
FG02398.1	1.120	*ZEB2*	bZIP transcription factor (AAD13811)	*Co. carbonum*	0.18

[a]The sources of each locus from the *G. zeae* genome databases can be found at either http://www.broad.mit.edu/annotation/genome/fusarium_graminearum/Home.html or http://mips.gsf.de/projects/fungi/Fgraminearum.html.
[b]Located at either the aurofusarin (Kim et al., 2006) or the zearalenone biosynthesis gene cluster (Kim et al., 2005b).
– Not yet assigned.
[c]Abbreviations used: *A, Aspergillus*; *B, Botryotinia*; *C, Candida*; *Ce, Cercospora*; *Cl, Claviceps*; *Co, Cochliobolus*; *Cr, Cryptococcus*; *D, Didymella*; *F, Fusarium*; *H, Hypocrea*; *M, Monascus*; *N, Neurospora*; *Sz, Schizosaccharomyces*.

deletion at the vector insertion site in Z43R606, the wild-type Z03643 strain was transformed with the plasmid pR606 digested with *Bgl*II to re-create the original vector insertion event of Z43R606 (Fig. 3A). All of the transformants that showed hybridization patterns identical to that of Z43R606 in DNA gel blot analysis (Fig. 3B) exhibited the same phenotypic changes as Z43R606. Other transformants carrying pR606 at an ectopic position were similar to Z03643 in all of the phenotypes examined (data not shown).

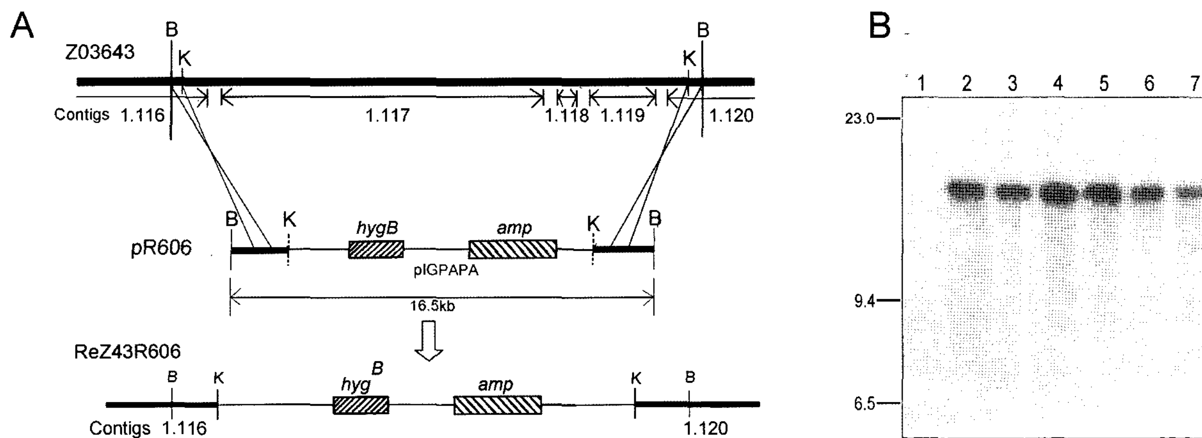

Fig. 3. Re-creation of the vector insertion event of Z43R606 in the wild-type Z03643 strain. (A) Re-creation strategy by double homologous recombination between the plasmid pR606 recovered from Z43R606 and the corresponding genomic regions in Z03643. Z03643 and ReZ43R606, genomic DNAs of Z03643 and a transgenic Z03643 strain carrying the same vector insertion event as Z43R606, respectively; K, *Kpn*I; B, *Bgl*II. (B) Blot of *Bgl*II-digested genomic DNAs of transformants generated using pR606, probed with pIGPAPA. Lane 1, Z03643; lane 2, Z43R606; lanes 3-7, re-created transformants.

Discussion

The Z43R606 strain was originally selected as an albino REMI mutant with the expectation that its phenotypic change was associated with a defect in the production of the red pigment aurofusarin. One possible explanation for the phenotype was that a REMI vector had been inserted in a genomic region containing the *PKS12* gene cluster, which is responsible for aurofusarin production (Kim et al., 2005a; Malz et al., 2005; Kim et al., 2006). Although genetic analyses revealed that the tagged mutation in Z43R606 maps to the *PKS12* gene cluster region, co-segregation of the albino and zearalenone deficiency phenotypes in Z43R606 with the *hygB* trait suggested that Z43R606 was genetically different from a *pks12*-deleted strain of *G. zeae* that is devoid of pigmentation but produces more zearalenone than normal (Jung et al., 2006). Molecular analysis suggested that during the REMI procedure, deletion of a large genomic region (~220 kb) occurs, deleting both the aurofusarin and zearalenone gene clusters and thus directly causing the *aur$^-$;zea$^-$* mutation in Z43R606. However, further investigations should be performed to confirm that a continuous ~220-kb genomic region is deleted from the Z43R606 genome. Although one of the merits of the REMI procedure is its potential to generate tagged mutations, making it simple to isolate the gene(s) under investigation, vector insertion-mediated deletion or genomic rearrangements during REMI have also been valuable for the isolation of genes from several filamentous fungi (Yun et al., 1998; Linnemannstons et al., 1999; Namiki et al., 2001). Similarly, a large genomic deletion that occurred in Z43R606 led us to re-confirm the functional requirement of both the aurofusarin- and zearalenone-*PKS* gene clusters for pigmentation and zearalenone biosynthesis in *G. zeae*, respectively. However, it is significant that other major phenotypes, such as sexual development, asexual sporulation, and fungal virulence, are not impaired by the large deletion in Z43R606. It is particularly noteworthy that more than 30 previously uncharacterized or unidentified putative genes located within the deleted DNA region, most of which appear to encode metabolic enzymes, are dispensable for these fungal phenotypes. This conclusion suggests that gene deletion generated by REMI could facilitate genome-wide functional analysis of *G. zeae*. Using REMI mutants carrying other large deletions, one could efficiently identify the genes that are not essential for specific fungal traits of interest. However, the successful recreation of the ~220-kb DNA deletion in the wild-type strain indicates that it may not be necessary to collect more REMI mutants for this purpose. Instead, one could generate a large deletion by a simple fungal transformation procedure using a vector or a PCR fragment carrying both the 5' and 3' flanking regions of the genomic region under investigation, fused to a fungal selectable marker. During the transformation procedure, a targeted large DNA replacement (deletion) could be achieved *via* a double homologous recombination between the transforming DNA and the corresponding genomic regions, as shown in this study.

In summary, we determined the functional requirement for important *G. zeae* traits of the genes located in a 220-kb genomic region, by characterization of the REMI mutant Z43R606, in which a unique vector integration event caused a large deletion.

Acknowledgements

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