A Natural Mutation Involving both Pathogenicity and Perithecium Formation in the Fusarium graminearum Species Complex

Haruhisha Suga,*1 Koji Kageyama,3 Masafumi Shimizu,4 and Misturo Hyakumachi†
*Life Science Research Center, †River Basin Research Center, and ‡Faculty of Applied Biological Sciences, Gifu University, 501-1193, Japan
ORCID ID: 0000-0002-7179-1765 (H.S.)

ABSTRACT Members of the Fusarium graminearum species complex (Fg complex or FGSC) are the primary pathogens causing Fusarium head blight in wheat and barley worldwide. A natural pathogenicity mutant (strain 0225022) was found in a sample of the Fg complex collected in Japan. The mutant strain did not induce symptoms in wheat spikes beyond the point of inoculation, and did not form perithecia. No segregation of phenotypic deficiencies occurred in the progenies of a cross between the mutant and a fully pathogenic wild-type strain, which suggested that a single genetic locus controlled both traits. The locus was mapped to chromosome 2 by using sequence-tagged markers; and a deletion of ~3 kb was detected in the mapped region of the mutant strain. The wild-type strain contains the FGSG_02810 gene, encoding a putative glycosylphosphatidylinositol anchor protein, in this region. The contribution of FGSG_02810 to pathogenicity and perithecium formation was confirmed by complementation in the mutant strain using gene transfer, and by gene disruption in the wild-type strain.

Fusarium head blight (FHB), or scab, is one of the most destructive and economically important diseases of wheat and barley worldwide. The major etiological agent, Fusarium graminearum Schwabe (F. graminearum Schwabe), is a haploid ascomycetous fungus. This pathogen causes quantitative losses in yield, and diseased grains may be contaminated with significant levels of mycotoxins such as deoxynivalenol (DON) and zearalenone, which are harmful to humans and animals (Desjardins et al. 1993; Windels 2000). F. graminearum can reproduce sexually by self-crossing, and also by outcrossing. Ascospores from sexual reproduction and conidia from asexual reproduction are believed to be the main F. graminearum inoculums infecting flowering wheat heads (Francl et al. 1999).

Copyright © 2016 Suga et al. doi: 10.1534/g3.116.033951
Manuscript received July 27, 2016; accepted for publication September 19, 2016; published Early Online September 27, 2016.
This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Supplemental material is available online at www.g3journal.org/lookup/suppl/doi:10.1534/g3.116.033951/-/DC1.
1Corresponding author: Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. E-mail: suga@gifu-u.ac.jp

KEYWORDS
Gibberella zeae ascomycetous fungus pathogenicity ascocarp gene mapping

Molecular phylogenetic analyses using worldwide collections of F. graminearum revealed that the F. graminearum species complex (Fg complex) includes at least 16 biogeographically structured species (O’Donnell et al. 2000, 2004, 2008; Starkey et al. 2007; Yli-Mattila et al. 2009; Sarver et al. 2011). Of these, only six species, and three species groups, are morphologically recognizable (Aoki et al. 2012). Reciprocal monophyly of the Fg complex species indicates that interspecific hybridization in nature is relatively limited; however, crossing between strains of different species has been attained in the laboratory (Bowden and Leslie 1999). Previously, a genetic map was constructed by interspecific crossing F. graminearum s. str. with F. asiaticum, and segregation distortion was observed on this crossing (Jurgenson et al. 2002). A genetic map with sequence-tagged markers was also generated by intraspecific crossing F. graminearum s. str. with F. graminearum s. str. to complement the whole genome sequence of F. graminearum s. str., resulting in the prediction of 11,640 genes (Gale et al. 2005; Cuomo et al. 2007).

Various forward and reverse genetic approaches have been applied to identify genes that contribute to Fg complex pathogenicity. Urban and Hammond-Kosack (2013) have summarized 159 known pathogenicity genes. These genes have been deduced by functional annotation to be involved mainly in (1) metabolism, (2) cellular communication/signal transduction, (3) cell rescue, defense, and pathogenicity/adaptation to nutrient conditions, and (4) transcription (Urban and Hammond-Kosack 2013).
Trichothecenes, such as DON, are known to inhibit eukaryotic protein synthesis (Pestka and Smolinski 2005). A non-DON-producing mutant, in which the tri5 gene is disrupted, can cause an initial infection on wheat spikes, but the infection does not extend beyond this site (Bai et al. 2002). At the molecular level, however, no single gene can fully account for the Fg complex’s wheat head infection and symptom development, and thus observation of aberrant phenotypic behavior for each gene disruptant would be required for comprehensive understanding of infection by the Fg complex.

Urban and Hammond-Kosack (2013) split the phenotypic process of infection (as observed for the wild-type Fg complex) into seven stages (A–G). Fungal invasion into the adjacent spikelet through the rachis node (from the initially infected wheat spikelet) is one of the critical stages for symptom development in the entire wheat head. The mutant that fails at stage E (entry into the rachis node as indicated by the internal rachis) stands out because visible signs of disease do not spread beyond the inoculated wheat spikelet when artificial point inoculation is performed. This type of mutant has been recognized in artificially created mutants, and sometimes also in natural Fg complex isolates. Associations between pathogenicity to wheat and phylogenetic lineages, trichothecene chemotype, and genetic diversity, have been investigated using Fg complex isolates (O’Donnell et al. 2000; Carter et al. 2002; Logrieco et al. 1990). Pathogenic variation among isolates has been reported, and a stage E mutant, one of several possible strains, was prominent (O’Donnell et al. 2000).

