Available online at www.sciencedirect.com
SCIENCE DIRECT
Fungal Genetics and Biology 43 (2006) 34–41
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Cryptic promoter activity in the coding region of the HMG-CoA reductase gene in *Fusarium graminearum*

Kyeyong Seong [a], Lei Li [a], Zhanming Hou [a], Miles Tracy [a], H. Corby Kistler [b], Jin-Rong Xu [a,*]

[a] *Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA*
[b] *USDA-ARS, Cereal Disease Laboratory, Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA*

Received 5 March 2005; accepted 7 October 2005

Abstract

Head blight or scab disease caused by *Fusarium graminearum* poses a major threat to wheat and barley production in North America and other countries. To better understand the molecular mechanisms of *F. graminearum* pathogenesis, we have generated a collection of random insertional mutants. In mutant 222, one of the transformants significantly reduced in virulence, the transforming vector was inserted at amino acid 269 of the hydroxymethyl-glutaryl CoA reductase gene (*HMR1*) that encodes a key enzyme in sterol and isoprenoid biosynthesis. The N-terminal transmembrane domains of *HMR1* were disrupted, but the C-terminal catalytic domain was intact in mutant 222. We failed to isolate mutants deleted of the *HMR1* gene, suggesting that *HMR1* is an essential gene. Mutants deleted of the N-terminal 254 amino acids of *HMR1* were viable and phenotypically similar to mutant 222. In both mutant 222 and the $hmr1^{\Delta 254}$ mutants, a 3-kb truncated *HMR1* transcript was detectable by northern blot analyses. In the wild-type strain, only the 5-kb messenger was observed. The initiation site of truncated *HMR1* transcripts was determined by 5′-RACE to be 507 bp upstream from the catalytic subunit. When a *HMR1* fragment corresponding to the DNA sequence of $HMR1^{269–641}$ was translationally fused to a promoter-less GFP construct, green fluorescent signals were detectable in vegetative hyphae of the resulting transformants. These data indicate that this region of *HMR1* ORF has cryptic promoter activity and can express the catalytic domain in *hmr1* mutants deleted of its N-terminal portion. Our results also illustrate the importance of the *HMR1* gene and the function of its transmembrane domains in *F. graminearum*.

Keywords: *Gibberella zeae*; HMG-CoA reductase; Cryptic promoter

1. Introduction

During the past decade, Fusarium head blight or scab has emerged as the plant disease with the greatest impact on wheat and barley (Bai and Shaner, 2004; Goswami and Kistler, 2004). The disease, caused by the filamentous fungus *Fusarium graminearum* (teleomorph *Gibberella zeae*), has reached epidemic proportions in the United States and across the world (De Wolf et al., 2003; O'Donnell et al., 2004). In addition to causing yield losses, Fusarium head blight can reduce grain quality and result in price discounts (McMullen et al., 1997). Infested cereals are often contaminated with trichothecene and estrogenic mycotoxins that are harmful to humans and animals (Desjardins et al., 1996; McMullen et al., 1997). Moreover, *F. graminearum* also causes stalk and ear rots of maize and infects other small grains (McMullen et al., 1997; Parry et al., 1995; White et al., 2003). Although it is naturally a haploid homothallic ascomycete, heterothallic *F. graminearum* strains have been generated by deleting the *MAT1-1* or *MAT1-2* mating type locus (Lee et al., 2003). Thousands of ESTs (Trail et al., 2003) and the genome sequence of *F. graminearum* are now available (www.broad.mit.edu/annotation/fungi/fusarium).

Fusarium graminearum infects wheat spikes from anthesis through soft dough stages of kernel development. Spikelets are primarily infected when ascospores or conidia (macroconidia) are deposited on or inside flowering spikelets. The infection pathway on wheat spikes is complex as

* Corresponding author. Fax: +1 765 494 0363.
E-mail address: jinrong@purdue.edu (J.-R. Xu).

1087-1845/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2005.10.002

the fungus penetrates through stomata or, more commonly, directly through floral bracts (Pritsch et al., 2000). From the infected floret, the fungus can spread up and down the spike and cause severe damage at conducive temperatures and humidity. Trichothecene mycotoxins are known to inhibit protein synthesis in eukaryotes and contribute to fungal virulence (Desjardins et al., 1996; Proctor et al., 2002). Mutants carrying loss-of-function mutations that prevent trichothecene biosynthesis are normal in growth and development but reduced in virulence on seedlings of Wheaten wheat, common winter rye, and maize (Desjardins et al., 1996, 2000; Harris et al., 1999). Further studies indicated that deoxynivalenol (DON) production is not necessary for initial infection facilitates spread of the fungus within colonized spikes (Bai et al., 2002).

Although *F. graminearum* is amenable to classical and molecular genetic manipulations (Bowden and Leslie, 1999; Proctor et al., 1995), there are only a limited number of virulence factors in addition to trichothecenes have been identified (Cumagun et al., 2004). Two mitogen-activated protein (MAP) kinase genes, *MGV1*, and *GPMK1*, are essential for pathogenicity in *F. graminearum* (Hou et al., 2002; Jenczmionka et al., 2003; Urban et al., 2003). The *gpmk1* mutants are defective in colonization of flowering wheat heads and in spreading from inoculated florets to neighboring spikelets (Jenczmionka et al., 2003; Urban et al., 2003). Further analysis indicated that Gpmk1 regulates the early induction of extracellular endoglucanase, xylanolytic, and proteolytic activities and is responsible for the overall induction of secreted lipolytic activities (Jenczmionka and Schafer, 2005). The *mgv1* deletion mutants are substantially reduced in virulence and DON production, and rarely spread beyond the inoculated floret (Hou et al., 2002). Disruption of the homolog of *Cochliobolus heterostrophus CPS1*, which represents a novel virulence factor in fungal pathogens, also causes reduced virulence in *F. graminearum* (Lu et al., 2003).

