EBR1, a Novel Zn₂Cys₆ Transcription Factor, Affects Virulence and Apical Dominance of the Hyphal Tip in *Fusarium graminearum*

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Zn₂Cys₆ transcription factors are unique to fungi and have been reported to be involved in different regulatory functions. Here, we characterized EBR1 (enhanced branching 1), a novel Zn₂Cys₆ transcription factor of *Fusarium graminearum*. Knocking out *EBR1* in *F. graminearum* PH-1 caused reduction of both radial growth and virulence. The conidia of knock-out strain PH-1Δ*ebr1* germinated faster than those of wild-type PH-1, but the conidiation of the mutant was significantly reduced. Detailed analysis showed that the reduced radial growth might be due to reduced apical dominance of the hyphal tip, leading to increased hyphal branching. Inoculation assays on wheat heads with a green fluorescent protein (GFP)-labeled PH-1Δ*ebr1* mutant showed that it was unable to penetrate the rachis of the spikelets. Protein fusion with GFP showed that EBR1 is localized in the nucleus of both conidia and hyphae. Knocking out the orthologous gene FOXG_05408 in *F. oxysporum* f. sp. *lycopersici* caused a much weaker phenotype than the PH-1Δ*ebr1* mutant, which may be due to the presence of multiple orthologous genes in this fungus. Transformation of FOXG_05408 into PH-1Δ*ebr1* restored the mutant phenotype. Similar to EBR1, FOXG_05408 is localized in the nucleus of *F. oxysporum* f. sp. *lycopersici*. Possible functions of EBR1 and its relation with other fungal transcription factors are discussed.

Gibberella zeae (anamorph *Fusarium graminearum*) is a worldwide-occurring pathogen causing a wide range of cereal diseases, including Fusarium head blight (FHB) on wheat and barley and ear and stalk rot on corn (Bluhm et al. 2007; Lee et al. 2001). High humidity and temperatures between 20 and 25°C at flowering time can result in serious FHB epidemics, causing yield losses of up to 90% in wheat. *Fusarium* infection can result in the accumulation of mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV) that are very harmful to humans and animals (Desjardins et al. 1993; McMullen et al. 1997; Proctor et al. 1995).

Several *F. graminearum* genes involved in virulence, growth, conidiation, and sexual development have been stud-

ied extensively. Using a gene knock-out strategy, genes in the trichothecene cluster involved in the production of DON have been reported to be required for wheat ear infection. Strains that no longer produce these mycotoxins can infect the initial inoculated spikelet but are blocked from spreading to neighboring spikelets (Bai et al. 2002; Desjardins et al. 1996, 2000). Also mitogen-activated protein (MAP) kinases such as *Gpmk1* have been shown to be involved in production of conidia, sexual development, and virulence (Jenczmionka et al. 2003). A mutation in *Mgv1*, another MAP kinase, led to reduced virulence, reduced vegetative growth, and increased sensitivity to the defensin MsDef1 (Hou et al. 2002; Ramamoorthy et al. 2007). Several additional genes involved in development and virulence have been identified. These include *FSR1*, encoding a putative multimodular protein and essential for virulence and perithecia formation (Shim et al. 2006), and *RAS2*, a Ras GTPase-encoding gene essential for spore germination, vegetative growth, and virulence (Bluhm et al. 2007). Also, disruption of *NPS6*, encoding a nonribosomal peptide synthetase, a conserved protein in plant pathogenic fungi such as *Cochliobolus miyabeanus*, *Fusarium graminearum*, and *Alternaria brassicicola*, caused reduced virulence and reduced tolerance to H₂O₂ (Oide et al. 2006). Finally, disruption of *FGL1*, encoding a secreted lipase, results in reduced extracellular lipolytic activity and decreased virulence (Voigt et al. 2005).

However, the number of genes that have been functionally analyzed is still small compared with the 14,000 genes predicted in the *F. graminearum* genome (Cuomo et al. 2007); many genes that are involved in virulence, vegetative growth, and sexual development in *F. graminearum* still need to be identified. To facilitate high-throughput functional analysis of *F. graminearum* genes, the transposable element *mimp1* was previously successfully employed to generate a transposon-inactivated gene pool (Dufresne et al. 2007, 2008). By this strategy, we identified *rev112*, a mutant that showed reduced virulence on wheat heads and reduced radial growth on potato dextrose agar. Thermal asymmetric interlaced polymerase chain reaction (PCR) showed that the transposable element *mimp1* was inserted in a putative Gal4-like Zn₂Cys₆ transcription factor, FGSG_10057 (Dufresne et al. 2008).

Zn₂Cys₆ transcription factors are unique for fungi and have been extensively studied in *Saccharomyces cerevisiae*, *Aspergillus flavus*, and *A. nidulans* (Chang et al. 1995; Johnston 1987; Shimizu et al. 2003; Vienken et al. 2005; Woloshuk et al. 1994; Yu et al. 1996). In *S. cerevisiae*, Gal4 is one of the best

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studied Zn₂Cys₆ transcription factors that regulates galactose metabolism (Johnston 1987). In *A. flavus*, *AflR* encodes a Zn₂Cys₆ transcription factor that is involved in the regulation of aflatoxin biosynthesis (Woloshuk et al. 1994). In *A. nidulans*, the Zn₂Cys₆ transcription factor RosA is a negative regulator of sexual development and overexpression of *RosA* causes reduced hyphal growth (Vienken et al. 2005).

In *Fusarium* species, only a few Zn₂Cys₆ transcription factors have been functionally characterized. In *F. graminearum*, GIP2 is a Zn₂Cys₆ transcription factor that regulates expression of genes involved in the biosynthesis of the toxin aurofusarin (Kim et al. 2006). Two Zn₂Cys₆ transcription factors, FUM and ZFR1, in *Fusarium verticillioides* are involved in the regulation of fumonisin biosynthesis (Brown et al. 2007; Flaherty and Woloshuk 2004). Fow2, a Zn₂Cys₆ transcription factor in *F. oxysporum* f. sp. *melonis*, was reported to be required for virulence but not for vegetative growth and conidiation (Imazaki et al. 2007).

In this study, we identified and characterized EBR1 (enhanced branching 1), a novel type of Zn₂Cys₆ transcription factor in *F. graminearum* that is required for radial growth, virulence, germination rate, and conidiation. In addition, we investigated the function of one of the orthologous genes in *F. oxysporum* f. sp. *lycopersici*, named FOXG_05408. We found that the FOXG_05408 knock-out mutant showed only a slight reduction of radial growth, but this gene could fully complement mutant PH-1 Δ *ebr1*, which suggests a conserved function of EBR1-like Zn₂Cys₆ transcription factors in different fungi.