Cumagun et al. (2004) used a natural nonpathogenic isolate of F. asiaticum to map the genomic region associated with pathogenicity. Cumagun et al. (2004), using quantitative trait locus (QTL) analysis, mapped one locus for pathogenicity, and two loci for aggressiveness on a previously constructed linkage group; however, the genes and mutations for these traits remain unknown. We also found several stage E mutants in our Japanese Fg complex sample (Suga et al. 2008). Disease symptoms were induced at the inoculated wheat spike and its rachis node, but the infection did not spread when their conidia were injected. In addition, these mutants also affected perithecial formation on carrot agar medium. In this study, we define these phenotypic mutations as nonpathogenic and nonperitecial-forming.

To comprehensively understand infection by the Fg complex, the objectives of this study were (1) to map the genomic region associated with nonpathogenic and nonperithetium-forming mutations, (2) to identify the gene that confers this phenotype by using the whole genome sequence of F. graminearum s. str. (Cuomo et al. 2007), and (3) to reveal what type of mutation occurs naturally in the Fg complex. As far as we know, this is the first report to clarify a natural mutation involving both pathogenicity and perithecial formation in the Fg complex.

MATERIALS AND METHODS

Strains and culture conditions

Regarding the Fg complex in Japan, the wild-type strain F. graminearum s. str. 0407011 (Fg0407011), showing pathogenicity and perithecial formation, and the mutant-type strain F. asiaticum 0225022 (Fa0225022), showing nonpathogenicity and nonperithecium formation, were used (Suga et al. 2008). Although they belong to different species, the Fg0407011 strain was chosen as a crossing partner of the Fa0225022 strain because crossing between F. graminearum s. str. and F. asiaticum has been confirmed (Jurgenson et al. 2002), thereby allowing molecular markers to be established on the basis of nucleotide polymorphisms.

Conidia used for the pathogenicity test were collected from 10-d-old cultures on synthetic nutrient agar (SNA) under black light, and suspended in sterile distilled water containing 1% (v/v) Triton X-100. F. asiaticum strain 0444002 (Fa0444002) (Suga et al. 2011) was used to obtain a wild-type sequence of the target region of F. asiaticum. The strains and transformants were maintained on potato dextrose agar (PDA) and kept at −80°C in 50% glycerol for long-term storage.

Crossing

Members of the Fg complex reproduce sexually. Both self-crossing and outcrossing are capable in the Fg complex (Bowden and Leslie 1999). Outcrossing with nitrate-nonutilizing (nit) mutants of the parental strain was conducted according to a mycelial plug crossing method (Bowden and Leslie 1999). The progenies derived from outcrossing were needed for mapping, and were phenotypically recognizable when different type of nit mutants were paired. Nit mutants were generated from the parental strains on PDA containing 1.5–5.0% KClO₃, and a nit1, nit3, or Nit M phenotype was determined by growth on different nitrogen sources (Correll et al. 1987). Plugs of a Nit M mutant of Fg0407011, and a nit1 mutant of Fa0225022, were placed on opposite sides of a 90-mm Petri dish containing carrot agar (Klittich and Leslie 1988).

After incubation at 25°C for 1 wk under black light, 1.5 ml of 2.5% (v/v) Tween 60 solution was added, and the aerial mycelium was knocked down with a glass rod. Thereafter, the Petri dish was kept under the same conditions to form perithecia. After 2 wk, the Petri dish was reversed, and coupled to another Petri dish of minimal medium containing 0.05% (v/v) tertgitol type NP-10, and 2% (w/v) L-sorbose instead of 3% sucrose (MMTS) (Bowden and Leslie 1999) to obtain colonies derived from discharged ascospores. Wild-type colonies, characterized by dense and aerial mycelium, and nit mutant-type colonies, with thin and little, or no, aerial mycelium, appeared on MMTS, and an individual wild-type colony was used as progeny in this study. Conidia produced on SNA under black light were spread on MMTS to obtain pure cultures of each progeny by single colony isolation.

Pathogenicity test

The pathogenicity test was performed using the rapid-maturing dwarf wheat cultivar Apogee, which has high susceptibility to FHB (Mackintosh et al. 2006). Each plant, cultivated in a small pot until one head remained, was inoculated just after heading out with ~1 x 10⁵ conidia added to the wounded lower spikelet. After inoculation, the plants were placed in a humidity box for 24 hr, and then transferred to a growth chamber (KG-50HLA; Koito Electric Industries, Shizuoka, Japan) maintained at 27°C.

Inoculation treatments consisted of one head for a progeny, and three heads for a transformant. Experimental treatments were repeated at least once for progenies and twice for transformants. Bleaching and/or necrosis of the spikelet 9 d after inoculation was observed. Pathogenicity was scored as pathogenic or nonpathogenic based on whether the symptoms were confined in the initially inoculated spikelet, as for the mutant Fa0225022, or whether they spread to the neighboring florets, as for the wild type, Fg0407011 (Figure 1). The pathogenicity of transformants was evaluated by the number of florets showing symptoms.

Perithecial formation

Perithecial formation in self-crossing was assayed on carrot agar plates (Klittich and Leslie 1988) as described above, except that the mycelial plug of a single progeny was placed in the center of the 90-mm plate. Perithecial formation was observed until 3 wk after aerial mycelium knockdown (Figure 1).
DNA extraction and PCR

Genomic DNA for PCR was extracted from 3- to 4-d-old mycelium (2–3 cm diameter) cultured on potato dextrose broth (PDB), as previously described (Suga et al. 2008). The final DNA pellet was dissolved in 200 μl of water, and 5 ng/μl yields of genomic DNA were estimated by comparison to DNA of known concentrations by agarose gel electrophoresis. Genomic DNA for Southern blot hybridization was extracted in a similar way with some modifications: >0.1 g of dry weight mycelium was ground in a mortar with a small amount of sea sand, and 5 ml of potassium ethyl xanthogenate (PEX) solution was added. RNase A treatment and phenol-chloroform extraction were performed after dissolving the DNA pellet in 200 μl of water.