To better understand the molecular mechanisms of *F. graminearum* pathogenesis, we have generated a collection of REMI (Restriction-Enzyme Mediated Integration) transformants and isolated 11 mutants reduced in virulence (Seong et al., 2005). In this study, we identified and characterized the HMG-CoA-reductase gene (*HMR1*) disrupted in one of these pathogenicity mutants, mutant 222. HMG-CoA reductase converts HMG-CoA to mevalonate and is a key enzyme of the mevalonate pathway involved in the synthesis of many primary and secondary metabolites, such as sterols, carotenoids, trichothecenes, and gibberellins (Woitek et al., 1997). We found that *HMR1* is an essential gene in *F. graminearum*. The insertion of the transforming vector in mutant 222 disrupted the N-terminal transmembrane domains of *HMR1*, but the C-terminal catalytic domain was intact. The DNA sequence corresponding to amino acid residues of 269–641 of *HMR1* possessed cryptic promoter activity and was sufficient for transcription of the catalytic domain in *hmr1* mutants.

2. Materials and methods

2.1. Culture conditions and fungal transformation

Fusarium graminearum wild-type strain PH-1 (NRRL 31084) and the transformants generated in this study were cultured at 25 °C on V8 juice agar or minimal medium (MM) plates (Hou et al., 2002; Trail et al., 2003). Protoplast preparation and fungal transformation were performed as described previously (Proctor et al., 1995). Complete medium (CM) supplemented with 250 μg/ml hygromycin B (Calbiochem, La Jolla, CA) or 200 μg/ml neomycin (Amresco, Solon, OH) was used for selecting transformants. For complementation assays, a cosmid clone (4D13) containing the full-length *HMR1* gene was isolated from the pMOcosX library of PH-1 and introduced into mutant 222 and ND3 by co-transformation with pSM334 (kindly provided by Dr. Seogchan Kang), a vector containing the neomycin phosphotransferase (Hou et al., 2002). Cracked corn cultures of *F. graminearum* (Hou et al., 2002) were used to measure DON and ergosterol production as previously described (Goswami and Kister, 2005). Genetic crosses and isolation of random ascospore progeny were performed as described (Bowden and Leslie, 1999; Cumagun et al., 2004). Approximately, six-week-old plants of wheat cultivar norm were used in infection assays with conidia collected from 7- to 10-day-old mung bean cultures (Gale et al., 2002).

2.2. Molecular manipulation

Standard molecular biology procedures were followed for Southern and Northern blot analyses and enzymatic manipulations of DNA (Sambrook et al., 1989). Proteins were extracted from mycelia collected from 2-day-old CM cultures as described (Bruno et al., 2004). Total proteins (≈20 μg) were separated on a 12% SDS–PAGE gel and transferred to nitrocellulose membranes. Antigen-antibody detection was performed with the ECL Supersignal System (Pierce, Rockford, IL) following the instructions provided by the manufacturer. A monoclonal anti-GFP antibody (Sigma, St. Louis, MO) and a monoclonal anti-actin antibody (Sigma) were used to detect GFP fusion and actin proteins, respectively. The BD SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA) was used to determine the transcription initiation site in mutant 222 and ND3 with primer RE1(TGGATCCTTGATACCCCA GCGGGCGACG) by 5′-RACE.

2.3. The HMR1 gene replacement constructs

The *HMR1* gene replacement vector pKY42 was constructed by the ligation-PCR approach (Zhao et al., 2004). Briefly, a 0.8-kb upstream fragment and a 0.8-kb downstream fragment were amplified with primers H1(ATCGAA TTCAGCGCTCGGTTTTATTGTCC)/H2(AAGGTT GG CCGGCCCGGTGTCGTTTACCACATTG) and primers H3(AAGGTTGGCGCGCCGGAGCTCTCCAAATCTC

TGT)/H4(ATCGGATCCGAGGGATATTCGACATGG AT) and digested with *Fse*I and *Asc*I, respectively. After ligating these PCR products with the *Asc*I–*Fse*I *hph* fragment, a 3.0-kb *HMR1* gene replacement construct was amplified with primers H1 and H4 and cloned into pGEM-T easy (Promega, Madison, WI) as pHY42. After digestion with *Bam*HI, pKY42 was transformed into protoplasts of PH-1. Hygromycin-resistant transformants were screened by PCR with primers H5 (ACCTTGAAAGTGGGAACGCT) and H6 (TGTTCCCATCAGCGTTATCC) that amplified a 0.5-kb fragment in PH-1. To delete the first 269 amino acid residues of *HMR1*, another gene replacement vector pKY48 (Fig. 2B) was generated with a similar approach. A 0.78-kb upstream flanking sequence of *HMR1* was amplified with primers R1 (TGCTCTAGATGATAAGGCAGACGGCA AAG) and R2 (AAGGTTGGCGCGCCAGGACAATAA AACCGAGCGC) and digested with *Asc*I. A 0.67-kb downstream fragment was amplified with primers R3 (AAGGT-TGGCCGGCCTGTTCCTCGGTTACATTGCC) and R4 (AAACTGCAGAAAGAGAAGAGTCGCCCTTG) and digested with *Fse*I. The 2.8-kb gene replacement construct was amplified with primers R1 and R4 after ligating these PCR products with the *hph* fragment, and subcloned into pGEM-T easy as pKY48 (Fig. 2B).

2.4. The GFP reporter construct pKY60

The construct for expressing GFP with the cryptic promoter of the *HMR1* ORF was generated by the yeast GAP-repair method (Bourett et al., 2002; Raymond et al., 1999, 2002). In brief, a 1.2-kb fragment of *HMR1* (Fig. 5A) was amplified with primers FP1 (GCGAATTGGGTACTCAAA TTGGTGGATTCAAGTTCTGGCTCGGT) and FP 2(GA ACAGCTCCTCGCCCTTGCTGTTTCGGTCGATGTT GTGCTC) and co-transformed with *Xho*I-digested pDL2 (Bourett et al., 2002) into *Saccharomyces cerevisiae* XK1-25 (Bruno et al., 2004). Plasmid pKY60 (Fig. 5A) was rescued from the resulting Trp^+ yeast transformant and confirmed by sequence analysis to carry the $HMR1^{269–641}$-GFP construct and transformed into PH-1 by selecting for hygromycin-resistant transformants.