RESULTS

EBR1, a Gal4-like Zn₂Cys₆ transcription factor, is conserved in fungi.

We identified a Gal4-like Zn₂Cys₆ domain in EBR1 in the region between amino acid (aa) 282 and 308, according to SMART analysis (Schultz et al. 1998). There are 35 annotated Zn₂Cys₆ transcription factors in the *F. graminearum* genome that vary both in the size and location of the Zn₂Cys₆ domain and in function (Supplementary Table S1). From those Zn₂Cys₆ transcription factors, EBR1 is the largest and the only protein with the Zn₂Cys₆ domain located after aa residue 280. The aa sequence of EBR1 was blasted against the Broad Institute database, and nine additional EBR1-like Zn₂Cys₆ transcription factors were identified from nine different fungi (Supplementary Table S2). By aligning these ten EBR1-like Zn₂Cys₆ transcription factors, we identified, apart from the six conserved cysteine residues, several other conserved amino acids (Fig. 1A). The overall length of EBR1-like Zn₂Cys₆ transcription factors is around 1,100 aa, and the Zn₂Cys₆ domain is located at around aa 230 to 310. The phylogenetic relationship between the EBR1-like Zn₂Cys₆ transcription factors is presented in Figure 1B.

Construction of a knock-out of EBR1 in *Fusarium graminearum*.

To confirm that the initially observed phenotype in *rev112* was only due to transposon inactivation and not to mutations elsewhere in the genome, we constructed a null mutant of *EBR1* by replacing the entire *EBR1* gene by homologous recombination in *F. graminearum* strains Fg820 and PH-1, using protoplast-mediated transformation (Fig. 2A). We selected 20 hygromycin-resistant colonies from both strains, and replacement of *EBR1* by the hygromycin cassette was confirmed by PCR in three transformants derived from Fg820 and one derived from PH-1 (PH-1 Δ *ebr1*) (Fig. 2B). All these knock-out strains exhibited reduced radial growth on complete medium (CM) (Leach et al. 1982) amended with 2% agar (CMA) me-

dium (Fig. 2C and D; Supplementary Fig. S1) and reduced virulence on wheat heads compared with the wild-type strain and ectopic transformants (Fig. 2E). In addition, all knock-out strains produced more pigment than wild-type strain and ectopic transformants when grown on CMA medium for 3 days (Fig. 2C). Mutant strain PH-1 Δ *ebr1* was used for further analysis.

PH-1 Δ *ebr1* is defective in conidiation.

The morphology of conidia from PH-1 Δ *ebr1* was similar to that of wild type and the ectopic transformant. To investigate whether mutant strain PH-1 Δ *ebr1* was affected in conidiation, we used both mycelium and conidia as inoculum to determine the production of conidia. With mycelium as starting inoculum, the same amounts of fresh mycelium of wild-type PH-1, PH-1 Δ *ebr1*, and an ectopic transformant were picked from CMA medium and were transferred to liquid mung bean medium for incubation. After 3 days of incubation, wild-type PH-1 and the ectopic transformant produced about 5×10^5 conidia per milliliter, whereas the knock-out mutant PH-1 Δ *ebr1* produced only 0.2×10^5 conidia per milliliter (Table 1). In contrast, when the same amount of conidia (10μ l of a 10^5 -conidia/ml suspension) of each strain was used as starting inoculum and was incubated in liquid mung bean medium for 3 days, no significant differences in production of conidia were observed between wild type and mutant (Table 1). These data indicate that the conidiation of mutant PH-1 Δ *ebr1* is significantly affected when mycelium was used as starting inoculum but not when conidia were used as starting inoculum.

The germination rate of conidia from PH-1 Δ *ebr1* is increased as compared with wild type.

To determine whether the reduced radial growth of mutant PH-1 Δ *ebr1* on CMA medium was due to a reduced germination rate of conidia, we analyzed the germination rate of conidia at 3, 4, 5, and 6 h after incubation on CMA medium. A total of 100 randomly picked conidia of each strain were analyzed for germination. After 3 h, the germination rate of conidia of PH-1 Δ *ebr1* (31%) appeared around three times as high as that of wild-type PH-1 and the ectopic transformant (10%) (Fig. 3). After 4 and 5 h, PH-1 Δ *ebr1* still showed a higher germination rate than wild-type PH-1 and the ectopic transformant. However, after 6 h of incubation, nearly all conidia of wild-type strain PH-1, mutant PH-1 Δ *ebr1*, and the ectopic transformant had germinated (Fig. 3). These data suggest that EBR1 might be a negative regulator of conidial germination.

Mycelium of PH-1 Δ *ebr1* shows increased branching compared with wild-type PH-1.

To determine which stage of growth was affected in PH-1 Δ *ebr1*, we monitored the growth of hyphae after the transfer of spores on CMA medium for 24 h. After 8 h, no significant differences between PH-1 Δ *ebr1*, wild-type PH-1, and the ectopic transformant were observed. The conidia of all these strains had germinated and grew over the surface, showing no significant differences in hyphal length. In contrast, at 24 h, PH-1 Δ *ebr1* showed more branched hyphae than wild-type PH-1 and the ectopic transformant (Fig. 4A and B). This suggests that EBR1 has a positive effect on the apical dominance of the hyphal tip and a negative effect on hyphal branching.

PH-1 Δ *ebr1* is impaired in infecting the rachis of wheat spikelets.

To uncover which stage of the infection process was affected in PH-1 Δ *ebr1*, we retransformed construct PSC001-*EBR1* into the wild-type PH-1 strain to obtain green fluorescent protein (GFP)-tagged *EBR1* knock-out strain PSC Δ *ebr1*.

A **B**

Fig. 1. Phylogenetic analysis of EBR1-like Zn₂Cys₆ transcription factors. **A**, Alignment of EBR1-like Zn₂Cys₆ transcription factors from 10 different fungi: *Aspergillus clavatus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *Fusarium graminearum*, *F. oxysporum* f. sp. *lycopersici*, *F. verticillioides*, *Magnaporthe oryzae*, *Neurospora crassa*, and *Botrytis cinerea*. The alignment shows several blocks of conserved amino acids in addition to the six conserved cysteine residues in the Gal4-like Zn₂Cys₆ domain. **B**, A phylogenetic tree constructed with the full-length protein sequences of 10 EBR1-like Zn₂Cys₆ transcription factors using the Clustal W program (Chenna et al. 2003).