Mapping

Thirty-two variable number tandem repeat sequence (VNTR) loci for rough mapping were selected from 54 VNTR loci in the whole genome sequence of F. graminearum s. str. PH1 strain based on the results of length polymorphisms of PCR products between the parental strains (Suga et al. 2004). The PCR comprised 0.5 units of AmpliTaq DNA polymerase (Life Technologies, Carlsbad, CA), in a 20-μl reaction mixture containing 1× reaction buffer, 200 μM dNTPs, each primer at 1 μM, and 5 ng of genomic DNA. PCR was performed in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) using the following cycling parameters: 94° for 2 min, 25 cycles of 94° for 1 min, 63 or 68° for 2 min, and 72° for 1 min. A single PCR product was confirmed by electrophoresis on 1% agarose gel before restriction enzyme digestion. If there was no polymorphism with the tested restriction enzyme, PCR products were sequenced directly by BigDye terminator v3.1 cycle sequencing kits (Life Technologies), and then run on an ABI 3100 genetic analyzer (Life Technologies), as previously described by Suga et al. (2008). Restriction enzymes were selected by computational PCR-RFLP with Genetyx version 4.0 software (Genetyx, Tokyo, Japan) based on the nucleotide sequence of the parental strains.

Detection of missing region

Similar to PCR-RFLP, PCR primers were designed upstream and downstream of the open reading frame of each gene in the genomic region between the HS369/HhaI and HS367/Aval markers. A NotI recognition sequence was added to the 5’ end of the primer for cloning the PCR product into plasmid vector pCB1004 (Carroll et al. 1994). The PCR comprised 0.5 units of AccuTaq LA DNA Polymerase (Sigma, St. Louis, MO) in a 20-μl reaction mixture, containing 1× reaction buffer, 500 μM dNTPs, 2% dimethyl sulfoxide (DMSO), each primer at 0.4 μM (Supplemental Material, Table S1), and 5 ng of genomic DNA. PCR was performed in an iCycler thermal cycler (Bio-Rad Laboratories) using the following cycling parameters: 94° for 30 sec, 30 cycles of 94° for 15 sec, 51° for 20 sec, and 68° for 5 min.

The PCR product generated with the primer pair HS484/HS451 was directly sequenced as described above by using primers HS484, HS451, HS512, HS513, HS514, HS515, HS516, and HS451 (Table S1). The sequences of Fa0225022 (HQ599308), Fg0407011 (HQ599310), and Fa0444002 (HQ599309) have been deposited in GenBank.

Construction of the transformation vector

The FGSG_02809, FGSG_02810, and FGSG_02811 genes, including regions upstream and downstream of the open reading frame, were amplified from Fg0407011 by PCR using the HS458/HS459, HS450/HS451, and HS442/Hs453 primer pairs, respectively (Table S1). PCR products treated with NotI were inserted into the NotI site in pCB1004 to create the respective transformation vectors pCB02809, pCB02810, and pCB02811 using DNA Ligation Kit Ver. 2 (Takara, Otsu, Japan).

The FGSG_02810 replacement vector pCR402810dis was constructed as follows. The PCR product from FGSG_02810 without NotI treatment was cloned into pCR4TOPO (Invitrogen, Carlsbad, CA).
according to the manufacturer’s instructions to obtain plasmid pCR402810. The hygromycin-resistance cassette region (TrpC promoter and hygromycin phosphotransferase coding sequence) in pCB1003 (Sweigard et al. 1997) was amplified by PCR using primers HS708 and HS709 in which XhoI and SacI recognition sequences were integrated, respectively (Table S1), using pCB1003 as the template DNA.

PCR was performed by using AmpliTaq DNA polymerase (Life Technologies), and the following cycling parameters: 94°C for 2 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplicons treated with XhoI and SacI were inserted into XhoI and SacI sites in pCR402810 (pCR402810dis) using DNA Ligation Kit Ver. 2.

**Fungal transformation**

Mung bean liquid media inoculated with tiny PDA blocks of fungal culture were reciprocally shaken for 2 d at 25°C to produce budding cells. The cells were harvested by centrifugation after filtration through Kim Wipes (Kimberly-Clark, Tokyo, Japan), and then suspended in PDB at a final concentration of 5.0 × 10^7/ml. The suspension was incubated at 25°C with gentle shaking until the length of the germ tubes reached 2–3 times the size of the budding cells. The germ tubes were harvested by filtration and washed with 1.2 M NaCl. For protoplasting, germ tubes were suspended in an enzyme solution containing 20 mg/ml of lysing enzyme (Sigma), 10 mg/ml of Cellulase Onozuka RS (Yakult, Tokyo, Japan), 10 mg/ml of Zymolyase 20T (Seikagaku Kogyo, Tokyo, Japan), 10 mg/ml of β-Glucuronidase type H-1 (Sigma), and a small amount of chitinase (Sigma) in 1.2 M NaCl, and agitated gently at 30°C for 4–6 h.

After filtration through four layers of Kim Wipes, a fivefold volume of SE (1 M sorbitol, 50 mM EDTA, pH 8.0) was added, and then protoplasts were harvested by centrifugation at 700 × g for 10 min. Protoplasts were washed first in SE, and then in STC (1 M sorbitol, 25 mM Tris-HCl, pH 7.0, 25 mM CaCl₂). The final protoplasts were suspended in STC at a concentration of ~5.0 × 10^7 per ml.