3. Results

3.1. Mutant 222 was disrupted in the HMG-CoA reductase gene

Mutant 222 was generated by transforming *Bam*HI-digested pCB1003 into the wild-type stain PH-1 (Seong et al., 2005). It had a reduced growth rate on V8 juice agar plates and was significantly reduced in virulence (Table 1). On V8 juice agar plates, mutant 222 produced short and compact aerial hyphae. While the wild-type PH-1 produced fluffy colonies, colonies formed by mutant 222 had granular surface appearance (Fig. 1A). The conventional plasmid rescue approach was used to identify the gene disrupted in mutant 222 by self-ligation of *Kpn*I-digested genomic

Table 1
Mutant 222 was reduced in vegetative growth and virulence

Strain	Growth rate (cm/day)		Disease index[a] (means ± SD)
PH-1	1.9 ± 0.1	1.9 ± 0.1	8.4 ± 1.4
222	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.3
222C	1.7 ± 0.2	2.4 ± 0.1	7.6 ± 0.7
ND3	0.7 ± 0.1	0.7 ± 0.2	1.4 ± 0.5
ND4	0.9 ± 0.0	0.6 ± 0.1	1.0 ± 0.0

[a] Mean and standard deviation of numbers of symptomatic spikelets at 14 days after inoculation in four independent infection assays.

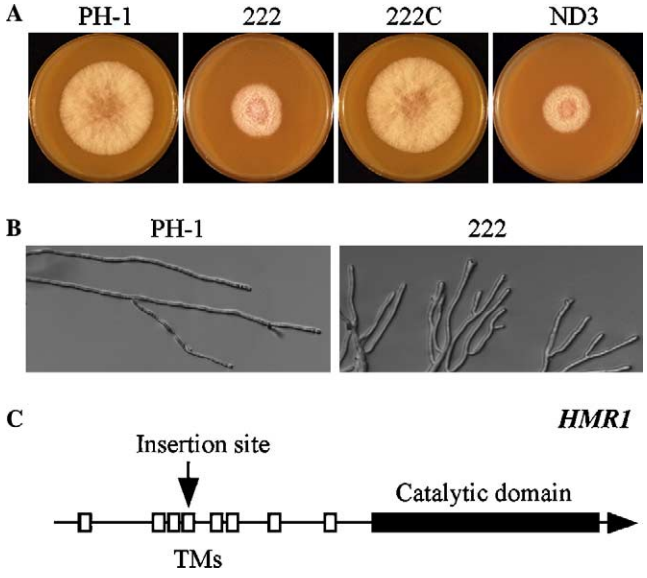

Fig. 1. Mutant 222 had distinct colony morphologies. (A) Colonies of the wild-type PH-1, REMI mutant 222, complemented strain 222C, and a $HMR1^{\Delta 254}$ deletion mutant ND3 grown on V8 juice agar plates. Typical colonies were photographed after incubation at 25 °C for 4 days. (B) Hyphal tips of PH-1 and mutant 222 cultures grown on complete medium. Vegetative hyphae of mutant 222 tended to have short branching distance. (C) The *HMR1* gene contains eight transmembrane domains (TMs, open boxes) and a C-terminal cytosolic catalytic domain (filled box). In mutant 222, the transforming vector was integrated at the 269 amino acid residue (marked with a vertical arrow) in the fourth transmembrane domain.

DNA. In three independent plasmid rescue attempts, only one identical clone pMT25 was isolated. Sequence analysis of the rescued 1.5-kb fragment in pMT25 indicated that pCB1003 was inserted at amino acid residue 269 (*Bam*HI site) of the HMG-CoA-reductase gene *HMR1* (GenBank Accession No. XP389373). The Hmr1 protein has eight transmembrane (TM) domains and a C-terminal catalytic domain (Fig. 1B). The transforming vector was inserted in the fourth TM domain of *HMR1* in mutant 222 (Fig. 1B).

To determine whether the phenotypes of mutant 222 were directly caused by the disruption of the *HMR1* gene, we isolated a cosmid (4D13) containing the wild-type *HMR1* gene and introduced it into mutant 222 by co-transformation with pSM334. One of the resulting geneticin-resistant transformants, 222C, displayed a normal vegetative growth rate and virulence on flowering wheat heads (Table 1). Southern blot analysis confirmed that 222C

harbored the wild-type *HMR1* gene carried on cosmid 4D13 (data not shown). In addition, we conducted co-segregation analysis by isolating 30 progeny from a cross between mutant 222 and a *nit1* mutant 11622 of PH-1. Similar to mutant 222, all 15 hygromycin-resistant progeny analyzed were significantly reduced in growth and virulence. In contrast, all 15 hygromycin-sensitive progeny were phenotypically similar to PH-1. These data indicate that the disruption of the *HMR1* gene was responsible for the reduction of virulence and growth rate in mutant 222.

3.2. HMR1 is essential for fungal growth

We constructed an *HMR1* gene replacement construct pKY42 (Fig. 2A) and attempted to delete the *HMR1* gene.