PSC Δ *ebr1* showed reduced radial growth on CMA medium similar to PH-1 Δ *ebr1*. As a control for the GFP-tagged wild-type strain, we transformed empty vector PSC001 into the wild-type strain to obtain the transformant GFPX19. Both PSC Δ *ebr1* and GFPX19 strains showed a clear GFP signal in their hyphae (Supplementary Fig. 2). In the wheat-head viru-

Table 1. Conidiation of *Fusarium graminearum* strains

Strain	Conidiation (10 ⁵ /ml) ^a	
	Mycelium	Conidia
PH-1	5.37 ± 0.67 a	4.26 ± 0.67
PH-1 Δ <i>ebr1</i>	0.17 ± 0.04 b	4.02 ± 1.55
Ectopic	6.32 ± 0.80 a	4.02 ± 0.61

^a The mycelium or conidia of each strain were used as original inoculums to produce conidia. The conidia were counted 3 days after incubation in liquid mung bean medium. The different lower case letters represent significant differences at $P \leq 0.01$. The experiment was repeated at least three times.

Fig. 2. Properties of replacement mutant strain PH-1 Δ *ebr1* of *Fusarium graminearum*. **A**, The construct containing upstream (LF) and downstream (RF) fragments of the *EBR1* gene flanking the *HygB* cassette was transformed into protoplasts of wild-type PH-1 strain to obtain replacement mutants. **B**, Reverse transcription-polymerase chain reaction was performed to confirm replacement of *EBR1* by the *HygB* cassette in PH-1 Δ *ebr1*. Actin expression was used as control. **C**, Strains PH-1, PH-1 Δ *ebr1*, and an ectopic transformant were incubated onto complete medium (Leach et al. 1982) amended with 2% agar (CMA) and were grown for 3 days. **D**, Radial growth of the various strains on CMA medium was measured at regular intervals until 3 days postinoculation (dpi). **E**, Wheat heads were inoculated with strains PH-1, PH-1 Δ *ebr1*, or ectopic transformant. Water-treated ears were used as control. Photographs were taken at 14 dpi. White arrows show the originally inoculated spikelets.

lence assay, *PSCΔebr1* displayed reduced virulence when compared with strain GFPX19 (Fig. 5A). The spikelets that were inoculated with either the *PSCΔebr1* or the GFPX19 strain were split into glume, lemma, palea, and rachis at 7 days postinoculation (dpi) (Fig. 5B). All tissues were analyzed by confocal microscopy to observe the presence of GFP signals. In total, 48 inoculated wheat spikelets for each isolate were used for analysis. At 7 dpi, wild-type strain GFPX19 generated GFP signals in the glume, lemma, palea, and rachis of nearly all inoculated spikelets, whereas *PSCΔebr1* had only penetrated lemma and palea and had partially penetrated glume, but nearly no GFP signal could be detected in the rachis (Fig. 5C;

Supplementary Fig. S3). These results indicate that mutant *PSCΔebr1* is able to infect the lemma and palea and, partially, glume but not the rachis.

EBR1 is exclusively localized in the nucleus.

EBR1 is responsible for conidial germination and hyphal growth on solid CMA medium. To further understand the function of EBR1 in hyphal growth, we studied the cellular location of EBR1 in conidia and hyphae. For this purpose, we tagged the *EBR1* genomic sequence with GFP under control of its native promoter and transformed it into wild-type strain PH-1. Several transformants were obtained, and a clear GFP signal was ob-

Fig. 4. Growth of wild-type PH-1, replacement strain PH-1Δebr1, and the ectopic transformant on complete medium (Leach et al. 1982) amended with 2% agar (CMA). **A**, Conidia of strains PH-1, PH-1Δebr1, and the ectopic transformant were incubated on CMA medium. The growth of hyphae was monitored microscopically. Until 8 h of incubation, no difference in growth pattern was observed, but at 24 h, the radial growth of PH-1Δebr1 was reduced compared with the PH-1 wild-type, with much shorter branch hyphae. This experiment was repeated three times with consistent differences between PH-1Δebr1 and PH-1. **B**, Mutant PH-1Δebr1 develops many short branches when compared with wild-type PH-1 and the ectopic transformant.

served by confocal microscopy. EBR1 appeared to be localized exclusively in the nucleus in both conidia and hyphae (Fig. 6A). Interestingly, during the process of conidial germination, we observed two nuclei in each conidial cell (Fig. 6B), with EBR1 located in both nuclei. To confirm that GFP-tagged EBR1 has

the proper function in the cell, we transformed the GFP-tagged full genomic sequence into the mutant PH-1 Δ ebr1. The transformants were selected on CMA medium containing geneticin (300 μ g/ml). In total, 21 transformants were obtained, and all of them did fully restore radial growth of mutant PH-1 Δ ebr1 to the level of wild-type strain PH-1 (Supplementary Fig. S4). These data confirm that EBR1, indeed, localizes in the nucleus and plays an important role in regulating hyphal growth.

Disruption of FOXG_05408 only slightly affects radial growth of *F. oxysporum* f. sp. *lycopersici*.

To determine whether the orthologous gene FOXG_05408 in *F. oxysporum* f. sp. *lycopersici* is also required for radial growth on CMA, it was replaced by the HygB cassette in *F. oxysporum* f. sp. *lycopersici* 4287, using homologous recombination (Fig. 7A). A total of 40 hygromycin-resistant transformants were obtained, as was confirmed by PCR. We continued further analysis with a transformant designated 4287 Δ FOXG_05408, in which the FOXG_05408 gene was replaced by the HygB cassette. Subsequently reverse transcription (RT)-PCR confirmed the absence of FOXG_05408 in strain 4287 Δ FOXG_05408 (Fig. 7B). The radial growth rate of 4287 Δ FOXG_05408 on CMA medium was only slightly affected as compared with wild type and the ectopic transformant (Fig. 7C; Supplementary Fig. S5). This could be caused by functional redundancy, as the *F. oxysporum* f. sp. *lycopersici* genome contains six potential paralogous genes of the FOXG_05408 gene (Fig. 7D), whereas no potential paralog of EBR1 was identified in the *F. graminearum* genome.

The FOXG_05408 gene can complement the mutant strain PH-1 Δ ebr1.

FOXG_05408 is one of seven homologous genes present in the genome of *F. oxysporum* f. sp. *lycopersici*. To examine whether FOXG_05408 could complement mutant strain PH-1 Δ ebr1, we retransformed PH-1 Δ ebr1 with FOXG_05408, under control of its own promoter, by protoplast-mediated transformation. Geneticin-resistant colonies were selected to confirm the presence of the FOXG_05408 gene, using FOXG_05408-specific primers (Fig. 8A). Transformant PH-1 Δ ebr1#FO could fully restore radial growth and virulence of PH-1 Δ ebr1 to wild type (Fig. 8B, C, and D). Also PH-1 Δ ebr1#FO showed restoration of conidiation and conidial germination of PH-1 Δ ebr1 to wild-type levels (Supplementary Table S3). These data prove that FOXG_05408 and EBR1 exhibit similar functions in the regulation of hyphal growth, conidiation, conidial germination, and virulence.