Transformation was performed according to the procedure of Wasmann and Van Etten (1996), with modifications as follows. Plasmid DNA was extracted by a Qiagen Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Transformation plates were incubated under light, and 5 ml of 1% water agar containing 10 mg/ml of hygromycin B (Wako, Osaka, Japan), 10 mg/ml of Zymolyase 20T (Seikagaku Kogyo, Tokyo, Japan), and 0.5 mg/ml of lysing enzyme (Sigma) in 1.2 M NaCl, and agitated gently at 30°C for 4–6 h.

**Reverse transcription (RT)-PCR**

Total RNA was extracted from mycelium cultured in PDB for 4 d, as well as from a wheat head inoculated with the strain; ~1 × 10^8 conidia were inoculated to each wounded spikelet and placed in a humidity box for 48 hr. RNA extraction was performed with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions, and then purified with Recombinant DNase I (Takara) and NucleoSpin RNA clean-up kit (Takara). RT-PCR was performed with a Titan One Tube RT-PCR Kit (Roche Diagnostics, Mannheim, Germany), and the primer pair HS601/HS602 (Table S1) according to the manufacturer’s instructions. PCR was performed in an iCycler thermal cycler (Bio-Rad) using the following cycling parameters: 50°C for 30 min and 94°C for 2 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 1 min.

Genomic DNA instead of RNA was also used to compare RT-PCR products on 1.0% agarose gel electrophoresis.

**Southern blot hybridization**

Southern blot hybridization was performed using a probe for a region in the FGSG_02810 gene that is missing in the Fa0225022 strain. A 186-bp DNA fragment amplified from the Fg0407011 genome by PCR using primer pair HS601/HS602 was labeled with digoxigenin (DIG) by using a PCR DIG Probe Synthesis Kit (Roche Diagnostics). The probe was used after purification by Mini Quick Spin DNA Columns (Roche Diagnostics). Genomic DNA digested with ClaI (New England Biolabs, Beverly, MA) was subjected to agarose gel electrophoresis together with DNA Molecular Weight Marker III, DIG-labeled (Roche Diagnostics), and transferred to a nylon membrane. The signal was detected by using DIG High Prime DNA Labeling and Detection Starter Kit I, according to the manufacturer’s instructions.

**Data availability**

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

**RESULTS**

**Rough mapping**

In total, 100 progenies were obtained from crossing between the wild-type strain (Fg0407011) and the mutant-type strain (Fa0225022). Sixty-four and 36 progenies showed pathogenicity and perithecium formation (i.e., wild-type), and nonpathogenicity and nonperithecium formation (i.e., mutant-type), respectively. Nonpathogenic and nonperithecium formation were not segregated in the progenies, indicating that a single gene was responsible for the traits, although 1:1 segregation of the wild type and mutant type was rejected (P = 0.005, χ^2 test).

Twenty-one progenies were analyzed with 32 VNTR markers. Three markers in chromosome 2 showed high LOD values (VNHK1049, 3.55; VNHK995, 4.57; and VNHK1069, 3.71) for the mutant phenotype (Figure 2). The mating type ideomorph (MAT) region essential for perithecium formation was found to be present at a distance of ~360 kb to VNHK995 in the whole genome sequence of _F. graminearum_ s. str., and the PCR-RFLP marker and HS296/ClaI in the MAT region had a higher LOD score (5.98) than that of VNHK995 (Figure 2).

**Fine mapping**

All the remaining progenies were analyzed with VNHK1049, VNHK995, and HS296/ClaI markers. The HS296/ClaI marker showed the highest LOD among these, but the phenotype of one progeny differed from the results of marker type; progeny 67P2169, with a mutant phenotype, had an Fg0407011-type allele at HS296/ClaI.

Fourteen progenies that had the Fg0407011-type allele at VNHK1049 and the Fa0225022-type allele at VNHK995, or vice versa, were used for further analysis. Five (HS355/MseI, HS353/HaeIII, HS351/MspA1I, HS349/Ddel, and HS347/HaeII) and two (HS308/HinfI and HS310/HaeII) PCR-RFLP markers were newly developed for the genomic regions between VNHK1049 and HS296/ClaI, and between HS296/ClaI and VNHK995, respectively. Among these, the HS351/MspA1 marker showed a perfect match between marker type and phenotype in the 14 progenies.

Therefore, PCR-RFLP markers (HS359/HinfI and HS357/HaeIII) were newly developed for the middle region between HS351/MspA1 and adjacent PCR-RFLP markers (HS353/HaeIII and HS349/Ddel). These markers did not show a perfect match between marker type
and phenotype in the 14 progenies; therefore, new marker development was repeated in the same way until six serial markers that showed a perfect match between marker type and phenotype in the 14 progenies were found: HS369/HhaI, HS371/TaqI, HS373/HaeIII, HS361/HincII, HS351/MspA1I, and HS367/AvaI (Figure 2). A perfect match between marker type and phenotype in the 100 progenies was confirmed with HS369/HhaI and HS367/AvaI.

