The Stress-Activated Protein Kinase FgOS-2 Is a Key Regulator in the Life Cycle of the Cereal Pathogen *Fusarium graminearum*

Thuat Van Nguyen, Wilhelm Schäfer, and Jörg Bormann

University of Hamburg, Biocenter Klein Flottbek, Department of Molecular Phytopathology and Genetics, Ohnhorststr. 18, D-22609 Hamburg, Germany

Submitted 22 February 2012. Accepted 10 May 2012.

Fusarium graminearum is one of the most destructive pathogens of cereals and a threat to food and feed production worldwide. It is an ascomycetous plant pathogen and the causal agent of Fusarium head blight disease in small grain cereals and of cob rot disease in maize. Infection with *F. graminearum* leads to yield losses and mycotoxin contamination. Zearalenone (ZEA) and deoxynivalenol (DON) are hazardous mycotoxins; the latter is necessary for virulence toward wheat. Deletion mutants of the *F. graminearum* orthologue of the *Saccharomyces cerevisiae* Hog1 stress-activated protein kinase, FgOS-2 (Δ FgOS-2), showed drastically reduced in planta DON and ZEA production. However, Δ FgOS-2 produced even more DON than the wild type under in vitro conditions, whereas ZEA production was similar to that of the wild type. These deletion strains are dramatically reduced in pathogenicity toward maize and wheat. We constitutively expressed the fluorescent protein dsRed in the deletion strains and the wild type. Microscopic analysis revealed that Δ FgOS-2 is unable to reach the rachis node at the base of wheat spikelets. During vegetative growth, Δ FgOS-2 strains exhibit increased resistance against the phenylpyrrole fludioxonil. Growth of mutant colonies on agar plates supplemented with NaCl is reduced but conidia formation remained unchanged. However, germination of mutant conidia on osmotic media is severely impaired. Germ tubes are swollen and contain multiple nuclei. The deletion mutants completely fail to produce perithecia and ascospores. Furthermore, FgOS-2 also plays a role in reactive oxygen species (ROS)-related signaling. The transcription and activity of fungal catalases is modulated by FgOS-2. Among the genes regulated by FgOS-2, we found a putative calcium-dependent NADPH-oxidase (*noxC*) and the transcriptional regulator of ROS metabolism, *atf1*. The present study describes new aspects of stress-activated protein kinase signaling in *F. graminearum*.

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is the causal agent of Fusarium head blight (FHB) disease in wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) and of cob rot in maize (*Zea mays* L.). It is a necrotrophic, filamentous ascomycete and one of

the most important phytopathogens worldwide in terms of crop losses and the accumulation of mycotoxins (Goswami and Kistler 2004; Sutton 1982). Some of these mycotoxins play a pivotal role in the world's food and feed production because they are toxic to animals and humans (McMullen et al. 1997). The polyketide zearalenone (ZEA), an oestrogen analogon, preferentially accumulates in stored grains. In contrast to the trichothecene deoxynivalenol (DON), it is not required for the infection of wheat (Gaffoor et al. 2005). DON has been described as a host-specific virulence factor for the infection of wheat; it is nonessential for pathogenicity toward maize (Jansen et al. 2005; Maier et al. 2006). Mutants defective in the first enzyme of the trichothecene-synthesis pathway, Tri5, show significantly reduced virulence toward wheat. Only the point-inoculated spikelet shows disease symptoms (Maier et al. 2006; Proctor et al. 1995).

The regulation of mycotoxin production in *F. graminearum* seems to be rather complex. Although several publications describe conditions under which DON synthesis is induced in vitro—for example, low pH (Gardiner et al. 2009b), polyamines (Gardiner et al. 2009a), H₂O₂-mediated oxidative stress (Ponts et al. 2006), carbon source (Jiao et al. 2008), and several other factors (Ochiai et al. 2007b; Tsuyuki et al. 2011)—to date, the underlying signaling pathways that regulate toxin production are not fully understood. DON biosynthesis is reduced in vitro under hyperosmotic conditions, indicating that the stress response pathway in *F. graminearum* is involved (Ochiai et al. 2007b). Stress-activated protein kinases (SAPK) act in response to changes in the environment and are, to a variable extent, necessary for survival under unfavorable stress conditions. These conditions include heat, salt and oxidative stresses. The SAPK of *Saccharomyces cerevisiae* (HOG1) is only necessary for growth under hyperosmotic stress conditions (O'Rourke et al. 2002), whereas the *Schizosaccharomyces pombe* homologue Spc1 is also crucial for coping with stresses such as UV light, heat stress, and oxidative stress.

The underlying SAPK signaling cascade starts with a sensor histidine kinase (Sln1 in *Saccharomyces cerevisiae*). An external signal is relayed via a phosphotransfer protein (Ypd1 in *S. cerevisiae*) to an array of response regulators (Ssk1 and Skn7 in *S. cerevisiae*). In its phosphorylated state, Ssk1 is active and the downstream mitogen-activated protein kinase (MAPK) cascade remains inactive. Upon stress-condition perception, Ssk1 is dephosphorylated and the MAPK cascade is activated (Li et al. 1998; Posas et al. 1996).