EBR1 is localized in the nucleus of both conidia and hyphae. To investigate whether FOXG_05408 is also localized in the nucleus, we transformed the GFP-tagged FOXG_05408 genomic sequence into *F. oxysporum* f. sp. *lycopersici* 4287 by protoplast-mediated transformation. As expected, FOXG_05408 is also localized in the nucleus of both conidia and hyphae (Supplementary Fig. S6), which confirms a similar location of EBR1-like Zn₂Cys₆ transcription factors in different fungi.

DISCUSSION

To sustain the continuous race with its host, timely development and a fast targeted growth are crucial for a fungal plant pathogen. *F. graminearum* is one of the fastest growing fungi, with a radial growth rate on potato dextrose agar (PDA) medium of 16 to 20 mm per day at 25°C in the dark (Leslie and Summerell 2006). During infection of wheat spikelets, fast directional growth allows the fungus to reach the rachis in less than 3 days after a conidium has landed on a spikelet. In this way, it will fully surround the developing seed and get access

Fig. 5. Wheat infection assay with green fluorescent protein (GFP)-tagged wild-type strain GFPX19 and replacement strain PSC Δ ebr1. **A**, Wheat spikelets were inoculated with strains GFPX19 and PSC Δ ebr1 for 7 days. Photographs show frontal (left) and side (right) views of infected heads. The arrows point to the originally inoculated spikelets. **B**, Photographs show the morphology of glume, lemma, palea, and spikelet rachis. The spikelet rachis and lower part of the glume, lemma, and palea (white rectangle) were observed using confocal microscopy. **C**, Many hyphae were detected at the lower part of the lemma and palea for both strains. However, hyphae were detected only in the glume when inoculated with wild-type strain GFPX19 but not with replacement strain PSC Δ ebr1. In addition, GFPX19 had penetrated the rachis at 7 days postinoculation. In contrast, nearly no GFP signal was detected in the rachis inoculated with PSC Δ ebr1. GFP shows the signal of hyphae. Red shows the autofluorescence of chloroplast (bar = 100 μ m).

to the rachis, a key factor in colonization of the remaining part of the wheat head and, possibly, other parts of the wheat plant (Jansen et al. 2005). The Zn₂Cys₆ transcription factor EBR1 appears to play a key role for *F. graminearum* in regulation of the infection process.

Zn₂Cys₆ transcription factors have been widely studied in various fungi, especially in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *A. flavus* (Anderson et al. 1995; Johnston 1987; Pan and Coleman 1990; Shimizu et al. 2003; Yu et al. 1996), but much less so in fungal plant pathogens. Previous studies have shown that Zn₂Cys₆ transcription factors, among

others, are involved in the regulation of the production of mycotoxins, secondary metabolites, meiosis, and nitrate metabolism (Anderson et al. 1995; Brown et al. 2007; Burger et al. 1991; Woloshuk et al. 1994). In this study, we found that the Zn₂Cys₆ transcription factor EBR1 in *F. graminearum* is involved in the regulation of hyphal growth and virulence.

Bioinformatic analysis showed the presence of orthologous *EBR1* genes in other fungi, including *A. clavatus*, *A. fumigatus*, *A. nidulans*, *A. niger*, *F. oxysporum* f. sp. *lycopersici*, *F. verticillioides*, *Magnaporthe oryzae*, *Neurospora crassa*, and *Botrytis cinerea*. In each fungus except *F. oxysporum*, the

Fig. 6. Expression of green fluorescent protein-tagged EBR1. **A**, Localization of EBR1 was examined by confocal microscopy. EBR1 protein was found to localize in the nucleus of both conidia and hyphae. 4',6-Diamidino-2-phenylindole staining was used as control (bar = 10 μ m). **B**, In germinating conidia, two nuclei were observed in each cell of the conidium and EBR1 was localized in the nucleus of the germinating conidia (bar = 10 μ m).

orthologous gene was identified as a single-copy gene encoding EBR1-like Zn₂Cys₆ transcription factors. Phylogenetic analysis showed that four EBR1-like Zn₂Cys₆ transcription factors from *Aspergillus* spp. and three EBR1-like Zn₂Cys₆ transcription factors from *Fusarium* spp. are categorized into one group. This suggests that EBR1-like Zn₂Cys₆ transcription factors could be candidates to study the evolutionary relationship between different fungi species.

Fig. 7. Replacement of FOXG_05408 in *Fusarium oxysporum* f. sp. *lycopersici* by the HygB cassette. **A**, Strategy to construct the replacement strain 4287ΔFOXG_05408. **B**, Reverse transcription-polymerase chain reaction analysis to confirm the absence of FOXG_05408 in the replacement mutant strain 4287ΔFOXG_05408. Actin was used as a control. **C**, Strains were incubated on complete medium (Leach et al. 1982) amended with 2% agar for determination of radial growth. Photographs were taken 3 days postincubation. Replacement mutant 4287ΔFOXG_05408 shows only a very slight reduction in radial growth when compared with wild-type FO 4287 and the ectopic transformant. **D**, Paralogous genes of FOXG_05408 occurring in *F. oxysporum* f. sp. *lycopersici*.

EBR1-like Zn₂Cys₆ proteins represent a novel type of Zn₂Cys₆ transcription factor that are clearly different from those that have been described previously. Gal4 is a typical Zn₂Cys₆ transcription factor that contains a Cys-X2-Cys-X6-Cys-X6-Cys-X2-Cys-X6-Cys motif, which is reported to bind two Zn (II) or Cd (II) ions (Pan and Coleman 1990). Another well-studied transcription factor is AflR of *A. nidulans*, a protein of 444 aa with the same motif as Gal4 (Shimizu et al. 2003). EBR1-like Zn₂Cys₆ transcription factors also contain a Zn₂Cys₆ DNA-binding domain with the conserved Cys-X2-Cys-X6-Cys-X5/6-Cys-X2-Cys-X6-Cys motif. However, unlike the Gal4 and AflR proteins that the Zn₂Cys₆ DNA-binding domain is located close to the N-terminus, the Zn₂Cys₆ DNA-binding domain of EBR1-like Zn₂Cys₆ transcription factors is located further downstream between aa residues 230 and 310. In addition to the Zn₂Cys₆ domain, Gal4-like proteins contain a fungus-specific domain. In EBR1-like Zn₂Cys₆ transcription factors, besides the Zn₂Cys₆ domain, additional domains consisting of groups of highly conserved aa residues are present. However, the possible functions of these domains are not yet known.