**PCR amplification of the target region**

PCR amplification was performed for the ORF and upstream and downstream regions of 16 genes present between the HS369/HhaI and HS367/AvaI markers based on annotation of the whole genome sequence of *F. graminearum* s. str. PH1 markers on chromosome 2 showed high LOD values (>3.5). The result of fine mapping is indicated below. Fourteen progenies (067P2102–067P2121) that showed the Fg0407011-type allele at VNHK1049 and the Fa0225022 allele at VNHK995 (or vice versa) were used. White and black circles on lines indicate the Fa0225022 and Fg0407011-type allele, respectively; white and black circles next to the progeny's name indicate mutant phenotype (non-pathogenic and non-peritheci um formation) and wild-type phenotype (pathogenic and peritheci um formation), respectively. The marker within the vertical dotted lines showed a perfect match between allele-type and phenotype in the 14 progenies. Genes within the region are shown as arrows above the FGSG gene ID number indicated. The dotted gray horizontal line below the arrows shows the PCR amplification test region for each gene, and the terminal bold lines represent the primers. The short, middle, and long dotted horizontal lines for FGSG_02810 indicate primer pairs HS450/HS451, HS484/HS485, respectively.
and FGSG_02811 genes were not amplified in Fa0225022 (Figure 3).

PCR amplification was performed with primers HS484 (forward) and HS485 (reverse) that had sequences complementary to the missing side primer of the FGSG_02812 and FGSG_02808 genes located next to the FGSG_02809 and FGSG_02811 genes, respectively, because successful PCR amplification was observed for these genes in Fa0225022 (Figure 3).

The expected size of DNA was amplified in Fg0407011, whereas a ~3-kb smaller DNA fragment was amplified in Fa0225022 (Figure 3). The HS451 primer for FGSG_02810 was located nearer to the missing side primer of the FGSG_02812 and FGSG_02808 genes in Fa0225022 also produced a ~3-kb smaller DNA fragment than Fg0407011. By contrast, a size of DNA fragment similar to that in Fg0407011 was amplified from another F. asiaticum strain (Fa0444002).

Sequencing of these DNA fragments, and the whole genome sequence of F. graminearum s. str. PH1 indicated that Fa0225022 has a deletion of 3194 bp between part of the FGSG_02811 gene and the upstream region of the FGSG_02809 gene, including the whole FGSG_02810 gene as compared with F. graminearum s. str. PH1 (Figure 4).

**Phenotype recovery by gene complementation**

The deficiency of perithecial formation and pathogenicity in Fa0225022 was assumed to be due to malfunction of the FGSG_02809, FGSG_02810, and FGSG_02811 genes. Transformants of Fa0225022 were generated with a plasmid carrying the FGSG_02809, FGSG_02810, or FGSG_02811 gene of the wild-type strain Fg0407011 (pCB02809, pCB02810, or pCB02811, respectively). Both pathogenicity and perithecial formation were recovered in the transformants of the FGSG_02810 gene, but not in those of the FGSG_02809 and FGSG_02811 genes (Table 1).

**FGSG_02810 gene disruption in the wild-type strain**

Disruption of FGSG_02810 was carried out to confirm that this gene is involved in both pathogenicity and perithecial formation in the Fg complex. Three disruption mutants of Fg0407011 were obtained by transformation with the replacement vector pCR402810dis, containing the hygromycin resistance gene (HygB).

Gene disruption was confirmed by PCR and Southern blot analysis using a probe for part of the FGSG_02810 gene (Figure 5). The disruption mutants showed a 4.7-kb hybridizing band instead of the 3.7-kb band in the wild-type strain (Figure 5). One ectopic transformant (Fg02810dis-1), and one FGSG_02810 gene complementation transformant (Fa02810-1), were also analyzed by Southern blot. The ectopic transformant showed several hybridization signals, in addition to the 3.7-kb band. Fa0225022 did not show any hybridization signal because the probe region is missing in this strain, while the FGSG_02810 gene complementation transformant showed a single hybridization band with ClaI digestion (Figure 5). The ectopic transformant showed a phenotype similar to the wild-type strain Fg0407011, namely, pathogenic and perithecial formation. The FGSG_02810 gene disruption mutants showed phenotypes similar to Fa0225022, namely, nonpathogenic and nonperithecial formation (Table 1).

**Expression of the FGSG_02810**

Transcription of FGSG_02810 in the wild-type strain Fg0407011 was confirmed by RT-PCR using HS601 and HS602 primers. Total RNA was isolated from the wheat spikelet infected with Fg0407011, and also from Fg0407011 mycelium grown on PDB. Both samples showed a 130-bp PCR product, corresponding to the size of the genomic DNA without a 56-bp intron (Figure 6).

**Function prediction of the FGSG_02810**

A conserved domain for the medium chain reductase/dehydrogenase (MDR)/zinc-dependent alcohol dehydrogenase-like family was detected at amino acids 134–214 of FGSG_02810 in strain Fg0407011 by using the conserved domain search in the National Center for Biotechnology Information (NCBI) database (2.20 e–09) (Figure S1) (Marchler-Bauer et al. 2015). The 17 N-terminal amino acids of FGSG_02810 were predicted as a signal peptide with SignalP 4.0 (D-Score: 0.834) (Petersen et al. 2011).
BLAST P search in the NCBI database indicated that FGSG_02810 has homology to the gene product of other *Fusarium* spp. for which whole genome sequences have been obtained, including *F. pseudograminearum* FPSE_05981, *F.avenaceum* FAVG1_10471, *F. oxysporum* FOXG_02761, *F. oxysporum* F. *s. *cubense race 4 FOCA_10001017, *F. verticillioides* FVEG_13122, *F. fujikuroi* FFUJ_05581, and *Nectria hematococa* mpV1 NECHADRAFT_92223 (E value: 2e^-101), both of which are putative glycosylphosphatidylinositol (GPI) anchored proteins. A GPI-modification site search with big-PI Fungal Predictor (Eisenhaber et al. 2004) indicated that serine 206 and glycine 207 in FGSG_02810 are predicted GPI-modification sites (p-value: 3.1e^-05) (Figure S1).

