A Mitogen-Activated Protein Kinase Gene (*MGV1*) in *Fusarium graminearum* Is Required for Female Fertility, Heterokaryon Formation, and Plant Infection

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Fusarium graminearum **is an important pathogen of small grains and maize in many areas of the world. Infected grains are often contaminated with mycotoxins harmful to humans and animals. During the past decade,** *F. graminearum* **has caused several severe epidemics of head scab in wheat and barley. In order to understand molecular mechanisms regulating fungal development and pathogenicity in this pathogen, we isolated and characterized a MAP kinase gene,** *MGV1,* **which is highly homologous to the** *MPS1* **gene in** *Magnaporthe grisea.* **The** *MGV1* **gene was dispensable for conidiation in** *F. graminearum* **but essential for female fertility during sexual reproduction. Vegetative growth of** *mgv1* **deletion mutants was normal in liquid media but reduced on solid media. Mycelia of the** *mgv1* **mutants had weak cell walls and were hypersensitive to cell wall degrading enzymes. Interestingly, the** *mgv1* **mutants were self-incompatible when tested for heterokaryon formation, and their virulence was substantially reduced. The ability of the mutants to accumulate trichothecene mycotoxins on inoculated wheat was also greatly reduced. Our data suggest that** *MGV1* **in** *F. graminearum* **is involved in multiple developmental processes related to sexual reproduction, plant infection, and cell wall integrity.**

Additional keywords: fungal pathogenicity, *Gibberella zeae*

Fusarium head blight (scab) is a disease of wheat and barley that can cause reduction in both crop yield and grain quality and has emerged in the past 10 years as an important constraint on small grain production (Windels 2000). In North America, the primary pathogen causing this disease is *Fusarium graminearum* Schwabe, lineage 7 (O'Donnell et al. 2000) (teleomorph *Gibberella zeae*). Infected grains are often contaminated with mycotoxins, such as deoxynivalenol (DON) and zearalenone, which are harmful to humans and animals (Desjardins et al. 1993; Windels 2000). In addition to being an important pathogen of wheat and barley, *F. graminearum* is also associated with stalk and ear rots of maize (Sutton 1982).

The development of ascospores is a vital process in the life cycle of the fungus and in disease development. The fungus overwinters on infected plant debris as saprophytic mycelia and produces spores when weather conditions favor fungal development in the spring. Ascospores and conidia formed by *F. graminearum* are believed to be the main inoculum infecting flowering wheat heads (Francl et al. 1999; McMullen et al. 1997). Ascospores are forcibly discharged from mature perithecia (Trail et al. 2002) formed on the surface of debris and possibly are dispersed by wind (Parry et al. 1995). Conidia may be dispersed primarily by rain splash and are important in spreading the disease in the field (Sutton 1982) and colonizing plant vegetative tissues, which serve as the substrate for overwintering mycelia (Wearing and Burgess 1977). *F. graminearum* is a homothallic ascomycete that can be readily forced to outcross (Bowden and Leslie 1999) and is amenable to classical and molecular genetic techniques such as fungal transformation and gene disruption (Proctor et al. 1995). However, there are few molecular studies of the development and pathogenicity of this important pathogen. The *TRI5* gene, involved in trichothecene production, is the only virulence gene characterized in *F. graminearum* (Proctor et al. 1995).

Signaling pathways involved in plant infection by the rice blast fungus, *Magnaporthe grisea,* have been well studied, and three mitogen-activated protein (MAP) kinases have been characterized (Xu 2000). The MAP kinase encoded by *MPS1,* a homologue of *Saccharomyces cerevisiae SLT2,* is important for maintaining cell wall integrity and essential for plant penetration (Xu et al. 1998). Appressoria formed by *M. grisea mps1* mutants neither penetrate plant cells nor elicit plant defense responses. Conidiation and aerial hyphae development are drastically reduced in *mps1* mutants, which are also female-sterile during sexual reproduction (Xu et al. 1998). Homozygous mutants of the human pathogen *Candida albicans* disrupted in *MKC1,* an *MPS1* homologue, produce less invasive growth on Spider medium and are less virulent in an experimental infection system using a murine model (Diez-Orejas et al. 1997). The *MPS1* and *MKC1* MAP kinase genes have 80% similarity, and both are involved in fungal pathogenesis.

In this study, we isolated and characterized the *F. graminearum* gene *MGV1* (map kinase for growth and virulence 1), a homologue of *MPS1*. *F. graminearum* does not form appressoria or other specialized penetration structures for plant infection, but ascospores and conidia are important in disease development and dispersal, and the production of these spores may be regulated by *MGV1*. Site-directed *mgv1* deletion mutants had reduced virulence and failed to produce perithecia and ascospores. However, hyphal growth and conidiation of the *mgv1* mutants in liquid culture were not affected, although their growth rate was reduced when they were cultured on solid media, and their mycelia appeared to have defective cell walls.

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RESULTS

MGV1 encodes a MAP kinase homologous to *M. grisea MPS1*.

The polymerase chain reaction (PCR) product amplified with primers M3K1 and M3K2 was cloned and sequenced. One PCR clone, pZMH2, was found to be homologous to *MPS1* by BLASTX searches and was used to screen *F. graminearum* cosmid and cDNA libraries. Sequence analysis of the corresponding cosmid and cDNA clones revealed an open reading frame (ORF) encoding a protein with 416 amino acids that is homologous to those encoded by *MPS1* and other fungal MAP kinase genes (Fig. 1). The predicted amino acid sequence of the *MGV1* gene is 86% identical and 92% similar to that of *M. grisea MPS1* and 51.2% identical and 76.9% similar to that of *S. cerevisiae SLT2* (Lee et al. 1993). Southern blot analysis showed that *MGV1* is a single-copy gene in *F. graminearum* (data not shown). The *MGV1* coding region contains all conserved protein kinase subdomains and the characteristic MAP kinase dual phosphorylation sites TEY (residues 174–176). Phylogenetic analysis indicated that *MGV1* is most similar to *MPS1,* and both *MPS1* and *MGV1* are in the same clade with the *S. cerevisiae SLT2* (Xu 2000).

Isolation of *mgv1* deletion mutants by gene replacement.