In mutant strain PH-1Δebr1, the production of conidia was significantly reduced when mycelium was used as inoculum. However, there was no difference between wild type and mutant when conidia were used as inoculum. We hypothesize that increased hyphal branching affects conidiation. Unexpectedly, the initial germination rate of conidia of mutant strain PH-1Δebr1 was higher than that of the wild type within 4 h after incubation on CMA medium. Until now, in *F. graminearum*, only a few genes have been reported to be associated with germination of conidia. A mutant disrupted in the Ras GTPase also showed delayed spore germination but did not cause a significant phenotypic change in emergence of germ tubes from conidia and rate of conidial germination (Bluhm et al. 2007). The reasons why conidial germination is faster in mutant strain PH-1Δebr1 than in wild type is not clear, but it might be that EBR1 represents a downstream component of the Ras GTPase signal transduction pathway that negatively regulates germination of conidia.

Detailed observation of hyphal development of mutant strain PH-1Δebr1 on solid CMA medium showed that its radial growth rate is not affected until 8 h after incubation but is significantly inhibited at 24 h, when increased branching of hyphae is clearly visible. We speculate that the reduced radial growth rate of mutant strain PH-1Δebr1 is caused by reduction of the apical dominance of the hyphal tip, leading to increased hyphal branching. The apical dominance of hyphal tips is a complex process that is regulated by various intracellular signals, including reactive oxygen species (Schmid and Harold 1988; Semighini and Harris 2008) and calcium ions (Silverman-Gavrila and Lew 2002, 2003). To date, no transcription factors have been reported to be involved in the apical dominance of the hyphal tip, and the Zn₂Cys₆ transcription factor EBR1 we identified represents the first example. The reason for reduced hyphal-tip growth of mutant PH-1Δebr1 is not clear. One possibility is that some genes that are directly regulated by EBR1 play an important role for hyphal-tip growth. In addition, it appears that mutant strain PH-1Δebr1 produces more pigment than wild type when grown on CMA medium. Previous studies have shown that the growth rate of a pigment-defective mutant (ΔGIP2) was increased approximately 30% relative to wild type when grown on PDA medium. In contrast, constitutive expression of GIP2 in *Gibberella zeae* leads to high accumulation of pigment and reduced radial growth on PDA medium (Kim et al. 2006). EBR1 seems to have the opposite effect, and we conclude that it may have a negative regulatory effect on the expression of GIP2.

Also virulence of mutant strain PH-1 Δ ebr1 is significantly reduced when assayed on wheat heads. After 14 dpi, wild-type PH-1 has colonized the entire wheat head, showing the typical symptoms of head blight, whereas mutant strain PH-1 Δ ebr1 only colonized the spikelets that were initially inoculated with conidia. The GFP-tagged PSC Δ ebr1 strain clearly showed absence of penetration of the rachis at 7 dpi, a phenotype that is reminiscent of naturally occurring resistance “type two” of wheat, in which colonization of the spikelets neighboring the initially inoculated one is blocked. Similarly, previous studies have reported that infection of wheat heads by mutant *top1* (topoisomerase I) was restricted to the inoculated florets, while wild-type strain had spread from inoculated spikelets to neighboring noninoculated spikelets after 20 days (Baldwin et al. 2010). Also a novel transcription factor, ZIF1, was reported to be important for *F. graminearum* to penetrate the rachis (Wang et al. 2011). There could be different causes leading to the failure to penetrate the rachis. Here, we speculate that reduced hyphal-tip growth of PH-1 Δ ebr1 might allow the host to mobilize effective defense responses in time to block further colonization of the fungus.

Replacing orthologous gene FOXG_05408 in *F. oxysporum* f. sp. *lycopersici* only slightly affected radial growth on CMA medium. This might be due to redundancy, as six homologous genes are present in *F. oxysporum* f. sp. *lycopersici*, whereas *F. graminearum* EBR1 contains a single-copy gene. Retransforming PH-1 Δ ebr1 with FOXG_05408 fully restored the mutant to wild type, confirming conserved functions between EBR1 and FOXG_05408 in *F. graminearum* and *F. oxysporum* f. sp. *lycopersici*, respectively.

GFP-tagged EBR1 showed that the protein is exclusively localized in the nucleus of both conidia and hyphae. Also the orthologous protein FOXG_05408 is localized in the nucleus of *F. oxysporum* f. sp. *lycopersici*. This suggests that EBR1-like Zn₂Cys₆ transcription factors are conserved proteins that are constitutively expressed. Previous reports have shown that the localization of Zn₂Cys₆ transcription factors is regulated by a G-protein signaling pathway or by extracellular signals. For example, in *A. nidulans*, the Zn₂Cys₆ transcription factor AflR is located in the cytoplasm when protein kinase A (*pkaA*) is overexpressed, while it resides in the nucleus when an RGS (regulator of G-protein signaling) gene *FlbA* is inactivated

Fig. 8. FOXG_05408 can complement the replacement strain PH-1 Δ ebr1. **A**, The genomic sequence of FOXG_05408 was transformed into replacement mutant strain PH-1 Δ ebr1. Geneticin-resistant transformants were selected for polymerase chain reaction confirmation using FOXG_05408-specific primers and using the HygB fragment as a control. **B** and **C**, FOXG_05408 restored radial growth of replacement mutant PH-1 Δ ebr1 on complete medium (Leach et al. 1982) amended with 2% agar to wild-type level. **D**, FOXG_05408 restored the virulence of replacement mutant PH-1 Δ ebr1 strain to wild-type level in the wheat-head assay. White arrows show the originally inoculated spikelets.

(Shimizu et al. 2003). AmyR is another Zn₂Cys₆ transcription factor that regulates the expression of amylolytic genes in *A. nidulans*. The location of AmyR is dependent on isomaltose. In the absence of isomaltose, AmyR locates in the nucleus and cytoplasm, but in the presence of isomaltose, AmyR locates exclusively in the nucleus (Makita et al. 2009). The mechanism of regulation varies between different types of Zn₂Cys₆ transcription factors. It is possible that both the germination of conidia and hyphal-tip growth require constitutive expression of *EBR1* in the nucleus. However, it remains to be investigated which domain of EBR1 is required for its nuclear localization.

MATERIALS AND METHODS

Plants.

The spring wheat cultivar Thassos was used in this study. Plants were grown in the greenhouse at 18°C with a 16-h-light and 8-h-dark regime. At anthesis, spikelets were inoculated with 5 µl of a conidial suspension (10⁵ conidia/ml) of *F. graminearum*.

Fungal strains and culture conditions.

F. graminearum strains Fg820 and PH-1 were used in this study. Fg820 was isolated from infected wheat plants in The Netherlands and PH-1 is a sequenced strain that originates from a wheat plot in Michigan in the United States (Cuomo et al. 2007). The sequenced *F. oxysporum* f. sp. *lycopersici* 4287 was also used in parallel experiments. All strains were cultured on solid PDA plates (25°C) and were stored in 2-ml Eppendorf tubes covered with 15% dimethyl sulfoxide in liquid nitrogen.