**DISCUSSION**

A natural mutation involving both pathogenicity and perithecium formation of the *Fg* complex has been revealed in this study. A deletion of ~3 kb in chromosome 2 was detected in the Fa0225022 mutant, and a lack of the FGSG_02810 gene encoding a putative GPI-anchored protein in the corresponding genomic region was shown to cause the deficiency of pathogenicity and perithecium formation in this strain. As far as we know, this is the first report to clarify a natural mutation responsible for the deficiency of both pathogenicity and perithecium formation in the *Fg* complex.

Previously, artificial random plasmid insertions in the genome, gene disruption, and transposon tagging (Dufresne et al. 2008) have been used to identify genes involved *Fg* complex pathogenicity. Pathogenicity of the *Fg* complex has been shown to involve genes for signal transduction (Jenczmionka et al. 2003; Hou et al. 2002; Yu et al. 2008), nonribosomal peptide synthetases (Lu et al. 2003), and amino acid synthase (Seong et al. 2005; Sce et al. 2007).

As summarized by Urban and Hammond-Kosack (2013), the artificial gene disruption method (Hou et al. 2002; Jenczmionka et al. 2003; Shim et al. 2006) has been used to identify 159 genes that contribute to the pathogenicity of the *Fg* complex, as well as several genes involved in both pathogenicity and perithecium formation. However, the contribution of FGSG_02810 to pathogenicity and perithecium formation has not previously been reported.

Cumagun et al. (2004) performed linkage mapping of pathogenicity with a natural mutant and observed close linkage between the pathogenicity locus (PATH1) and the perithecium formation locus (PERI1), although the gene(s) was not revealed. In addition to Fa0225022, we found another nonpathogenic mutant strain (Fa0233007) in our *Fg* complex collection (Suga et al. 2008), and it is also unable to form the perithecium. However, phenotype recovery by transformation of this mutant strain with pCB02810 failed, and successful PCR amplification of FGSG_02810 in the mutant strain (data not shown), suggested that another gene mutation is responsible for nonpathogenic and nonperithecium formation in Fa0233007.
The FGSG_02810 gene encodes a putative GPI anchor protein. GPI anchor proteins are known to be present in the cell wall (Kapteyn et al. 1996; Schoffelmeer et al. 2001; De Groot et al. 2005) and may be involved in hyphal fusion. Disruption of the mitogen-activated protein (MAP) kinase genes MGV1 and Gpmk1 in the Fg complex was found to result in deficiencies of both pathogenicity and peritheium formation (Hou et al. 2002; Jenczmionka et al. 2003). Modification of cell wall structure and loss of self-hyphal fusion were observed in the MGV1 disruption mutant, and this mutant was both female-sterile and male-sterile (Hou et al. 2002). Similarly, Fa0225022 and the natural mutant used in the study of Cumagun et al. (2004) were female-sterile and male-sterile because the outcrossing succeeded. Some Fusarium species are known to have a female sterile strain that is frequently isolated in nature, but the responsible mutation(s) has not yet been revealed. Mutation of a FGSG_02810 homolog is a possible candidate for female sterilization, although natural female sterile strains are uncommon in the Fg complex.

The involvement of a GPI anchor protein gene in pathogenicity has been revealed in the rice fungal pathogen Pyricularia oryzae (Ahn et al. 2004), and the human fungal pathogen Aspergillus fumigatus (Li et al. 2007). GPI anchor protein genes, FGSG_00576, FGSG_001588, and FGSG_08844, are predicted in F. graminearum s. str., but their disruption did not alter the phenotype (including pathogenicity), although disruption of the phosphoethanolamine transferase gene (gpi7) resulted in cell wall structure abnormalities, macroconidium production, and pathogenicity to wheat in F. graminearum s. str. (Rittenour and Harris 2013).

Rittenour and Harris (2013) detected 57 genes encoding putative GPI anchor proteins in the F. graminearum s. str. genome that are similar to proteins with known functions; several genes encode putative carbohydrate-modifying enzymes. A conserved domain for the medium chain reductase/dehydrogenase (MDR)/zinc-dependent alcohol dehydrogenase-like family was also detected in FGSG_02810, although this gene was not included by Rittenour and Harris (2013). According to Rittenour and Harris (2013), no phenotypic change could be due to the gene redundancy of FGSG_001588 and FGSG_08844, while our results indicate that FGSG_02810 is a single gene. The gpi7 might affect FGSG_02810 gene function, although alteration of macroconidium production was not detected in the FGSG_02810 complement transformant or FGSG_02810 disruption mutants (data not shown).

Natural pathogenic variation has been observed in the Fg complex, but the cause of this variation is poorly understood at a molecular level. In this study, using genetic mapping aided by whole genome sequence (Cuomo et al. 2007), we identified a natural mutation involving pathogenicity of the Fg complex. Although the function of FGSG_02810 remains unclear, our data suggest that it plays a role in breaking the rachis nodes in wheat head and peritheium formation.

Further study will lead to a better understanding of the molecular mechanisms involved in host infection and production of inoculum in the Fg complex. Considerable bioinformatics analysis data (such as transcriptome and proteome) are now available on the Fg complex. Moreover, combinational analyses of genetic
mapping and bioinformatics data are also useful for further elucidation of phenotypic variations of the Fg complex at the molecular level.

ACKNOWLEDGMENTS

We thank H. C. Kistler (United States Department of Agriculture–Agricultural Research Service) for providing some primers, and M. Funasaka (Gifu University, Japan) and T. Scott (Gifu University, Japan) for technical support. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (No. 21580055).