The *MGV1* gene replacement vector pZMH6 was constructed by replacing the first 680 bp of the *MGV1* ORF and 770 bp of its upstream region with the *hph* gene (Fig. 2A). Over 100 hygromycin-resistant transformants were isolated and screened with PCR primers 17F and H855R (data not shown). Two putative *mgv1* deletion mutants were identified and confirmed by Southern blot analysis (Fig. 2B). When hybridized with a fragment amplified with primers 13F and K1R as the probe (Fig. 2B), only the wild-type PH-1 and a randomly selected ectopic transformant, N47, had the 1-kb *Hin*dIII band. The *mgv1* deletion transformants, C6 and N35, did not have any hybridization signal, because the probe sequence was deleted in pZMH6. When the same blot was stripped and rehybridized with the *hph* gene as the probe, C6, N35, and N47, but not PH-1, had distinct hybridization bands. Only a single 7.5-kb band in C6 hybridized to *hph,* but multiple bands hybridized in N35, indicating that both homologous recombination and multiple ectopic integrations of pZMH6 occurred in this transformant. The deletion of the *MGV1* gene in C6 and N35 was further confirmed by Southern blot analysis with DNA samples digested with *Kpn*I (data not shown). Even though N35 was identical to C6 in colony morphology, to avoid unexpected interference

Fig. 1. *MGV1* encodes a mitogen-activated protein (MAP) kinase in *Fusarium graminearum.* **A,** Alignment of the predicted amino acid sequence encoded by *MGV1* with those of its homologues. The GenBank accession numbers are *F. graminearum* Mgv1 (AF492766), *Magnaporthe grisea* Mps1 (AF02316), *Colletotrichum lagenarium* Maf1 (AY064246), *Blumeria graminis* Mpk2 (AF301165), *Aspergillus nidulans* MpkA (U59214), *Schizosaccharomyces pombe* Spm1 (U65405), and *Saccharomyces cerevisiae* Slt2 (X59262). **B,** Phylogenetic comparison of *MGV1* MAP kinase with other fungal MAP kinases. The amino acid sequences of MAP kinases from *S. cerevisiae* (sc), *S. pombe* (sp), *Candida albicans* (ca), *M. grisea* (mg) and other fungi were analyzed by the Clustal X program (Thompson et al. 1994) to create a progressive alignment dendrogram. The branch lengths are proportional to the mean number of differences per residue along each branch. Mps1 and Mgv1 cluster together with the Slt2 MAP kinase from *S. cerevisiae.*

resulting from the ectopic integration of pZMH6 in N35, only C6 was used in most of the experiments for characterizing the phenotypes of the *mgv1* mutants.

Reduced vegetative growth of *mgv1* mutants on solid media but not in liquid media.

On V8 juice agar plates, colonies of the *mgv1* mutants were smaller and more compact than those of the wild-type strain PH-1 and often had uneven margins (Fig. 2C). While the wild-type strain produced abundant aerial hyphae on V8 juice agar, the *mgv1* mutants produced fewer and shorter aerial hyphae (Fig. 2C). Aerial hyphae produced by the *mgv1* mutants also had less pigment. One-week-old colonies on V8 juice agar plates were whitish, in contrast to the pinkish colonies formed by PH-1. After incubation on V8 juice medium for 5 days, the average diameters of PH-1 and C6 colonies were 7.7 and 2.3 cm, respectively (Table 1). Vegetative growth of the *mgv1* mu-

Fig. 2. *Fusarium graminearum MGV1* gene replacement construct and mutants. **A,** Physical map of the *MGV1* genomic region and gene replacement vector pZMH6. Arrows indicate the orientation of the *MGV1* and *hph* genes. The restriction enzymes are *Bgl*II (Bg), *Eco*RI (E), *Hind*III (H), *Mbo*I (M), *Sal*I (S), and *Stu*I (St). **B,** Southern blot of wild-type *F. graminearum* PH-1, the ectopic transformant N47, and the *mgv1* mutants C6 and N35. All DNA samples were digested with *Hind*III. The blot was hybridized with the fragment amplified with 13F and K1R as the probe. **C,** Colony morphology of PH-1 and *mgv1* mutant C6 cultured on V8 juice agar plates. The photograph was taken after 3 days of incubation at 25°C.

tant C6 was also significantly reduced when it was cultured on CM and 5×YEG media (Table 1), indicating that the reduction in growth rate was not related to the specific media used. Similarly, different media had no effect on the production of aerial hyphae and pigment in the *mgv1* mutants, because C6 produced whitish compact colonies on all media tested.

We also determined hyphal growth of an *mgv1* mutant in liquid CMC medium by measuring the fungal biomass accumulated after incubation for 3 days. No significant differences were observed between PH-1, C6, and N47 (Table 1). When cultured in nutritionally rich yeast extract–peptone–dextrose (YEPD) medium, both the wild type and the *mgv1* mutant produced more abundant vegetative mycelia. However, there was no significant difference in fungal biomass accumulation between the wild-type strain and the *mgv1* mutant (data not shown). Thus, the *mgv1* deletion mutant had no defect in vegetative growth in liquid media.

Conidiation in *mgv1* mutants.

On V8 juice agar plates, both PH-1 and C6 produced conidia, but not efficiently. Because vegetative growth of the *mgv1* mutant on V8 juice agar plates was reduced, quantitative comparison of the conidiation of C6 and PH-1 colonies was difficult. In liquid CMC medium, the *mgv1* mutant produced abundant conidia (Fig. 3A). On average, PH-1, N47, and C6 all produced about 1.5×10^5 conidia/ml after 5 days of incubation (Fig. 3A), indicating that the deletion of *MGV1* had no obvious effect on conidiation.

Conidia produced by the *mgv1* mutant C6 were normal in size and morphology. No defect was observed in the germination of conidia of C6 and N35 (data not shown). Old cultures of wild-type *F. graminearum* strains produce a few thick-walled cells within conidia. These cells do not resemble the globose chlamydospores, which are formed only rarely in *F. graminearum* culture and have been described by many authorities (Booth 1971; Gerlach and Nirenberg 1982; Nelson et al. 1983). The *mgv1* mutant was also able to form thick-walled cells within conidia in CMC cultures more than 7 days old (Fig. 3B). However, the number of C6 and N35 conidia that developed wall thickenings was less than 5% of the number of PH-1 and N47 conidia that developed them (data not shown). In a small portion (less than 0.1%) of the C6 conidia having wall thickenings, the apical and basal cells of macroconidia were disarticulated, resulting in spore fragmentation (Fig. 3B). These fragmented conidia germinated normally when transferred to CM or 5×YEG media (data not shown). No fragmented spores were observed in macroconidia produced by PH-1 and N47 cultures grown under the same conditions. Most likely conidia with wall thickenings also have weakened cell-to-cell junctions as a result of the *mgv1* deletion.

mgv1 mutants are female-sterile.