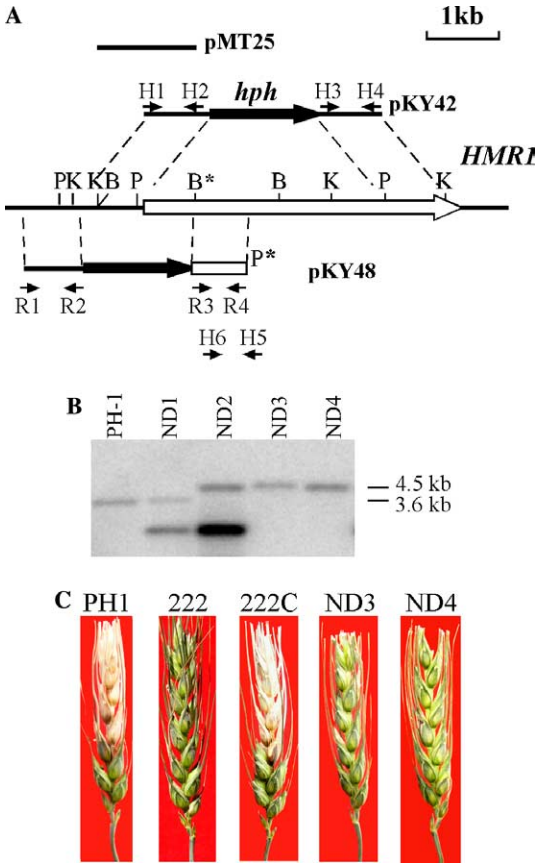

Fig. 2. Deletion of the N-terminal region of the *HMR1* gene. (A) Gene replacement constructs pKY42 and pKY48 were generated by ligation-PCR. The thick arrows indicated the directions of the *HMR1* and *hph* genes. The majority of *HMR1* ORF was replaced by *hph* in pYK42, but only the promoter region and the first 254 amino acids of *HMR1* were deleted in pKY48. PCR primers H1, H2, H3, H4, H5, H6, R1, R2, R3, and R4 were used in constructing pKY42 and pKY48 and mutant screenings. B: *Bam*HI; K: *Kpn*I; P: *Pst*I; B*: the insertion site in mutant 222; P*: *Pst*I site derived from the cloning vector. (B) Southern blot of genomic DNAs from the wild-type strain (PH-1), ectopic transformants (ND1 and ND2), and $hmr1^{\Delta 254}$ deletion mutants (ND3 and ND4) were hybridized with a probe amplified with primers R3 and R4. All DNA samples were digested with *Pst*I. (C) Scab disease developed on flowering wheat heads inoculated with conidia PH-1, mutant 222, complemented strain 222C, ND3, and ND4. Photos were taken 2 weeks after inoculation.

Over 357 transformants isolated in three independent transformations were screened with PCR primers H5 and H6 (Fig. 2A), but none of them was an *hmr1* deletion mutant. We then generated an additional 210 hygromycin-resistant transformants with 5 mg/ml mevalonic acid added to the regeneration medium but again failed to identify any putative *hmr1* deletion mutants in these transformants. These data suggest that *HMR1* is an essential gene in *F. graminearum*. It is likely that the insertion of pCB1003 in the N-terminal region of *HMR1* ORF in mutant 222 did not generate a null allele.

To determine whether the N-terminal portion disrupted in mutant 222 was essential for *HMR1* function, we generated a gene replacement construct pKY48 (Fig. 2A) to delete the first 254 amino acids of the *HMR1* gene. After transforming PH-1, twelve hygromycin-resistant transformants were isolated. Two of them, ND3 and ND4, were confirmed to have the first 254 amino acids of *HMR1* replaced by the *hph* gene (Fig. 2B). On Southern blots probed with a fragment amplified with R3 and R4, a 3.6-kb *Pst*I band was observed in the wild-type strain PH-1 and a randomly selected ectopic transformant RD1. In the $hmr1^{\Delta 254}$ deletion mutants RD4 and RD5, a 4.5-kb *Pst*I band was detected with the same probe (Fig. 2B), indicating that a gene replacement event had occurred in these two transformants. Transformant RD2 had the 4.5-kb *Pst*I band, but it also contained the 2.5-kb band derived from the ectopic integration of pKY48 (Fig. 2B). The $hmr1^{\Delta 254}$ mutants ND3 and ND4 were reduced in the growth rate and produced small compact colonies on V8 juice agar plates (Fig. 1A). Similar to mutant 222, ND3 and ND4 also were significantly reduced in virulence on flowering wheat heads and failed to cause necrosis beyond the point of inoculation (Fig. 2C). When the wild-type *HMR1* allele carried on cosmid 4D13 was introduced into ND3, all the defects associated with the $hmr1^{\Delta 254}$ mutation were complemented (data not shown).

3.3. The catalytic domain of HMR1 was transcribed in mutant 222 and ND3

Since the carboxyl catalytic domain of *HMR1* was intact in mutant 222 and ND3, it might be still expressed and functional. To determine whether the catalytic domain of *HMR1* was transcribed in mutant 222, we isolated RNA samples from mycelia grown in CM for three days. When probed with a fragment amplified with primers H3 and H4 (Fig. 2A), a 3-kb band was detected in mutant 222. The 5-kb band observed in the wild-type strain PH-1 was absent in mutant 222. In the complemented strain 222C, both 3-kb and 5-kb transcripts were detected (Fig. 3A). These data indicated that the C-terminal portion of the *HMR1* gene was still transcribed in mutant 222. In mutants ND3 and ND4, the truncated 3-kb *HMR1* transcript also was detected (data not shown).

To determine the transcription initiation site in mutant 222 and ND3, we performed 5′-RACE with primer RE1

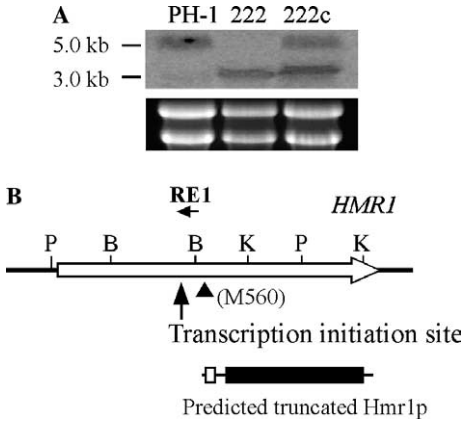

Fig. 3. Truncated *HMR1* transcripts. (A) Northern blot analysis of RNAs isolated from the wild-type strain PH-1, mutant 222, its complemented strain 222C, and *hmr1*$^{\Delta 254}$ mutant ND3. When hybridized with a PCR fragment amplified with primer H3 and H4, mutant 222 and ND3 had a 3.0-kb band but not the 5.0-kb band present in PH-1. In 222C, both 5.0 and 3.0-kb bands were detected. The bottom panel was the RNA gel stained with ethidium bromide. (B) Primer RE1 was used in the 5′-RACE reactions to identify the initiation site of the truncated *HMR1* transcript (marked with a vertical arrow) in mutants 222 and ND3. The triangle indicated the position of the ATG codon M560 of *HMR1*. The predicted truncated Hmr1 protein in mutants 222 and ND3 has the entire catalytic domain (filled box) and one transmembrane domain (empty box). Restriction enzymes are: B: *Bam*HI; K: *Kpn*I; P: *Pst*I.