LITERATURE CITED

Ahn, N., S. Kim, W. Choi, K. H. Im, and Y. H. Lee, 2004 Extracellular matrix protein gene, EMPI, is required for appressorium formation and pathogenicity of the rice blast fungus, Magnaporthe grisea. Mol. Cells 17: 166–173.

Aoki, T., T. Ward, C. Kistler, and K. O’Donnell, 2012 Systematics, phylogeny and trichothecene mycotoxin potential of Fusarium head blight cereal pathogens. Mycotoxins 62: 91–102.

Bai, G. H., A. E. Desjardins, and R. D. Plattner, 2002 Deoxynivalenol-nonproducing Fusarium graminearum causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153: 91–98.

Bowden, R. L., and J. F. Leslie, 1999 Sexual recombination in Gibberella zeae. Phytopathology 89: 182–188.

Carroll, A. M., J. A. Swiegaard, and B. Valetn, 1994 Improved vectors for selecting resistance to hygromycin. Fungal Gen. Newsl. 41: 20–21.

Carter, J. P., H. N. Rezanoor, D. Holden, A. E. Desjardins, R. D. Plattner et al., 2002 Variation in pathogenicity associated with the genetic diversity of Fusarium graminearum. Eur. J. Plant Pathol. 108: 573–583.

Correll, J. C., C. J. R. Kliiitch, and J. F. Leslie, 1987 Nitrature nonutilizing mutant of Fusarium oxyosporum and their use in vegetative compatibility tests. Phytopathology 77: 1640–1646.

Cumagun, C. J. R., R. L. Bowden, J. E. Jurgenson, J. F. Leslie, and T. Miedaner, 2004 Genetic mapping of pathogenicity and aggressiveness of Gibberella zeae (Fusarium graminearum) toward wheat. Phytopathology 94: 520–526.

Cuono, C. A., U. Guldener, J.-R. Xu, F. Trail, B. G. Turgeon et al., 2007 The Fusarium graminearum genome reveals a link between localized polymorphism and pathogen specialization. Science 317: 1400–1402.

De Groot, P. W. J., A. F. Ram, and F. M. Klis, 2005 Features and functions of covalently linked proteins in fungal cell walls. Fungal Genet. Biol. 42: 657–675.

Desjardins, A. E., T. M. Hohn, and S. P. McCormick, 1993 Trichothecene biosynthesis in Fusarium species: chemistry, genetics, and significance. Microbiol. Rev. 57: 593–604.

Dufrere, M., T. d. Lee, S. B. M'Barek, X. Xu, X. Zhang et al., 2008 Transposon-tagging identifies novel pathogenicity genes in Fusarium graminearum. Fungal Genet. Biol. 45: 1552–1561.

Eisenhaber, B., G. Schneider, M. Wildpaner, and F. Eisenhaber, 2004 A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for Aspergillus nidulans, Candida albicans, Neurospora crassa, Saccharomyces cerevisiae and Schizosaccharomyces pombe. J. Mol. Biol. 337: 243–253.

Francl, L., G. Shaner, G. Bergstrom, J. Gilbert, W. Pedersen et al., 1999 Daily inoculum levels of Gibberella zeae on wheat spikes. Plant Dis. 83: 662–666.

Gale, L. R., J. D. Bryant, S. Calvo, H. Giese, T. Katan et al., 2005 Chromosome complement of the fungal plant pathogen Fusarium graminearum based on genetic and physical mapping and cytological observations. Genetics 171: 985–1001.

Hou, Z., C. Xue, Y. Peng, T. Katan, H. C. Kistler et al., 2002 A mitogen-activated protein kinase gene (MGVI) in Fusarium graminearum is required for female fertility, heterokaryon formation and plant infection. Mol. Plant Microbe Interact. 15: 1119–1127.

Jenczmionka, N. J., F. J. Maier, A. P. Losch, and W. Schafapr, 2003 Mating, conidiation and pathogenicity of Fusarium graminearum, the main causal agent of the head-blight disease of wheat, are regulated by the MAP kinase gmpk1. Curr. Genet. 43: 87–95.

Jurgenson, J. E., R. L. Bowden, K. A. Zeller, J. F. Leslie, N. J. Alexander et al., 2002 A genetic map of Gibberella zeae (Fusarium graminearum). Genetics 160: 1451–1460.

Kapteyn, J. C., R. C. Montiitian, E. Vink, J. de la Cruz, A. Llobell et al., 1996 Retention of Saccharomyces cerevisiae cell wall proteins through a phosphodiester-linked β-1,3-/β-1,6-glucan heteropolym. Glycolobiology 6: 337–345.

Klittich, C. J. R., and J. F. Leslie, 1988 Nitrate reduction mutants of Fusarium moniliforme (Gibberella fujikuroi). Genetics 118: 417–423.

Li, H., H. Zhou, Y. Luo, H. Ouyang, H. Hu et al., 2007 Glycosylphosphatidylinositol (GPI) anchor is required in Aspergillus fumigatus for morphogenesis and virulence. Mol. Microbiol. 64: 1014–1027.

Logrieco, A., C. Altmare, A. Bottalo, and M. Manka, 1990 Pathogenicity of Fusarium graminearum chemotypes towards corn, wheat, triticale and rye. J. Phytopathol. 130: 197–204.

Lu, S. W., S. Kroken, B. N. Lee, B. Robbertse, A. C. Churchill et al., 2003 A novel class of gene controlling virulence in plant pathogenic ascomycete fungi. Proc. Natl. Acad. Sci. USA 100: 5980–5985.