To determine the potential function of *MGV1* in sexual reproduction, the *mgv1* mutants were assayed for the production of perithecia on carrot agar medium under conditions that would
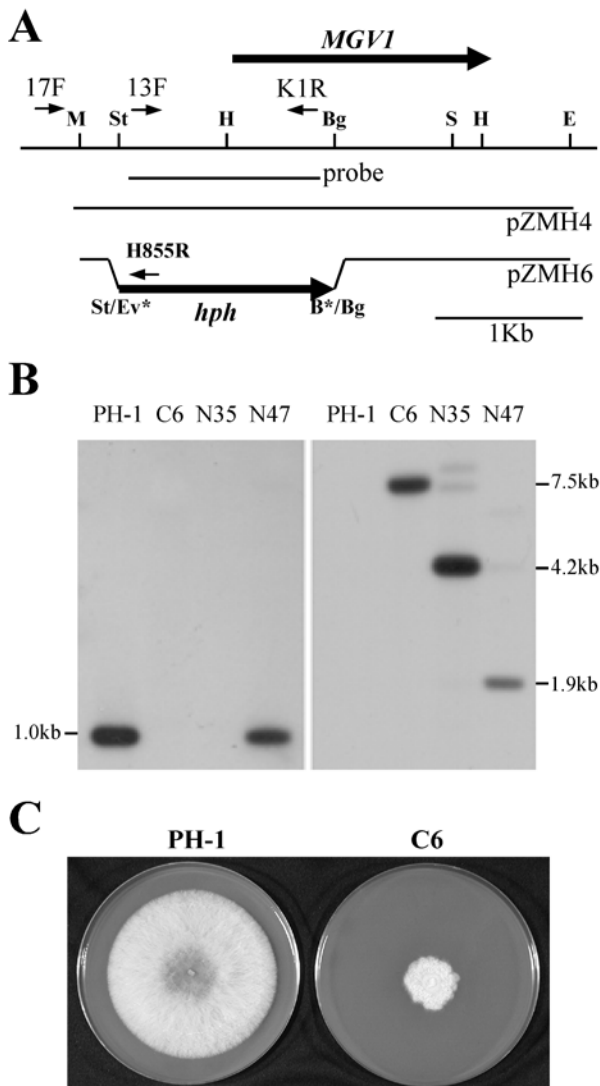
Table 1. Vegetative growth of *Fusarium graminearum* strains on different media

Strain	Colony diameter (cm)[a] on:			Dry weight (mg)[b]
	V8 juice	5×YEG	CM	
PH-1 (wild type)	7.7 ± 0.2	6.6 ± 0.1	9.1 ± 0.2	136.3 ± 5.3
C6 (*mgv1* mutant)	2.3 ± 0.2	1.7 ± 0.1	3.6 ± 0.2	138.8 ± 9.3
N47 (ectopic)	8.4 ± 0.1	6.6 ± 0.1	8.6 ± 0.4	140.5 ± 8.8

[a] Colony diameter was measured after incubation for 5 days. The mean and error were calculated from at least three independent experiments.
[b] Dry weight of fungal biomass grown in liquid CMC medium.

promote selfing or outcrossing. As a homothallic fungus, both the wild-type PH-1 and the ectopic transformant N47 alone produced abundant perithecia after 2–3 weeks. However, the *mgv1* mutant C6 failed to produce any perithecia under selfing conditions, even after 1 month of incubation (Fig. 4A). No perithecia or protoperithecia were observed in cultures of C6 (Fig. 4A) or N35, indicating that autonomous sexual reproduction was abolished in the *mgv1* mutants.

On outcrossing plates with the *mgv1* mutant C6 or N35 and the PH-1 *nit1* mutant 11622 growing side-by-side, perithecia were observed only on the *nit1* mutant colonies (Fig. 4B). No perithecia were observed on the C6 or N35 colonies 3 weeks or more after cross-fertilization (Fig. 4B). Random ascospores collected from perithecia formed on the *nit1* mutant colonies in C6 × 11622 outcross plates were cultured on minimal medium containing hygromycin (250 μg/ml) and 1% potassium chlorate. All 20 randomly selected ascospore progeny that grew on this medium had the colony morphology of *mgv1* mutants and

were confirmed by Southern blot analysis to be *mgv1* mutants (data not shown). Because they were resistant to both hygromycin and potassium chlorate, we conclude that these recombinant colonies were from outcrosses between the hygromycin-resistant *mgv1* and the chlorate-resistant *nit1* mutants. Thus, the *mgv1* deletion mutant was unable to form the female fruiting structure on it own (female-sterile) but was still able to contribute to a sexual outcross (male-fertile). All of the 20 hygromycin-sensitive progeny isolated from the same outcross grew at the same rate as PH-1 and formed normal colonies with abundant aerial hyphae, indicating that the hygromycin-resistant marker co-segregated with the colony phenotype observed in the *mgv1* deletion mutants.

Cell wall defects in *mgv1* mutants.