For production of conidia, strains were grown on CMA for 3 days, after which a small piece of CMA with mycelium (25 mm²) was transferred to 3 ml of liquid mung bean medium (Bai and Shaner 1996) and was incubated on a rotary shaker for 3 to 5 days (25°C, 200 rpm). The number of conidia was determined microscopically, using a GLASSTIC SLIDE 10 with GRIDS (Hycor Biomedical, Inc., Garden Grove, CA, U.S.A.).

Growth, conidiation, and germination assays.

For the growth assay of *F. graminearum*, conidia were diluted to a concentration of 10⁵ conidia per milliliter, and 500 conidia (5 µl) were transferred to 9-cm petri plates containing CMA. The petri plates were placed in an incubator (25°C) in the dark, and the radial growth of *F. graminearum* was measured at 24, 48, and 72 h after incubation. To determine the radial growth of *F. oxysporum* f. sp. *lycopersici*, conidia were diluted to 10⁶ conidia per milliliter, a 5-µl conidial suspension was spotted in the center of CMA plates, and growth-rate measurements were conducted after 3 days.

For conidiation assays using mycelium as initial inoculum, an equal volume (3 mm² from CMA medium) of fresh mycelium of each strain was transferred into 3 ml of liquid mung bean medium and was incubated for 3 days in a rotary shaker (25°C, 200 rpm; THZ-C-1, Taicang, Jiangsu, China). For conidiation assays using conidia as initial inoculum, a 10-µl conidial suspension (10⁵ conidia/ml) of each strain was transferred to 3 ml of liquid mung bean medium and was incubated for 3 days in a rotary shaker (25°C, 200 rpm). The number of conidia was determined microscopically, using a GLASSTIC SLIDE 10 with GRIDS (Hycor Biomedical, Inc.).

For germination assays, 5-µl conidial suspensions (10⁵ conidia/ml) were pipetted onto a glass slide containing a thin layer of CMA and were incubated in a 25°C incubator. The germination rate of 100 randomly selected conidia was counted microscopically (Olympus CX31) 4 h after incubation. The same samples were also used to monitor the growth of hyphae

at 8 and 24 h after incubation. The experiments were repeated twice.

Virulence assays.

When the first anthers extruded from the wheat heads, 5 µl of conidial suspension (10⁵ conidia/ml) was pipetted into the floral cavity between the lemma and palea in one single floret of a spikelet. For each head, two spikelets (the ninth and tenth spikelets from the bottom of the wheat head) were inoculated and the plants were kept in a greenhouse at 20°C with a 16-h-light and 8-h-dark regime. The symptoms were observed at 14 dpi.

To follow the infection process in wheat heads by GFP-tagged strains, the infected wheat heads were cut at 7 dpi, and the spikelets were carefully split into glume, lemma, palea, and rachis. For glume, lemma, and palea, the lower part of tissue was analyzed, respectively. For rachis, the tissue was first cut vertically into a thin piece. All tissues were placed onto a microscopic slide, and the GFP signal was detected by confocal microscopy.

Bioinformatic analysis.

Nine orthologous genes of *EBR1* were identified by BLAST analysis of the complete protein sequence of EBR1 against nine known fungal genomes available in the BROAD database. The sequences of the 10 proteins were aligned using Megalign software (DNASTAR, Inc., Madison, WI, U.S.A.), and the alignment was edited with Genedoc software (Nicholas et al. 1997). To construct a phylogenetic tree, 10 EBR1-like transcription factors were aligned using the Clustal W program (Thompson et al. 1994).

DNA and RNA isolation.

Genomic DNA was extracted from fresh mycelium by the cetyltrimethylammonium bromide method, as described previously (Saghai-Marroof et al. 1984). For RNA isolation, fresh hyphae were collected from 3-day-old cultures grown on CMA medium. RNA was extracted, using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions.

Generation of transformation constructs.

To prepare a construct for knocking out the *EBR1* gene, two fragments upstream and downstream of the *EBR1* gene were amplified from genomic DNA, using primers Fg10057 P1 and P2 and Fg10057 P3 and P4, respectively (primers are listed in Supplementary Table S4). PCR was conducted according to the instruction of Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). The amplified fragments were isolated from agarose gel and were ligated into the vector pShortHyg K7 (Supplementary Fig. S7), using the restriction sites *Bam*HI/*Xba*I and *Kpn*I/*Xho*I, respectively. The resulting construct was named pShortHyg K7-*EBR1* (Fig. 2A). The knock-out construct for the orthologous gene FOXG_05408 of *F. oxysporum* f. sp. *lycopersici* was prepared in a similar way. Primers FO05408 P1 and P2 and FO05408 P3 and P4 were used to amplify an upstream and downstream fragment of FOXG_05408, respectively. The same PCR protocol was used for amplification and both fragments were ligated into pShortHyg K7 to obtain the knock-out construct pShortHyg K7-FOXG_05408 (Fig. 7A). To construct the GFP-tagged *EBR1* gene, the *EBR1* genomic sequence, together with its 1,613-bp promoter region, was amplified from genomic DNA using primers Fg10057 P11 and P12. The PCR product was digested with *Spe*I and was ligated to the *Spe*I site of vector pShortHyg K7-GFP-Tnos. The obtained construct pShortHyg K7-*EBR1*-GFP-Tnos was used for transformation. For GFP-tagged *EBR1* knock-out strain, vector PSC001 was used to ob-

tain the final construct PSC001-*EBRI*, using the same procedure described for construct pShortHyg K7-*EBRI*. For the complementation assay, genomic sequence of FOXG_05408 under control of its native promoter (1,570 bp) was amplified using primers FO05408 P7 and P8. The PCR product was ligated to the *SpeI* site of vector pShortGen K7-Tnos, which contains the geneticin cassette.

Protoplast-mediated transformation.