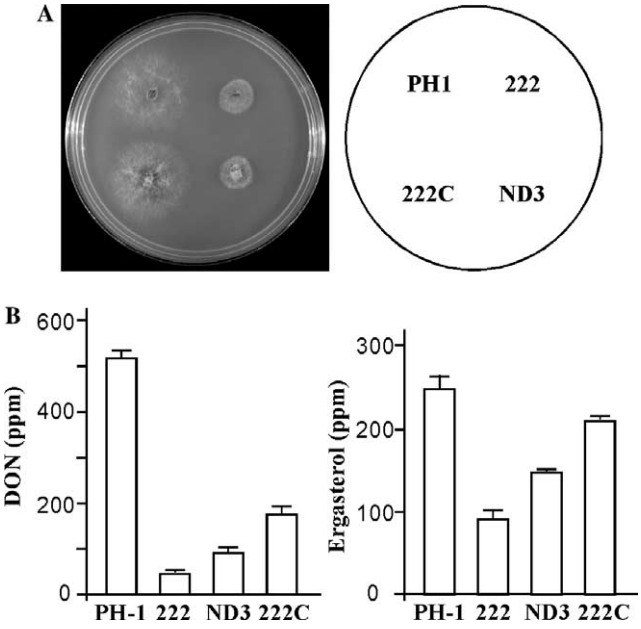

Fig. 4. Growth and mycotoxin production in mutants 222 and ND3. (A) Colonies formed by the wild-type strains PH-1, mutant 222, its complemented strain 222C, and the *hmr1*$^{\Delta 254}$ mutant ND3 on the minimal medium (MM). Photos were taken after incubation at 25 °C for 2 days. (B) Production of deoxynivalenol (DON, left panel) and ergosterol (right panel) by PH-1, 222, ND3, and 222C on cracked corn seeds. Mean and standard errors were calculated from three independent experiments.

(Fig. 3B) and isolated a 210-bp fragment. Sequence analysis of this fragment indicated that transcription of the truncated *HMR1* transcript was initiated 1763 bp downstream from the start codon of the native *HMR1* ORF and 454 bp upstream from the catalytic domain (Fig. 3B). We identified five in-frame ATG codons between this transcription initiation site and the catalytic domain of *HMR1*. Only the ATG codon (Hmr1^{M560}, Fig. 3B) located 141 amino acids upstream from the catalytic domain was conserved in *HMR1* homologs from *Magnaporthe grisea* and *Neurospora crassa*. A putative Kozak sequence (Bruchez et al., 1993) also was identified in front of Hmr1^{M560}. Therefore, it is likely that the entire catalytic domain was transcribed and translated in mutant 222 and ND3. The eighth transmembrane domain of *HMR1* (amino acid residues 575–592) also might be expressed in mutants 222 and ND3 (Fig. 3B).

3.4. The catalytic domain of HMR1 was functional in mutant 222 and ND3

To determine whether mutants 222 and ND3 could grow without a sterol source, we assayed their growth on minimal medium (MM). Similar to their growth on V8 juice agar, both 222 and ND3 formed compact colonies on MM (Fig. 4A) with a reduced growth rate (Table 1). These mutants also produced morphologically normal conidia on MM (data not shown). Therefore, vegetative growth and asexual reproduction in mutant 222 and ND3 did not require supplemental sterol source. Mutant 222 and ND3 were female sterile but fertile when out-crossing with the *nit1* mutant 11622 as the male (data not shown). Since trichothecene mycotoxins are derived from the mevalonate pathway (Kimura et al., 2003; Sweeney and Dobson, 1999), we assayed the production of DON in mutants 222 and ND3. In cracked corn cultures (Fig. 4B) and infected wheat spikelets (data not shown), mutants 222 and ND3 still produced DON, although at reduced levels in comparison with those of PH-1. These data suggest that the catalytic domain of *HMR1* may be partially functional in mutant 222 and produce a residual HMG-CoA reductase activity. Since the production of ergosterol also was reduced in cracked corn cultures inoculated with mutant 222 and ND3, it is likely that reduction of DON production is related with reduced fungal growth in these *hmr1* mutants.

3.5. The N-terminal portion of the HMR1 gene has promoter activity

Since mutants 222 and ND3 had similar phenotypes and shared the same initiation site for transcription of the catalytic domain of *HMR1*, it is likely that the N-terminal portion of the *HMR1* gene had promoter activity in *F. graminearum*. To test this hypothesis, a 1.2-kb fragment containing the nucleotide sequence between amino acid residue 269 (the insertion site of pCB1003 in 222) and 641 of *HMR1* was amplified with primers FP1 and FP2 (Fig. 5A). This fragment was cloned into vector pDL2 in front of the GFP ORF followed by the TrpC terminator by the yeast in vivo recombination approach (Bourett et al., 2002; Raymond et al., 2002). The resulting *HMR1*$^{269–641}$-GFP

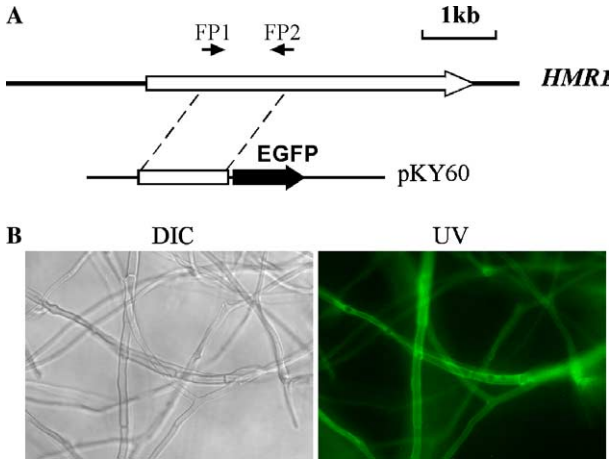

Fig. 5. Expression of GFP with a portion of the *HMR1* coding region. (A) The *HMR1*$^{269–641}$-GFP reporter construct pKY60 was generated by cloning a DNA fragment amplified with primers FP1 and FP2 (corresponding to the region between amino acid residue 269 and 641) in front of the promoter-less GFP. (B) Vegetative hyphae of transformant HG1 were examined under DIC (left panel) and epifluorescence microscopy (right panel). GFP signal was observed in the cytoplasm without any special distribution patterns.

construct pKY60 (Fig. 5A) was transformed into PH-1. All 20 hygromycin-resistant transformants examined had GFP signals (data not shown). Transformants HG1 and HG2 contained a single copy of pKY60 based on Southern analyses (data not shown). GFP signals were observed in the cytoplasm of vegetative hyphae of transformants HG1 and HG2 (Fig. 5B). No special cellular localization patterns of GFP signals were observed. No GFP signal was detected in conidia and young germ tubes (data not shown), indicating that *HMR1* is not expressed or expressed at a relatively low level.