Mackintosh, C. A., D. F. Garvin, L. E. Radmer, S. J. Heinen, and G. J. Muehlbauer, 2006 A model wheat cultivar for transformation to improve resistance to Fusarium head blight. Plant Cell Rep. 25: 313–319.

Marchler-Bauer, A., M. K. Derbyshire, N. R. Gonzales, S. Lu, F. Chitsaz et al., 2015 CDD: NCBI’s conserved domain database. Nucleic Acids Res. 43: D222–D226.

O’Donnell, K., H. C. Kistler, B. K. Tacke, and H. H. Casper, 2004 Genenealogies reveal global phylogeographic structure and reproductive isolation among lineages of Fusarium graminearum, the fungus causing wheat scab. Proc. Natl. Acad. Sci. USA 97: 7905–7910.

O’Donnell, K., T. J. Ward, D. M. Geiser, H. C. Kistler, and T. Aoki, 2004 Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the Fusarium graminearum clade. Fungal Genet. Biol. 41: 600–623.

O’Donnell, K., T. J. Ward, D. Abera, H. C. Kistler, T. Aoki et al., 2008 Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the Fusarium graminearum species complex from Ethiopia. Fungal Genet. Biol. 45: 1514–1522.

Pestka, J. J., and A. T. Smolinski, 2005 Deoxynivalenol: toxicology and potential effects on humans. J. Toxicol. Environ. Health B Crit. Rev. 8: 39–69.

Petersen, T. N., S. Brunak, G. von Heijne, and H. Nielsen, 2011 SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat. Methods 8: 785–786.

Rittenour, W. R., and S. D. Harris, 2013 Glycosylphosphatidylinositol-anchored proteins in Fusarium graminearum: inventory, variability, and virulence. PLoS One 8: e81603.

Serov, B. A., J. T. Ward, L. R. Gale, K. Broz, H. Corby Kistler et al., 2011 Novel Fusarium head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. Fungal Genet. Biol. 48: 1096–1107.

Schofflmeier, E. A. M., J. H. Vossen, A. A. van Doorn, B. J. C. Cornelissen, and M. A. Haring, 2001 FEM1, a Fusarium oxysporum glycoprotein that is covalently linked to the cell wall matrix and is conserved in filamentous fungi. Mol. Genet. Genomics 265: 143–152.

Seo, B.-W., H.-K. Kim, Y.-W. Lee, and S.-H. Yun, 2007 Functional analysis of a histidine auxotrophic mutation in Gibberella zeae. Plant Pathol. J. 23: 51–56.

Seong, K., Z. Hou, M. H. Tracy, C. Kistler, and J.-R. Xu, 2005 Random insertional mutagenesis identifies genes associated with virulence in the wheat scab fungus Fusarium graminearum. Phytopathology 95: 744–750.
Shim, W. B., U. S. Sagaram, Y. E. Choi, J. So, H. H. Wilkinson et al., 2006 FSR1 is essential for virulence and female fertility in *Fusarium verticillioides* and *F. graminearum*. Mol. Plant Microbe Interact. 19: 725–733.

Starkey, D. E., T. J. Ward, T. Aoki, L. R. Gale, H. C. Kistler et al., 2007 Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. Fungal Genet. Biol. 44: 1191–1204.

Suga, H., L. R. Gale, and H. C. Kistler, 2004 Development of VNTR markers for two *Fusarium graminearum* clade species. Mol. Ecol. Notes 4: 468–470.

Suga, H., G. W. Karugia, T. Ward, L. R. Gale, K. Tomimura et al., 2008 Molecular characterization of the *Fusarium graminearum* species complex in Japan. Phytopathology 98: 159–166.

Suga, H., T. Nakajima, K. Kageyama, and M. Hyakumachi, 2011 The genetic profile and molecular diagnosis of thiophanate-methyl resistant strains of *Fusarium asiaticum* in Japan. Fungal Biol. 115: 1244–1250.

Sweigard, J. A., F. Chumley, A. M. Carroll, L. Farrall, and B. Valent, 1997 A series of vectors for fungal transformation. Fungal Genet. Newsl. 44: 52–53.

Urban, M., and K. E. Hammond-Kosack, 2013 Molecular genetics and genomic approaches to explore *Fusarium* infection on wheat floral tissue, pp. 43–79 in *Fusarium: Genomics, Molecular and Cellular Biology*, edited by D. W. Brown and R. H. Proctor. Caister Academic Press, Norfolk.

Van Ooijen, J. W., and R. E. Voorrips, 2001 *JoinMap* 3.0. Software for the Calculation of Linkage Maps. Plant Research International, Wageningen, The Netherlands.

Wasmann, C. C., and H. D. VanEtten, 1996 Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of *Nectria haematococca* on pea. Mol. Plant Microbe Interact. 9: 793–803.

Windels, C. E., 2000 Economic and social impacts of *Fusarium* head blight: changing farms and rural communities in the northern great plains. Phytopathology 90: 17–21.

Yli-Mattila, T., T. Gagkaeva, T. J. Ward, T. Aoki, H. C. Kistler et al., 2009 A novel Asian clade within the *Fusarium graminearum* species complex includes a newly discovered cereal head blight pathogen from the Russian far east. Mycologia 101: 841–852.

Yu, H.-Y., J.-A. Seo, J.-E. Kim, K.-H. Han, W.-B. Shim et al., 2008 Functional analyses of heterotrimeric G protein Go and Gβ subunits in *Gibberella zeae*. Microbiology 154: 392–401.

Communicating editor: J. D. Faris