Transformation of *F. graminearum* and *F. oxysporum* f. sp. *lycopersici* was performed as described previously (Turgeon et al. 1987), with some minor modifications. Approximately 10^5 conidia grown in liquid mung bean medium were transferred to 50 ml of liquid CM medium. The conidia were incubated for 2 days on a rotary shaker (25°C, 150 rpm) to produce mycelium. The mycelium was homogenized by vortexing, and 10 ml of the homogenate was transferred to 200 ml of fresh CM liquid medium and was incubated overnight (25°C, 150 rpm). The mycelium was collected by filtration on sterile cheese cloth and was washed twice with sterile water. The mycelium was transferred to 20 ml of Driselase/glucanase (2.5:1.5% in 700 mM NaCl, pH 5.6) in a 50-ml tube and was incubated for 2.5 to 3 h (22°C, 43.5 rpm). When the number of protoplasts was higher than 10^7 per milliliter, the protoplasts were filtered (48 µm) and 20 ml of 700 mM NaCl was added to the protoplast filtrate. The protoplasts were collected by centrifugation at $1,300 \times g$ for 5 min (Centrifuge 5810 R; Eppendorf, Hamburg, Germany) and were resuspended in 10 ml of ice-cold 700 mM NaCl. The protoplasts were washed and collected by centrifugation at $830 \times g$ for 5 min. The washing step was repeated. Finally, the protoplasts were collected by centrifugation ($830 \times g$ for 5 min) and were resuspended in STC (0.8M sorbitol, 50 mM Tris HCl, pH 8.0, 50 mM CaCl₂) with SPTC (4:1) (0.8 M sorbitol, 40% polyethylene glycol 4000, 50 mM Tris-HCl, pH 8.0, 50 mM CaCl₂) at a final protoplast concentration of 0.5×10^8 to 2×10^8 per milliliter. To 100 µl of protoplast suspension, 30 µg of plasmid and 5 µl of heparin (5 mg/ml in STC) were added in 2-ml Eppendorf tubes. The mixture was incubated on ice for 30 min, after which 1 ml of SPTC was added to the mixture, which was incubated for 20 min at 22°C. The mixture was added gently to 200 ml of regeneration medium (yeast extract [0.1%], casein hydrolysate [0.1%], sucrose [34.2%], granulated agar [1.6%]) at 43°C. The regeneration medium was poured in 20-ml portions in 94-mm petri dishes. The protoplasts were incubated for 12 to 24 h at 28°C, after which the selection top layer was added (1.2% granulated agar in water containing 300 µg of hygromycin B or geneticin per milliliter) to the plates. The plates were incubated at 28°C for 3 days. Colonies that grew through the selection medium were transferred to CMA medium containing 100 µg of hygromycin B or geneticin per milliliter and were incubated at 28°C.

Validation of the knock-out transformants by PCR.

DNA was isolated from the mycelium of transformants as described above. The DNA was used as template for the PCR amplification, using the primers from the HygB cassette to confirm the presence of the *HygB* gene and the primers from the open reading frame region of the genes to confirm whether the targeted gene had been knocked out. Positive knock-out strains were selected for RNA isolation, and RT-PCR was performed to further confirm the absence of mRNA.

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LITERATURE CITED

- Anderson, S. F., Steber, C. M., Esposito, R. E., and Coleman, J. E. 1995. UME6, a negative regulator of meiosis in *Saccharomyces cerevisiae*, contains a C-terminal Zn₂Cys₆ binuclear cluster that binds the URS1 DNA sequence in a zinc-dependent manner. *Prot. Sci.* 4:1832-1843.
- Bai, G. H., and Shaner, G. 1996. Variation in *Fusarium graminearum* and cultivar resistance to wheat scab. *Plant Dis.* 80:975-979.
- Bai, G. H., Desjardins, A. E., and Plattner, R. D. 2002. Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia* 153:91-98.
- Baldwin, T. K., Urban, M., Brown, N., and Hammond-Kosack, K. E. 2010. A role for topoisomerase I in *Fusarium graminearum* and *F. culmorum* pathogenesis and sporulation. *Mol. Plant-Microbe Interact.* 23:566-577.
- Bluhm, B. H., Zhao, X., Flaherty, J. E., Xu, J. R., and Dunkle, L. D. 2007. RAS2 regulates growth and pathogenesis in *Fusarium graminearum*. *Mol. Plant-Microbe Interact.* 20:627-636.
- Brown, D. W., Butchko, R. A., Busman, M., and Proctor, R. H. 2007. The *Fusarium verticillioides* FUM gene cluster encodes a Zn(II)2Cys6 protein that affects FUM gene expression and fumonisin production. *Eukaryot. Cell* 6:1210-1218.
- Burger, G., Strauss, J., Scazzocchio, C., and Lang, B. F. 1991. *nirA*, the pathway-specific regulatory gene of nitrate assimilation in *Aspergillus nidulans* encodes a putative GAL4-type zinc finger protein and contains four introns in highly conserved regions. *Mol. Cell Biol.* 11:5746-5755.
- Chang, P. K., Ehrlich, K. C., Yu, J., Bhatnagar, D., and Cleveland, T. E. 1995. Increased expression of *Aspergillus parasiticus aflR*, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 61:2372-2377.
- Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. 2003. Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* 31:3497-3500.
- Cuomo, C. A., Guldener, U., Xu, J. R., Trail, F., Turgeon, B. G., Di Pietro, A., Walton, J. D., Ma, L. J., Baker, S. E., Rep, M., Adam, G., Antoniw, J., Baldwin, T., Calvo, S., Chang, Y. L., Decaprio, D., Gale, L. R., Gnerre, S., Goswami, R. S., Hammond-Kosack, K., Harris, L. J., Hilburn, K., Kennell, J. C., Kroken, S., Magnuson, J. K., Mannhaupt, G., Mauceli, E., Mewes, H. W., Mitterbauer, R., Muehlbauer, G., Munsterkotter, M., Nelson, D., O'Donnell, K., Ouellet, T., Qi, W., Quesneville, H., Roncero, M. I., Seong, K. Y., Tetko, I. V., Urban, M., Waalwijk, C., Ward, T. J., Yao, J., Birren, B. W., and Kistler, H. C. 2007. The *Fusarium graminearum* genome reveals a link between localized polymorphism and pathogen specialization. *Science* 317:1400-1402.
- Desjardins, A. E., Hohn, T. M., and McCormick, S. P. 1993. Trichothecene biosynthesis in *Fusarium* species: Chemistry, genetics, and significance. *Microbiol. Rev.* 57:595-604.
- Desjardins, A. E., Proctor, R. H., Bai, G., McCormick, S. P., Shaner, G., Beuchley, G., Hohn, T. M. 1996. Reduced virulence of trichothecenes antibiotic-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Mol. Plant-Microbe Interact.* 9:775-781.
- Desjardins, A. E., Bai, G., Plattner, R. D., and 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.
- Dufresne, M., Hua-Van, A., El Wahab, H. A., Ben M'Barek, S., Vasnier, C., Teyssset, L., Kema, G. H., and Daboussi, M. J. 2007. Transposition of a fungal miniature inverted-repeat transposable element through the action of a Tc1-like transposase. *Genetics* 175:441-452.
- Dufresne, M., van der Lee, T., Ben M'Barek, S., Xu, X., Zhang, X., Liu, T., Waalwijk, C., Zhang, W., Kema, G. H., and Daboussi, M. J. 2008. Transposon-tagging identifies novel pathogenicity genes in *Fusarium graminearum*. *Fungal Genet. Biol.* 45:1552-1561.
- Flaherty, J. E., and Woloshuk, C. P. 2004. Regulation of fumonisin biosynthesis in *Fusarium verticillioides* by a zinc binuclear cluster-type gene, ZFR1. *Appl. Environ. Microbiol.* 70:2653-2659.
- Hou, Z., Xue, C., Peng, Y., Katan, T., Kistler, H. C., and 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.
- Imazaki, I., Kurahashi, M., Iida, Y., and Tsuge, T. 2007. Fow2, a Zn(II)2Cys6-type transcription regulator, controls plant infection of the vascular wilt fungus *Fusarium oxysporum*. *Mol. Microbiol.* 63:737-753.
- Jansen, C., von Wettstein, D., Schafer, W., Kogel, K. H., Felk, A., and Maier, F. J. 2005. Infection patterns in barley and wheat spikes inocu-