4. Discussion

HMG-CoA is converted to mevalonate and then isopentenyl diphosphate, which serves as a precursor for isoprenoid and sterol biosynthesis (Parrish et al., 1995; Woitek et al., 1997). As the key enzyme of the mevalonate pathway, HMG-CoA reductase is the rate limiting enzyme of cholesterol biosynthesis in humans (Goldstein and Brown, 1990). In REMI mutant 222, the transforming vector was inserted in the HMG-CoA-reductase gene *HMR1*. Since we failed to isolate an *hmr1* deletion mutant, it is likely that *HMR1* is an essential gene in *F. graminearum*. HMG-CoA reductase is encoded by a single copy essential gene in the fission yeast *Schizosaccharomyces pombe* (Lum et al., 1996), many mammals, and bacterial pathogens (Lum et al., 1996; Wilding et al., 2000a,b). The budding yeast *S. cerevisiae* has two HMG-CoA-reductase genes, *HMG1* and *HMG2* (Basson and Rine, 1986). Although deletion of any one of them is not lethal, the *hmg1 and hmg2* double mutant is not viable. There is only a single copy of the HMG-CoA-reductase gene in the *F. graminearum*, *N. crassa*, *Aspergillus nidulans*, and *M. grisea* genomes. In some eubacteria and plants, there is an alternative pathway for isopentenyl diphosphate biosynthesis independent of mevalonate (Dubey et al., 2003; Eisenreich et al., 2004). However, none of the key enzymes involved in the non-mevalonate terpenoid biosynthesis were found in the *F. graminearum* genome.

In eukaryotic cells, HMG-CoA reductase has a complex transmembrane region and a cytosolic catalytic domain at the C-terminus (Liscum et al., 1985; Roitelman et al., 1991). The hydrophilic catalytic domain responsible for conversion of HMG-CoA to mevalonate is highly conserved (Frimpong and Rodwell, 1994). In contrast, the hydrophobic N-terminal region has various numbers of transmembrane segments, and the sequences are relatively divergent in different eukaryotes (Hampton et al., 1996). The transmembrane domains direct the localization of the HMG-CoA reductase to the endoplasmic reticulum (Gil et al., 1985; Parrish et al., 1995) and are also involved in its degradation (Hampton et al., 1996). In REMI mutant 222, the catalytic domain was intact and a 3-kb transcript was detectable. Without the transmembrane domains, the carboxyl catalytic domain of the *HMR1* gene may be defective in sub-cellular localization but partially functional in the cytoplasm. In bacteria, the HMG-CoA reductase has no transmembrane domains. It is likely that one of the *HMR1* transmembrane domains (Fig. 5B) was expressed together with the catalytic domain in mutants 222 and ND3. Interestingly, when a C-terminal-truncated fragment of the *Ustilago maydis* HMG-CoA-reductase gene was expressed in *Escherichia coli*, it was in a catalytically active form (Croxen et al., 1994). Similarly, the 1658-bp region of the yeast *HMG1* gene encoding the catalytic domain had the HMG-CoA-reductase activities when expressed under control of strong constitutive or inducible promoters and increased carotenoid biosynthesis in *N. crassa* (Wang and Keasling, 2002). Therefore, it is not surprising that the truncated Hmr1 without the transmembrane domains still has HMR-CoA reductase activities in *F. graminearum* mutants. We attempted to detect HMG-CoA reductase with an anti-human HMG-CoA reductase antibody (Upstate Cell Signaling Solutions, Lake Placid, NY) in the wild-type PH-1 and *hmr1* mutants. Unfortunately, this antibody did not react to the Hmr1 protein in *F. graminearum*.

The mevalonate pathway is involved in the biosynthesis of many primary and secondary metabolites (Holstein and Hohl, 2004; Woitek et al., 1997). While sterols are essential components of cytoplasm membranes, some secondary metabolites derived from the mevalonate pathway may be involved in fungal–plant interactions, such as isoprenoids and gibberellins (Tudzynski, 2005). In *F. graminearum*, trichothecene DON has been shown to be a virulence factor on spreading within colonized spikes (Bai et al., 2002; Desjardins et al., 1996). It is also possible that *HMR1*, as the key enzyme of the mevalonate pathway, is involved in the biosynthesis of other un-identified phytotoxic compounds in *F. graminearum*. Therefore, *HMR1* may be important for plant infection. In filamentous fungi, no HMG-CoA reductase has been functionally characterized. However, *HMR1*

appears to be an essential housekeeping gene in *F. graminearum*. Mutants 222 and ND3 were significantly reduced in vegetative growth. The defect of these *hmr1* mutants in plant infection could be directly related with their reduced growth rate. Therefore, further studies are necessary to clarify the role of *HMR1* and the mevalonate pathway in fungal pathogenesis.

Our data suggested that the N-terminal portion of the *HMR1* coding region (807–1714 bp) has cryptic promoter activity and is responsible for transcription of the catalytic domain of *HMR1* in mutant 222 and ND3. Since the truncated *HMR1* transcript was not observed in the wild-type strain, this promoter activity must be inactive and probably suppressed by transcription of *HMR1* from its native promoter. However, this suppression is likely to be *cis*-active because when pYK60 was integrated ectopically in PH-1 transformants, the wild-type *HMR1* had no effect on the expression of GFP by this cryptic promoter carried on pKY60 (ectopically integrated). Cryptic promoter activities in 5′-UTR, introns or intergenic sequences have been reported in plants and animals (Fobert et al., 1994; Han and Zhang, 2002; Terrinoni et al., 2001; Wu et al., 2003). In *S. cerevisiae*, the single intron of the *ACT1* actin gene contains a cryptic promoter (Irniger et al., 1992), which is inactive due to the transcriptional interference posed by the native promoter of the wild-type *ACT1* gene. When the native promoter is deleted, the second exon of *ACT1* is transcribed by this cryptic promoter but its biological function is not clear (Irniger et al., 1992). To our knowledge, cryptic promoters inside the coding sequences have not been reported in filamentous fungi. Interestingly, the DNA sequence corresponding to $HMR1^{259–641}$ is well conserved among the *HMR1* homologs from *N. crassa* and *M. grisea* (79 and 80% identity, respectively).