Because the central part of *mgv1* colonies sometimes underwent autolysis in cultures more than 3–4 weeks old, we suspected that *mgv1* mutants had defects in hyphal cell walls. To confirm this, we treated *F. graminearum* germlings collected from conidia germinated in liquid YEPD medium for 14 h with the cell wall degrading enzyme Driselase. After incubation at 30°C for only 30 min, few protoplasts were observed among the wild-type germlings, while germlings from *mgv1* mutant C6 were well digested and released abundant protoplasts (Fig. 5A).
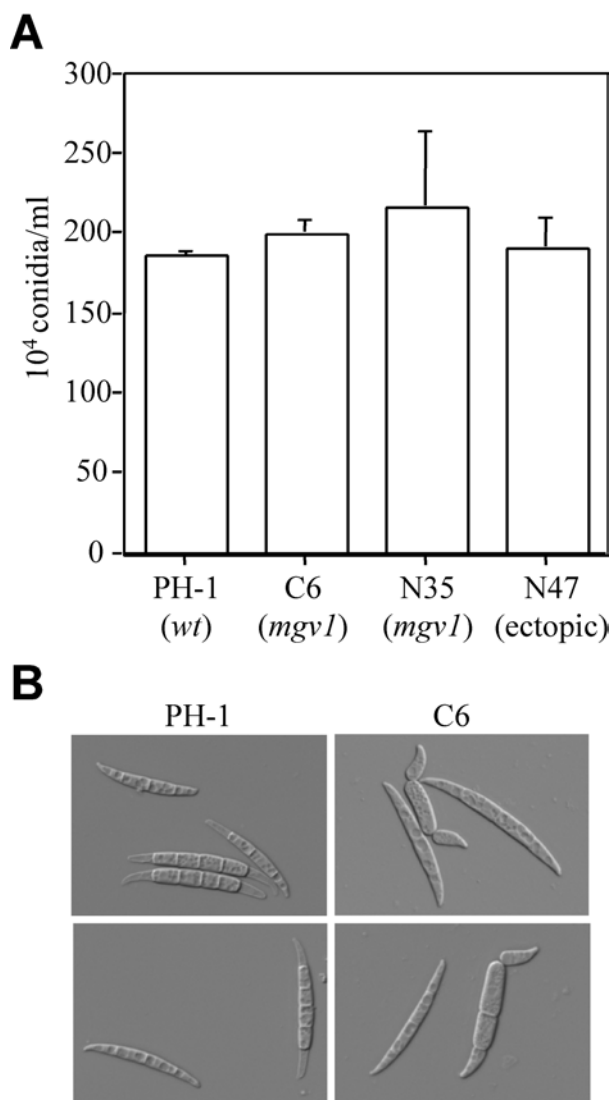
Fig. 3. Conidia produced by *mgv1* deletion mutants of *Fusarium graminearum*. **A,** Conidiation in liquid CMC medium. Concentrations of conidia produced by cultures of the wild-type strain PH-1, *mgv1* mutants C6 and N35, and the ectopic transformant N47 were determined after incubation for 5 days. Means and standard errors were calculated from three independent experiments. **B,** Morphology of conidia and thick-walled conidia produced by PH-1 and *mgv1* mutant C6. A small portion of conidia became fragmented in C6 cultures more than 7 days old.
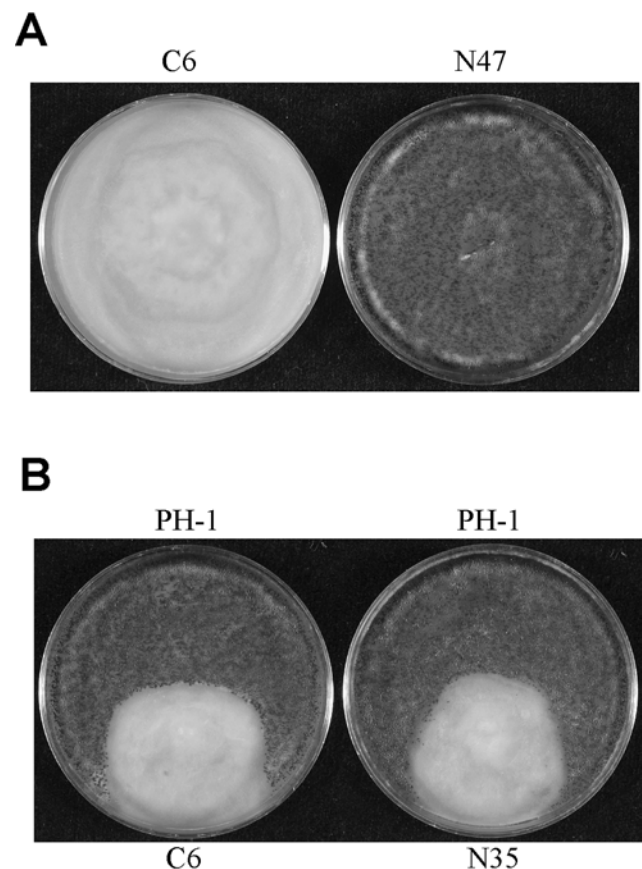

Fig. 4. Sexual reproduction in *mgv1* mutants of *Fusarium graminearum*. **A,** The *mgv1* mutants were self-sterile. Abundant perithecia (black dots) were produced by the ectopic transformant N47 (right) 2 weeks after fertilization on carrot agar plates. The *mgv1* mutant C6 (left) failed to form any perithecia under the same conditions. **B,** Outcrossing between the wild-type strain PH-1 (in the upper part of the plates) and the *mgv1* deletion mutants C6 and N35 (in the lower part of the plates). Only the colonies of PH-1, and not those of C6 and N35, developed perithecia on co-inoculated carrot agar plates. The photographs were taken 2 weeks after cross-fertilization.

When conidia germinated in liquid CMC medium for 18 h at 25°C were shifted to 32°C and incubated for another 12 h, terminal and intercalary swollen bodies developed in many germlings from the *mgv1* mutants C6 and N35 (Fig. 5). When 18-h germlings were shifted to 37°C and incubated for over 12 h, most of the *mgv1* germ tubes had swollen bodies, and many had burst. We also observed that incubation temperatures higher than 32°C significantly inhibited the formation of conidia in the *mgv1* mutants (data not shown). These observations suggest that the *mgv1* mutants have an impaired cell wall and are sensitive to elevated incubation temperatures (above 32°C). This defect in cell wall integrity appeared to be more severe when the *mgv1* mutants were cultured on nutritionally rich media, such as 5xYEG and TB3. Intercalary and apical hyphal swollen bodies could be observed in some mycelia even when the mutants were cultured at 25°C in these media (data not shown).

Virulence is reduced in *mgv1* mutants.

To determine whether the virulence of the *mgv1* mutants is affected, flowering wheat heads were inoculated with conidia from PH-1, C6, N35, and N47. Most of the basal spikelets inoculated with *mgv1* spore suspensions developed typical scab symptoms 14 days after inoculation, similar to those inoculated with conidia from PH-1 and N47 (Fig. 6). However, the number of uninoculated spikelets that developed scab symptoms on wheat heads basally inoculated with the *mgv1* mutant C6 or N35 was significantly reduced (Table 2). At 14 days after inoculation, only one or two spikelets were blighted on most wheat heads inoculated with the *mgv1* mutants. Under the same conditions, about half of the spikelets were blighted on wheat heads inoculated with PH-1 or N47 (Fig. 6). These data indicate that the ability of the *mgv1* mutants to colonize and spread in flowering wheat heads was reduced.