- lated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. Proc. Natl. Acad. Sci. U.S.A. 102:16892-16897.
- Jenczmionka, N. J., Maier, F. J., Losch, A. P., and 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.
- Johnston, M. 1987. A model fungal gene regulatory mechanism: The GAL genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 51:458-476.
- Kim, J. E., Jin, J., Kim, H., Kim, J. C., Yun, S. H., and Lee, Y. W. 2006. GIP2, a putative transcription factor that regulates the aurofusarin biosynthetic gene cluster in *Gibberella zeae*. Appl. Environ. Microbiol. 72:1645-1652.
- Leach, J., Lang, B. R., and Yoder, O. C. 1982. Methods for selection of mutants and in vitro culture of *Cochliobolus heterostrophus*. J. Gen. Microbiol. 128:1719-1729.
- Lee, T., Oh, D. W., Kim, H. S., Lee, J., Kim, Y. H., Yun, S. H., and Lee, Y. W. 2001. Identification of deoxynivalenol- and nivalenol-producing chemotypes of *Gibberella zeae* by using PCR. Appl. Environ. Microbiol. 67:2966-2972.
- Leslie J., and Summerell B. 2006. The Fusarium Laboratory Manual. Blackwell Publishing, Ames, IA, U.S.A.
- Makita, T., Katsuyama, Y., Tani, S., Suzuki, H., Kato, N., Todd, R. B., Hynes, M. J., Tsukagoshi, N., Kato, M., and Kobayashi, T. 2009. Inducer-dependent nuclear localization of a Zn(II)(2)Cys(6) transcriptional activator, AmyR, in *Aspergillus nidulans*. Biosci. Biotechnol. Biochem. 73:391-399.
- McMullen, M., Jones, R., Gallenberg, D. 1997. Scab of wheat and barley: A re-emerging disease of devastating impact. Plant Dis. 81:1340-1348.
- Nicholas, K. B., Nicholas, H. B. J., and Deerfield, D. W. I. 1997. GeneDoc: Analysis and visualization of genetic variation. EMBNEW News 4:14.
- Oide, S., Moeder, W., Krasnoff, S., Gibson, D., Haas, H., Yoshioka, K., and Turgeon, B. G. 2006. NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. Plant Cell 18:2836-2853.
- Pan, T., and Coleman, J. E. 1990. GAL4 transcription factor is not a "zinc finger" but forms a Zn(II)2Cys6 binuclear cluster. Proc. Natl. Acad. Sci. U.S.A. 87:2077-2081.
- Proctor, R. H., Hohn, T. M., and 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.
- Ramamoorthy, V., X. Zhao, A. K. Snyder, J.-R. Xu, and D. M. Shah. 2007. Two mitogen-activated protein kinase signalling cascades mediate basal resistance to antifungal plant defensins in *Fusarium graminearum*. Cell Microbiol. 9:1491-1506.
- Saghai-Marouf, M. A., Soliman, K. M., Jorgensen, R. A., and Allard, R. W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. U.S.A. 81:8014-8018.
- Schmid, J., and Harold, F. M. 1988. Dual roles for calcium ions in apical growth of *Neurospora crassa*. J. Gen. Microbiol. 134:2623-2631.
- Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. 1998. SMART, a simple modular architecture research tool: Identification of signaling domains. Proc. Natl. Acad. Sci. U.S.A. 95:5857-5864.
- Semighini, C. P., and Harris, S. D. 2008. Regulation of apical dominance in *Aspergillus nidulans* hyphae by reactive oxygen species. Genetics 179:1919-1932.
- Shim, W.-B., Sagaram, U. S., Choi, Y.-E., So, J., Wilkinson, H. H., and Lee, Y.-W. 2006. *FSR1* is essential for virulence and female fertility in *Fusarium verticillioides* and *F. graminearum*. Mol. Plant-Microbe Interact. 19:725-733.
- Shimizu, K., Hicks, J. K., Huang, T. P., and Keller, N. P. 2003. Pka, Ras and RGS protein interactions regulate activity of AflR, a Zn(II)2Cys6 transcription factor in *Aspergillus nidulans*. Genetics 165:1095-1104.
- Silverman-Gavrila, L. B., and Lew, R. R. 2002. An IP3-activated Ca²⁺ channel regulates fungal tip growth. J. Cell. Sci. 115:5013-5025.
- Silverman-Gavrila, L. B., and Lew, R. R. 2003. Calcium gradient dependence of *Neurospora crassa* hyphal growth. Microbiology 149:2475-2485.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680.
- Turgeon, B. G., Garber, R. C., and Yoder, O. C. 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell Biol. 7:3297-3305.
- Vienken, K., Scherer, M., and Fischer, R. 2005. The Zn(II)2Cys6 putative *Aspergillus nidulans* transcription factor repressor of sexual development inhibits sexual development under low-carbon conditions and in submerged culture. Genetics 169:619-630.
- Voigt, C. A., Schafer, W., and Salomon, S. 2005. A secreted lipase of *Fusarium graminearum* is a virulence factor required for infection of cereals. Plant J. 42:364-375.
- Wang, Y., Liu, W., Hou, Z., Wang, C., Zhou, X., Jonkers, W., Ding, S., Kistler, H. C., and Xu, J. R. 2011. A novel transcriptional factor important for pathogenesis and ascosporeogenesis in *Fusarium graminearum*. Mol. Plant-Microbe Interact. 24:118-128.
- Woloshuk, C. P., Foutz, K. R., Brewer, J. F., Bhatnagar, D., Cleveland, T. E., and Payne, G. A. 1994. Molecular characterization of aflR, a regulatory locus for aflatoxin biosynthesis. Appl. Environ. Microbiol. 60:2408-2414.
- Yu, J. H., Butchko, R. A., Fernandes, M., Keller, N. P., Leonard, T. J., and Adams, T. H. 1996. Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. Curr. Genet. 29:549-555.

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