Acknowledgments

We thank Dr. Larry Dunkle, Dr. Charles Woloshuk, and Dr. Joseph Flaherty for critical reading of this manuscript, Dr. Yan-Hong Dong for DON and ergosterol measurements, Dr. Gregory Shaner and Dr. George Buechley for assistance with wheat infection assays. This work was supported by a grant from the US Wheat and Barley Scab initiative to H.C.K. and J.X. and a grant (to J.X.) from the USDA National Research Initiative (#2003-01130). This is journal article No. 17825 of the Purdue University Agricultural Experiment Station.

References

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

Bai, G.H., Shaner, G., 2004. Management and resistance in wheat and barley to Fusarium head blight. Annu. Rev. Phytopathol. 42, 135–161.

Basson, T.M., Rine, J., 1986. *Saccharomyces cerevisiae* contains two functional genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Proc. Natl. Acad. Sci. USA, 5563–5567.

Bourett, T.M., Sweigard, J.A., Czymmek, K.J., Carroll, A., Howard, R.J., 2002. Reef coral fluorescent proteins for visualizing fungal pathogens. Fungal Genet. Biol. 37, 211–220.

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

Bruchez, J.J.P., Eberle, J., Russo, V.E.A., 1993. Regulatory sequences involved in the translation of *Neurospora crassa* mRNA: Kozak sequences and stop codons. Fungal Genet. Newsl. 40, 85–88.

Bruno, K.S., Tenjo, F., Li, L., Hamer, J.E., Xu, J.R., 2004. Cellular localization and role of kinase activity of *PMK1* in *Magnaporthe grisea*. Eukaryot Cell 3, 1525–1532.

Croxen, R., Goosey, M.W., Keon, J.P.R., Hargreaves, J.A., 1994. Isolation of an *Ustilago maydis* gene encoding 3-hydroxy-5-methylglutaryl-coenzyme-a reductase and expression of a c-terminal-truncated form in *Escherichia coli*. Microbiology 140, 2363–2370.

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

De Wolf, E.D., Madden, L.V., Lipps, P.E., 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. Phytopathology 93, 428–435.

Desjardins, A.E., Bai, G.H., Plattner, R.D., Proctor, R.H., 2000. Analysis of aberrant virulence of *Gibberella zeae* following transformation-mediated complementation of a trichothecene deficient (Tri5) mutant. Microbiology 146, 2059–2068.

Desjardins, A.E., Proctor, R.H., Bai, G.H., McCormick, S.P., Shaner, G., Buechley, G., Hohn, T.M., 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Mol. Plant Microbe Interact. 9, 775–781.

Dubey, V.S., Bhalla, R., Luthra, R., 2003. An overview of the non-mevalonate pathway for terpenoid biosynthesis in plants. J. Biosci. 28, 637–646.

Eisenreich, W., Bacher, A., Arigoni, D., Rohdich, F., 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell Mol. Life Sci. 61, 1401–1426.

Fobert, P.R., Labbe, H., Cosmopoulos, J., Gottlobmchugh, S., Ouellet, T., Hattori, J., Sunohara, G., Iyer, V.N., Miki, B.L., 1994. T-DNA tagging of a seed coat-specific cryptic promoter in tobacco. Plant J. 6, 567–577.

Frimpong, K., Rodwell, V.W., 1994. The active-site of hamster 3-hydroxy-3-methylglutaryl-CoA reductase resides at the subunit Interface and incorporates catalytically essential acidic residues from separate polypeptides. J. Biol. Chem. 269, 1217–1221.

Gale, L.R., Chen, L.F., Hernick, C.A., Takamura, K., Kistler, H.C., 2002. Population analysis of *Fusarium graminearum* from wheat fields in eastern China. Phytopathology 92, 1315–1322.

Gil, G., Faust, J.R., Chin, D.J., Goldstein, J.L., Brown, M.S., 1985. Membrane-bound domain of HMG-CoA reductase is required for sterol-enhanced degradation of the enzyme. Cell 41, 249–258.

Goldstein, J.L., Brown, M.S., 1990. Regulation of the mevalonate pathway. Nature 343, 425–430.

Goswami, R.S., Kistler, H.C., 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol. Plant Pathol. 5, 515–525.

Goswami, R.S., Kister, H.C., 2005. Pathogenicity and in planta mycotoxin accumulation among members of the *Fusarium graminearum* species complex on wheat and rice. Phytopathology 95, 1397–1404.

Hampton, R., DimsterDenk, D., Rine, J., 1996. The biology of HMG-CoA reductase: the pros of contra-regulation. TIBS 21, 140–145.

Han, B.G., Zhang, J.T., 2002. Regulation of gene expression by internal ribosome entry sites or cryptic promoters: the eIF4G story. Mol. Cell. Biol. 22, 7372–7384.

Harris, L.J., Desjardins, A.E., Plattner, R.D., Nicholson, P., Butler, G., Young, J.C., Weston, G., Proctor, R.H., Hohn, T.M., 1999. Possible role of trichothecene mycotoxins in virulence of *Fusarium graminearum* on maize. Plant Dis. 83, 954–960.

Holstein, S.A., Hohl, R.J., 2004. Isoprenoids: remarkable diversity of form and function. Lipids 39, 293–309.

Hou, Z.M., Xue, C.Y., Peng, Y.L., Katan, T., Kistler, H.C., Xu, J.R., 2002. A mitogen-activated protein kinase gene (*MGV1*) in *Fusarium graminearum* is required for female fertility, heterokaryon formation, and plant infection. Mol. Plant Microbe Interact. 15, 1119–1127.

Irniger, S., Egli, C.M., Kuenzler, M., Braus, G.H., 1992. The yeast actin intron contains a cryptic promoter that can be switched on by preventing transcriptional interference. Nucleic Acids Res. 20, 4733–4739.

Jenczmionka, N.J., Maier, F.J., Losch, A.P., Schafer, W., 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 *gpmk1*. Curr. Genet. 43, 87–95.