To determine whether the reductions in virulence and growth rate on solid media were directly related to the deletion of *MGV1*, a 9-kb *Bam*HI fragment containing the wild-type *MGV1* gene was cloned in pBCSK and introduced into *mgv1* mutant C6 by co-transformation with pSM334. Neomycin-resistant transformants were screened by PCR with primers 13F and K1R, and the presence of the wild-type *MGV1* allele was confirmed by Southern blot analysis. Complemented *MGV1* transformants grew at rates comparable to that of the wild-type strain PH-1 (Table 1) and had normal virulence on flowering wheat heads (data not shown). Thus, reintroduction of the wild-type *MGV1* allele restored the growth and virulence defect in the *mgv1* mutants.

Production of DON in *mgv1* mutants.

To date, DON is the only known virulence factor in *F. graminearum* (Proctor et al. 1995). To determine whether DON accumulation in plants is affected by the *mgv1* deletion, spikelets of the wheat cultivar Norm were inoculated with N35, C6, or the ectopic transformant N47. The trichothecene content of

the plants was measured 2 weeks after inoculation. While DON and 15-acetyl-DON accumulated to levels reaching above 200 ppm in samples inoculated with N47, trichothecene concentrations in samples inoculated with the *mgv1* mutants N35 and C6 were less than 1.5 ppm or not detectable (Table 3).

mgv1 mutants are self-incompatible in heterokaryon formation.

To determine whether *mgv1* mutants are capable of normal cell fusion and heterokaryon formation, several independent auxotrophic (*nit1*) mutants were made from *mgv1* strains C6 and N35 and then paired on minimal medium with a PH-1 strain having a nonallelic auxotrophic mutation (*nitM*) also in the pathway for nitrate reduction. As a control, *nit1* mutations were made from the ectopic transformant strain N47 and also paired with the PH-1 *nitM* strain. Wild-type growth was readily observed (Fig. 7) at the convergence of colonies of N47 (*MGV1 nit1*) and PH-1 (*MGV1 nitM*), indicating successful cell fusion and heterokaryon formation. Repeated pairing of independent C6 (*mgv1 nit1*) and N35 (*mgv1 nit1*) strains with PH-1 (*MGV1 nitM*) never resulted in wild-type growth at the convergence of the colonies (Fig. 7). When samples were examined under a microscope, hyphal fusion was not observed in the *mgv1* mutants C6 and N35 (data not shown). Thus, while the ectopic transformant N47 maintained its capacity for hyphal fusion and heterokaryon formation with its isogenic parent PH-1, the *mgv1* mutants lost this capacity.

DISCUSSION

The *MGV1* gene has all the characteristic features of MAP kinases and is closely related to the *MPS1* gene of *M. grisea*. Like *M. grisea mps1* mutants, *F. graminearum mgv1* mutants are hypersensitive to cell wall degrading enzymes, suggesting a weaker cell wall in mutants in which the MAP kinase gene has been deleted. Intercalary or apical swollen bodies were formed on germ tubes cultured at an elevated incubation temperature (e.g., 32°C) or in rich media. In *S. cerevisiae, slt2* mutants are defective in cell wall integrity and display enhanced sensitivity to cell wall degrading enzymes. On rich media such as YPD, *slt2* mutants are unable to grow at 37°C in the absence of osmotic stabilizers (Lee et al. 1993). In *Schizosaccharomyces pombe, spm1* mutants have cells of abnormal shape and are sensitive to elevated incubation temperature (Toda et al. 1996). In *C.*

Fig. 5. Weakening of hyphal cell walls in *mgv1* mutants of *Fusarium graminearum.* After incubation for 30 min in protoplasting solution at 30°C (upper panels), the wild-type strain (PH-1) released protoplasts but still had many germlings remained intact. Germlings of the *mgv1* mutant (C6) were usually completely digested and released abundant protoplasts under the same conditions. When germlings grown in complete medium at 25°C were shifted to 32°C, swollen and burst hyphal cells were observed in C6 but not in PH-1.

Table 2. Spread of head scab symptoms from basal spikelets inoculated with various strains of *Fusarium graminearum*

Strain	No. of heads inoculated	Disease index[a]
C6 (*mgv1* mutant)	33	1.97 ± 1.26
N35 (*mgv1* mutant)	31	1.81 ± 1.54
N47 (ectopic)	32	5.56 ± 2.00
PH-1 (wild type)	35	7.09 ± 2.86

[a] Disease was rated by the number of symptomatic spikelets 14 days after inoculation of a basal spikelet. C6 and N35 are significantly less virulent than either N47 or PH-1 ($p = 0.0001$).
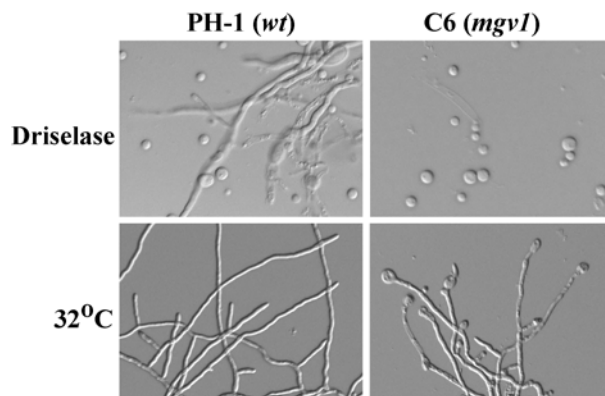
albicans, homozygous *mkc1* deletion mutants are sensitive to high incubation temperature (42°C), cell wall degrading enzymes, and antifungal compounds that interfere with 1,3-b-glucan and chitin synthesis (Navarro-Garcia et al. 1998). Hyphal cells produced by *mkc1* mutants are shorter than those of the wild type, and agar penetration by *mkc1* hyphae is reduced on Spider medium (Navarro-Garcia et al. 1998). It is likely that the *MGV1/MPS1* MAP kinase is well conserved in various fungi for regulating cell wall integrity and possibly nutrient sensing.

One intriguing phenomenon is that hyphal growth of *mgv1* mutants is reduced on solid media but normal in liquid media. The mutants have shortened aerial hyphae and form whitish, compact colonies. Similarly, in *M. grisea, mps1* mutants have been reported to form fewer aerial hyphae and conidia on oatmeal agar plates, while producing normal mycelial growth in liquid media (Xu et al. 1998). In *Aspergillus nidulans, mpkA* mutants also have a reduced growth rate and form fewer aerial hyphal (Bussink and Osmani 1999). In *Botrytis cinerea,* mutants disrupted in the *MPS1* homologue also have significantly reduced aerial hyphae development, conidiation, and growth rate on solid media but grow normally in liquid media (L. Zheng and J.-R. Xu, *unpublished data*). Thus, this MAP kinase may be important for the development and elongation of aerial hyphae of *F. graminearum* and possibly other filamentous fungi. Modifications of cell wall structure and surface involved

Fig. 6. Infection assay on wheat heads. Scab disease developed on flowering wheat heads inoculated in a basal spikelet with conidia from the wild-type strain PH-1 (top), *mgv1* mutant C6 (middle), and *mgv1* mutant N35 (bottom). The photographs were taken 2 weeks after inoculation. In both *mgv1* mutants, the ability to cause necrosis beyond the point of inoculation was reduced.

in aerial hyphae formation may be defective in the *mgv1* mutant. In *M. grisea* and *B. cinerea,* conidiation is also reduced in mutants that lack the MAP kinase gene, because conidiophores are produced on aerial hyphae. This is not the case in *F. graminearum,* which forms conidiophores in submerged cultures or very close to the surface on solid media. The *F. graminearum mgv1* mutant, like the *M. grisea mps1* mutant (Xu et al. 1998), is male-fertile but female-sterile. In *F. graminearum,* ascospores are thought to be important as primary inoculum and for long-distance dispersal (Trail and Common 2000). Chlamydospores are assumed to be one type of survival structure. It will be interesting to test whether the defects in female fertility and cell wall thickening have any effect on the survival and dispersal of the *mgv1* mutants in the field.

Virulence is severely reduced in the *mgv1* mutants. Because the vegetative growth rate is reduced on solid media, it is possible that the reduction in virulence of *mgv1* mutants may be solely a consequence of the reduced growth rate. However, the growth rate of *mgv1* mutants in liquid media is unaltered, and it is not clear whether growth conditions *in planta* may be more similar to solid media or liquid media. Furthermore, in many wheat heads inoculated with *mgv1* conidia, symptoms were absent, or only the inoculated spikelets developed symptoms. These observations therefore may not be explained simply by a reduction in growth rate. Thus, the *mgv1* mutants may be defective in producing some other virulence factors required for efficiently colonizing and spreading in wheat heads. As potent inhibitors of eukaryotic protein synthesis, trichothecenes may interfere with the induced resistance response of plants. DON production has been shown to contribute to the aggressiveness of *F. graminearum* in wheat (Proctor et al. 1995). The ability of *mgv1* mutants to produce trichothecene mycotoxins in infected plants was greatly reduced (Table 3), and this may contribute to reduced levels of disease produced by these strains. In *M. grisea, MPS1* is essential for appressorial penetration and infection of healthy rice leaves (Xu et al. 1998). The *MPS1* homologue is also important for pathogenicity in *B. cinerea* (Zheng and Xu, *unpublished data*). In *C. albicans,* the homozygous *mkc1* deletion mutants have reduced virulence in mice (Navarro-Garcia et al. 1998). In the obligate powdery mildew fungus *Blumeria graminis,* quantitative reverse transcription PCR indicated that the *MPK2* gene, a homologue of *MGV1,* is not expressed in ungerminated conidia, but its expression increases as germinated conidia form mature appressoria (Zhang and Gurr 2001), suggesting that *MPK2* may be involved in plant penetration processes.

The wild-type *F. graminearum,* like many filamentous fungi, can undergo hyphal fusion between compatible isolates to form heterokaryons (Glass et al. 2000; Leslie 1993). Mutants generated from the same strain are usually compatible with each other because they have identical *het* loci, which determine vegetative compatibility. Interestingly, the *mgv1* mutant is self-incompatible in heterokaryon formation. This phenotype has been reported as heterokaryon self-incompatibility (hsi) in other fungi (Correll et al. 1989; Glass et al. 2000). A single genetic locus, *hsi-1,* that determines heterokaryon self-incompatibility in *Gibberella fujikuroi* has been identified but not characterized (Correll et al. 1989). In *Neurospora crassa,* heterokaryon formation is delayed and reduced in the *ham-1* and *ham-2* (hyphal anastomosis) mutants, which are defective in self- and non-self-hyphal fusions (Wilson and Dempsey 1999; Xiang et al. 2002). The *HAM-2* gene encodes a protein that is highly conserved but of unknown function among eukaryotes (Xiang et al. 2002). Interestingly, *ham-2* deletion mutants also have pleiotropic defects, including female sterility, shortened aerial hyphae, and reduced growth rate on solid medium (Xiang et al. 2002). It is possible that the *MGV1* MAP kinase pathway
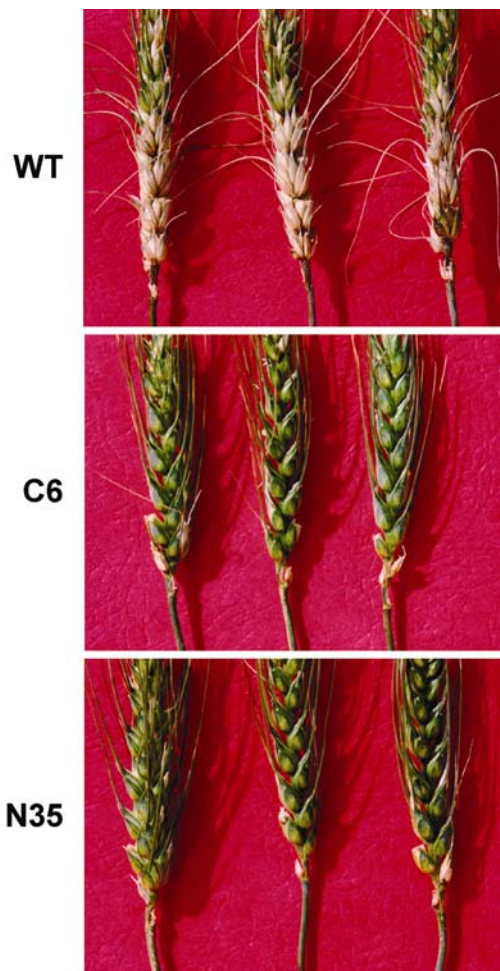
is somehow connected with *HAM-2* and is involved in hyphal fusion and heterokaryon formation in filamentous fungi.