Jenczmionka, N.J., Schafer, W., 2005. The Gpmk1 MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. Curr. Genet. 47, 29–36.

Kimura, M., Tokai, T., O'Donnell, K., Ward, T.J., Fujimura, M., Hamamoto, H., Shibata, T., Yamaguchi, I., 2003. The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. FEBS Lett. 539, 105–110.

Lee, J., Lee, T., Lee, Y.W., Yun, S.H., Turgeon, B.G., 2003. Shifting fungal reproductive mode by manipulation of mating type genes: obligatory heterothallism of *Gibberella zeae*. Mol. Microbiol. 50, 145–152.

Liscum, L., Finermoore, J., Stroud, R.M., Luskey, K.L., Brown, M.S., Goldstein, J.L., 1985. Domain structure of 3-hydroxy-3-methylglutaryl coenzyme-A reductase, a glycoprotein of the endoplasmic reticulum. J. Biol. Chem. 260, 522–530.

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

Lum, P.Y., Edwards, S., Wright, R., 1996. Molecular, functional and evolutionary characterization of the gene encoding HMG-CoA reductase in the fission yeast, *Schizosaccharomyces pombe*. Yeast 12, 1107–1124.

McMullen, M., Jones, R., Gallenberg, D., 1997. Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis. 81, 1340–1348.

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., Aoki, T., 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.

Parrish, M.L., Sengstag, C., Rine, J.D., Wright, R.L., 1995. Identification of the sequences in HMG-CoA reductase required for karmellae assembly. Mol. Biol. Cell 6, 1535–1547.

Parry, D.W., Jenkinson, P., McLeod, L., 1995. Fusarium ear blight (Scab) in small grain cereals - a review. Plant Pathol. 44, 207–238.

Pritsch, C., Muehlbauer, G.J., Bushnell, W.R., Somers, D.A., Vance, C.P., 2000. Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. Mol. Plant Microbe Interact. 13, 159–169.

Proctor, R.H., Desjardins, A.E., McCormick, S.P., Plattner, R.D., Alexander, N.J., Brown, D.W., 2002. Genetic analysis of the role of trichothecene and fumonisin mycotoxins in the virulence of Fusarium. Eur. J. Plant Pathol. 108, 691–698.

Proctor, R.H., Hohn, T.M., McCormick, S.P., 1995. Reduced Virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. Mol. Plant Microbe Interact. 8, 593–601.

Raymond, C.K., Pownder, T.A., Sexson, S.L., 1999. General method for plasmid construction using homologous recombination. Biotechniques 26, 134–139.

Raymond, C.K., Sims, E.H., Olson, M.V., 2002. Linker-mediated recombinational subcloning of large DNA fragments using yeast. Genome Res. 12, 190–197.

Roitelman, J., Barnun, S., Inoue, S., Simoni, R.D., 1991. Involvement of calcium in the mevalonate accelerated degradation of 3-hydroxy-3-methylglutaryl CoA reductase. J. Biol. Chem. 266, 16085–16091.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Seong, K., Hou, Z., Tracy, M., Kistler, H.C., Xu, J.R., 2005. Random insertional mutagenesis identifies genes associated with virulence in the wheat scab fungus *Fusarium graminearum*. Phytopathology 95, 744–750.

Sweeney, M.J., Dobson, A.D.W., 1999. Molecular biology of mycotoxin biosynthesis. FEMS Microbiol. Lett. 175, 149–163.

Terrinoni, A., Dell'Arciprete, R., Fornaro, M., Stella, M., Alberti, S., 2001. Cyclin D1 gene contains a cryptic promoter that is functional in human cancer cells. Genes Chromosomes Cancer 31, 209–220.

Trail, F., Xu, J.R., San Miguel, P., Halgren, R.G., Kistler, H.C., 2003. Analysis of expressed sequence tags from *Gibberella zeae* (anamorph *Fusarium graminearum*). Fungal Genet. Biol. 38, 187–197.

Tudzynski, B., 2005. Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. Appl. Microbiol. Biotechnol. 66, 597–611.

Urban, M., Mott, E., Farley, T., Hammond-Kosack, K., 2003. The *Fusarium graminearum MAP1* gene is essential for pathogenicity and development of perithecia. Mol. Plant Pathol. 4, 347–359.

Wang, G.Y., Keasling, J.D., 2002. Amplification of HMG-CoA reductase production enhances carotenoid accumulation in *Neurospora crassa*. Metab. Eng. 4, 193–201.

White, E.M., McGarel, A.S.L., Ruddle, O., 2003. The influence of variety, year, disease control and plant growth regulator application on crop damage, yield and quality of winter oats (*Avena sativa*). J. Agric. Sci. 140, 31–42.

Wilding, E.I., Brown, J.R., Bryant, A.P., Chalker, A.F., Holmes, D.J., Ingraham, K.A., Iordanescu, S., Chi, Y.S., Rosenberg, M., Gwynn, M.N., 2000a. Identification, evolution, and essentiality of the mevalonate pathway for isopentenyl diphosphate biosynthesis in Gram-positive cocci. J. Bacteriol. 182, 4319–4327.

Wilding, E.I., Kim, D.Y., Bryant, A.P., Gwynn, M.N., Lunsford, R.D., McDevitt, D., Myers, J.E., Rosenberg, M., Sylvester, D., Stauffacher, C.V., Rodwell, V.W., 2000b. Essentiality, expression, and characterization of the class II 3-hydroxy-3-methylglutaryl coenzyme A reductase of *Staphylococcus aureus*. J. Bacteriol. 182, 5147–5152.

Woitek, S., Unkles, S.E., Kinghorn, J.R., Tudzynski, B., 1997. 3-Hydroxy-3-methylglutaryl-CoA reductase gene of *Gibberella fujikuroi*: isolation and characterization. Curr. Genet. 31, 38–47.

Wu, K.Q., Hu, M., Martin, T., Wang, C.M., Li, X.Q., Tian, L.N., Brown, D., Miki, B., 2003. The cryptic enhancer elements of the tCUP promoter. Plant Mol. Biol. 51, 351–362.

Zhao, X., Xue, C., Kim, Y., Xu, J.R., 2004. A ligation-PCR approach for generating gene replacement constructs in *Magnaporthe grisea*. Fungal Genet. Newsl. 51, 17–18.