MATERIALS AND METHODS

Strains and culture conditions.

F. graminearum (lineage 7) wild-type strain PH-1 (Trail and Common 2000) (NRRL 31084) and all the mutants generated in this study were routinely cultured at 25°C on V8 juice agar (Zheng et al. 2000). A *nit1* mutant 11622 of PH-1 was obtained from R. Bowden at Kansas State University. To compare growth rates on V8 juice, CM (Correll et al. 1987), TB3 (0.3% yeast extract, 0.3% casamino acids, and 3% glucose), and 5×YEG (Zheng et al. 2000) agar media, the diameters of colonies formed by monoconidial cultures on 150 × 15 mm petri plates were measured after incubation for 5 days. To assay conidiation, five conidia were introduced into 100 ml of liquid CMC medium (Cappellini and Peterson 1965), the suspension was shaken at 250 rpm for 5 days, and the concentration of conidia was determined with a hemacytometer. For dry weight measurements, 4.5×10^5 conidia were introduced into 100 ml of liquid CMC or YEPD (0.3% yeast extract, 1% peptone, and 2% dextrose) medium in a 250-ml flask, and the suspension was incubated at 25°C while being shaken. Mycelia were collected from 3-day-old cultures by filtration through two layers of Whatman no. 1 filter paper, baked at 100°C for 2 days, and then weighed. To assay degradation of cell walls and release of protoplasts, germlings collected from 14-h YEPD cultures were treated with Driselase (20 mg/ml) (Sigma, St. Louis, MO, U.S.A.) in 1.4 M KCl at 30°C.

DNA manipulations and sequence analysis.

Standard molecular biology procedures were followed for gel electrophoresis and Southern blot analysis (Sambrook et al. 1989). Fungal DNA was extracted by the CTAB protocol as described previously (Proctor et al. 1995). Plasmid and cosmid DNA samples were purified with Qiagen plasmid preparation kits (Qiagen, Valencia, CA, U.S.A.) and sequenced with the BigDye Terminator kit (PE Applied Biosystems, Foster City, CA, U.S.A.). An ordered cosmid library consisting of 9,216 clones was constructed with PH-1 genomic DNA in the cosmid vector pMOcosX as described previously (Orbach 1994). RNA isolated from PH-1 mycelia starved for nitrogen was used to construct a cDNA library with a Lambda Uni-ZAP II cDNA synthesis kit (Stratagene, La Jolla, CA, U.S.A.) following the instructions provided by the manufacturer. Homologue searches of DNA/protein sequence databases were performed with the BLAST programs (Altschul et al. 1997). Amino acid sequence comparisons and alignments were made with the BESTFIT, PILEUP and BOXSHADE programs in the GCG Wisconsin software package (Accelrys, San Diego, CA, U.S.A.) and with Clustal X (Thompson et al. 1994).

Table 3. Trichothecene accumulation in wheat spikelets inoculated with *mgv1* mutants

Sample	Trichothecene concentration (ppm)[a]		
	DON	3-A DON	15-A DON
N35-A	0.9	nd	nd
N35-B	1.2	nd	nd
C6-A	nd	nd	nd
C6-B	nd	nd	nd
N47-A	431	6.7	285
N47-B	317	3.3	151

[a] Concentration of deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-A DON) or 15-acetyldeoxynivalenol (15-A DON) in spikelet tissue, 14 days after inoculation. nd = not detectable (less than 0.5 ppm).

Isolation of the *MGV1* gene.

Degenerate PCR primers M3K1 (GGNGCNTAYGGNATHGTNTG) and M3K2 (ATYTCNGGNGCNCKRTACCA) were designed according to the conserved amino acid residues in fungal MAP kinases *S. cerevisiae SLT2, Schizosaccharomyces pombe SPM1,* and *M. grisea MPS1* (Xu et al. 1998). These two primers were used to amplify the *MPS1* homologue from *F. graminearum* by PCR consisting of 30 cycles of 1 min at 94°C, 1 min at 48°C and 2 min at 72°C followed by 5 min at 72°C. First-strand cDNA synthesized with RNA isolated from nitrogen-starved PH-1 mycelia was used as the template. The PCR product amplified with M3K1 and M3K2, about 0.5 kb, was cloned into the pGEM-T vector (Promega, Madison, WI, U.S.A.). The insert of a PCR clone homologous to *MPS1* was amplified with PCR primers T7 and SP6 and used as the probe to screen the pMOcosX cosmid library and the Lambda ZAP II cDNA library constructed in this study. Seven *MGV1*-containing cosmid clones, including 5P24, 17O9, and 21E24, were isolated. The genomic sequence of the *MGV1* gene and its flanking regions was determined by primer walking. Two *MGV1* cDNA clones, pC3K3 and pC3K4, were also isolated and sequenced.

Construction of *MGV1* gene replacement vector and mutants.

The 3.4-kb *Eco*RI fragment containing the entire *MGV1* ORF was isolated from cosmid clone 17O9 and cloned into the *Eco*RI site of the pBluescript KS (Stratagene) as pZMH4 (Fig. 2). The pZMH4 DNA was then digested with *Stu*I and *Bgl*II and ligated with a 1.4-kb *Eco*RV-*Bam*HI fragment containing the bacterial hygromycin phosphotransferase gene (*hph*) from pAhd5 (Zheng et al. 2000). The resulting gene replacement vector, pZMH6, was linearized by digestion with *Not*I and transformed into the wild-type strain PH-1 as described previously (Proctor et al. 1995). Monoconidial cultures of transformants
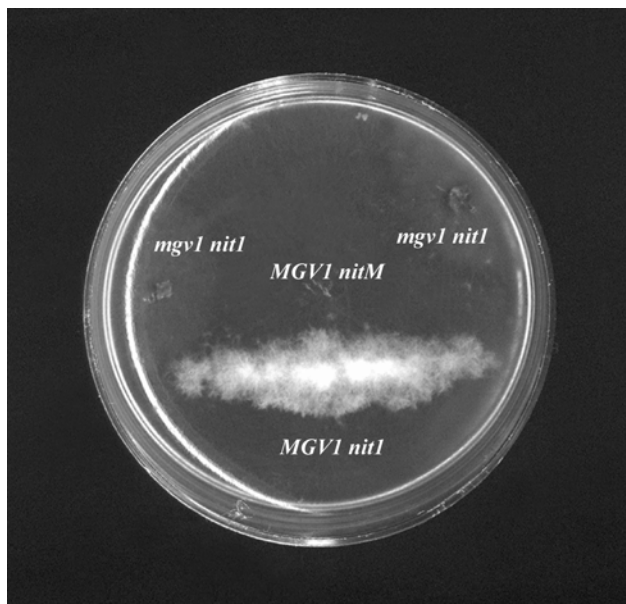

Fig. 7. Self-incompatibility in heterokaryon formation by strains of *Fusarium graminearum* The petri plate contains minimal medium inoculated with four strains derived from the wild-type strain PH-1: the *nitM* mutant of PH-1 (*MGV1 nitM*) (center), C6 (*mgv1 nit1*) (upper left), N35 (*mgv1 nit1*) (upper right), and N47 (*MGV1 nit1*) (lower center). Only the *MGV1* strains formed a heterokaryon characterized by the regeneration of wild-type growth due to complementation of auxotrophic mutations. The *mgv1* mutants were unable to form heterokaryons with isogenic *MGV1* strains.

resistant to hygromycin B (Calbiochem, La Jolla, CA, U.S.A.) at a concentration of 250 μg/ml on V8 juice agar plates were screened by PCR with primers 17F (TGGGTTGACCTG-ATAATG) and H855R (GCTGATCTGACCAGTTGC). The primer 17F sequence is located in the upstream region of *MGV1* and is not included in pZMH6. Only transformants with homologous recombination between pZMH6 and the endogenous *MGV1* gene will have the 450-bp PCR product. Putative *mgv1* deletion mutants identified in the PCR screens were further confirmed by Southern blot analysis. For complementation assays, the wild-type *MGV1* gene was reintroduced into the *mgv1* mutant C6 by co-transformation with pSM334 (kindly provided by Seogchan Kang at Pennsylvania State University), a vector containing the neomycin phosphotransferase fused to the promoter of the *Cochliobolus heterostrophus* glyceraldehyde-3-phosphate dehydrogenase gene (Van Wert and Yoder 1992). Neomycin-resistant transformants were selected on CM containing 200 μg of neomycin per ml (Amresco, Solon, OH, U.S.A.) and screened by PCR with primers K1R (GAGTGG-ATGTACTTGAGA) and 13F (ATACACCACCACACAA-ATAC).

Infection assays on flowering wheat heads.

Conidia were collected from 5-day-old CMC cultures by filtration through two layers of Miracloth (Calbiochem) and resuspended in sterile distilled water to a concentration of 0.5×10^5 to 1×10^5 conidia/ml. Approximately 6-week-old plants of the wheat cultivars Norm and Pioneer 2375 with flowering heads were used for infection assays. A floret in the basal spikelet of the flowering wheat head was injected with 10 μl of the conidial suspension. Inoculated plants were placed in a humidity chamber for 3 days and then transferred to a greenhouse with normal growth conditions. Symptomatic spikelets in each head were counted 14 days after inoculation. For the co-inoculation assay, conidia from PH-1 and C6 were mixed in equal ratios and used to inoculate flowering wheat heads. Analysis of variance was used to compare the mean number of symptomatic spikelets. As the wheat cultivar had no significant effect on the mean number of symptomatic spikelets ($P = 0.74$), results from both cultivars were pooled. The number of inoculated wheat heads per treatment ranged from 15 to 18. All tests were repeated once.

Sexual reproduction and vegetative incompatibility assays.

Perithecium formation in selfing and outcrossing cultures was assayed on modified carrot agar plates as described previously (Bowden and Leslie 1999). Ascospore cirri from perithecia on outcross plates, inoculated with C6 and 11622, were collected and resuspended in distilled water. Random ascospores were analyzed for resistance to hygromycin and 1% potassium chlorate on minimal medium. The *nit1* mutants of C6, N35, and N47 and a *nitM* mutant of PH-1 were obtained as described previously (Correll et al. 1987). To assay vegetative incompatibility, strains with complementary auxotrophic mutations were paired on minimal medium in 60×15 mm petri plates. The plates were incubated at ambient temperature under continuous fluorescent and near-UV light and allowed to grow together for 3 weeks as described previously (Correll et al. 1987).

Measurement of DON production.

The directly inoculated spikelets from five plants per treatment were pooled for DON analysis. The treatments were repeated once. Trichothecene levels in infected tissues were determined according to the method of Mirocha et al. (1998) with modifications. Briefly, 1 g of the pooled inoculated spikelets was extracted with 10 ml of a mixture of acetonitrile and water (84 : 16 v/v) in a 50-ml centrifuge tube for 1 h. After column

cleanup, 1 ml of the extract was placed in a 0.5-dram vial and evaporated to dryness under nitrogen. The sample was derivatized with 20 μl of trimethylsilyl ether (TMS) reagent (TMSI/TMCS 100 : 1, Sigma) and diluted with 200 μl of isooctane. Gas chromatography–mass spectroscopy (GC-MS) sample analysis was carried out on a Varian Saturn 3 GC-MS system with a Varian 3400 CX gas chromatograph and 8200 CX Auto-Sampler. The quantitative analysis was done in selected ion monitoring mode. Ions at *m/z* 235.10, 259.10, 295.10, 392.15, and 412 were monitored. A full-scan GC-MS analysis (with a scan range of *m/z* 180 to 440) was carried out for toxin identification in a wheat head sample. The gas chromatograph column was a J&W DB-5ms, 30 m in length, with 0.25-μm film thickness and 250-μm i.d. The GC temperature program was an initial temperature of 150°C for 1 min, ramped to 280°C at 25°C/min, followed by isothermal conditions for 5 min.

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