Homologues of the yeast HOG1 can be found and have diverse and distinct functions in several filamentous fungi (Xu 2000). In *Aspergillus nidulans*, the HOG1 homologue SakA is

Corresponding author: J. Bormann; Telephone: +49 40 42816 309; E-mail: bormannj@botanik.uni-hamburg.de

*The e-Xtra logo stands for “electronic extra” and indicates that 13 supplementary figures and two supplementary tables are published online.

involved in stress signaling after exposure to oxidative and heat stresses but not osmotic stress. Furthermore, SakA deletion mutants show impaired sexual development and the spores are less resistant to stress and are unable to survive long storage periods (Kawasaki et al. 2002). The importance of HOG1 homologues regarding pathogenic development varies between different fungal organisms (Heller and Tudzynski 2011). The *Magnaporthe oryzae* HOG1 homologue, Osm1, is dispensable for virulence toward rice but necessary for proper growth in hyperosmotic media (Dixon et al. 1999). The latter also applies for mutants defective in the putative upstream response regulator of the osmotic stress signal transduction pathway in *F. graminearum*, FgRrg1 (Jiang et al. 2011). The *Botrytis cinerea* HOG1 homologue Sak1 also shows increased sensitivity toward osmotic stress (Segmüller et al. 2007). Δ BcSak1 mutants, furthermore, show drastically reduced virulence. They are unable to produce pseudoappressoria and fail to penetrate unwounded plant tissue. Wounded surfaces can be colonized, although at a significantly slower rate compared with the wild-type strain (Segmüller et al. 2007). In addition, whereas BcSak1-deletion strains show greater sensitivity to H₂O₂, they also exhibit greater resistance to the dicarboximide antibiotic iprodione. *F. proliferatum* HOG1 deletion mutants are retarded in growth and show an upregulation of genes involved in programmed cell death (Ádám et al. 2008). Therefore, in spite of the conserved stress-activated protein kinase genes, the resulting regulation is diverse and shows pronounced differences among fungal species.

In the present study, we describe the *F. graminearum* HOG1-orthologue FgOS-2 as a central regulator during the life cycle of this pathogen. It is involved in numerous cellular processes, including the regulation of secondary metabolism, sexual development, pathogenicity toward wheat and maize, and reactive oxygen species (ROS) metabolism. In contrast to previous findings (Ochiai et al. 2007a), we found an almost wild-type-like production of mycotoxins under in vitro conditions and, for the first time, showed a lack of toxin production in planta. Furthermore, we present data on the pathogenicity and possible downstream targets of FgOS-2.

RESULTS

Generation of Δ FgOS-2 mutants.

In order to study the impact of OS-2 on the pathogenicity and development of *F. graminearum*, the gene was deleted using a gene replacement approach (Supplementary Fig. S1A). For this purpose, the 5'- and the 3'-flanking regions were amplified by polymerase chain reaction (PCR) and subsequently fused to a hygromycin resistance cassette. The excised replacement fragment was used to transform protoplasts of *F. graminearum* strain PH1. Hygromycin-resistant colonies were screened by diagnostic PCR; six of 45 independent transformants showed homologous integration of the replacement fragment. All transformants showed amplification of the wild-type sequence, indicating that these strains were heterokaryotic. Single-spore isolations were performed to obtain homokaryotic deletion mutants, from which four stable homokaryotic deletion mutants were selected. One mutant was selected which showed the intact wild-type gene and ectopic integration of the vector. All of the selected mutants and the wild type were analyzed in a Southern blot experiment. When hybridized with a probe for the hygromycin gene, all mutants showed a single integration of the fragment. No signal was obtained for the wild type. A probe amplified from the wild-type allele of FgOS-2 generated a signal in the wild type and in one mutant, confirming this strain as being an ectopic strain. Four mutant strains showed no signal.

FgOS-2 deletion mutants are sensitive

to osmotic stress but not to oxidative stress or fungicides.

To test the vegetative growth on different media, a plate assay was conducted using three independent deletion mutants, one ectopic strain, and the wild type, respectively. When grown on agar plates containing basic medium (e.g., potato dextrose agar [PDA]), the mutant strains showed slightly retarded growth compared with the wild type (Fig. 1). Growth of the mutant strains completely ceased on agar plates supplemented with osmotic stress agents (0.3 to 0.8 M NaCl and KCl or 0.6 to 1.2 M sorbitol), whereas the wild type and ectopic strain showed no growth retardation (Fig. 1; Supplementary Fig. S2). The germination of the mutant's conidia in the presence of 0.8 M NaCl was different compared with the wild type: the characteristic swelling of the conidial compartments immediately before and during germination (Seong et al. 2008) was not observed in the mutant (Fig. 2B and C). Also, the germination rate was reduced to approximately 65% (Fig. 2A). Conidia of the Δ FgOS-2 strains always germinated out of one compartment close to the center of the spore, whereas the wild-type conidia germinated out of two to three compartments (Fig. 2B and C). The germ tubes of the deletion mutants were malformed and growth ceased after only a few micrometers of growth (Fig. 2B and C). 4',6-Diamidino-2-phenylindole (DAPI) staining revealed that the germ tube of the deletion mutants was polyenergid (>10 nuclei) and not septated whereas, in the wild type, one compartment contained two to three nuclei (Fig. 2C). On the non-osmotic medium, no obvious difference in conidial germination or initial mycelial growth was apparent. In order to test the response to oxidative stress, complete medium (CM) plates were supplemented with 0, 10, 15, and 20 mM H₂O₂. With increasing concentrations of H₂O₂ in the media, growth of the wild type diminished, whereas growth of the mutant strains was only slightly inhibited (Fig. 3; Supplementary Fig. S3). On plates supplemented with 20 mM H₂O₂, the wild type was significantly reduced in growth compared with the Δ FgOS-2 strains (Fig. 3). Thus, the mutant grew better than the wild type under oxidative stress conditions. This result contradicts previous findings, that FgOS-2 mutants are more sensitive toward oxidative stress (Ochiai et al. 2007a). No differences in growth performance between wild-type and mutant strains were observed on agar plates containing different nitrogen sources (NaNO₃ and (NH₄)₂SO₄, each 5 mM; Supplementary Fig. S4), on medium buffered to certain pH values (pH 5, 7 and 9; Supplementary Fig. S5) and on plates incubated at different temperatures (18, 30, and 32°C; Supplementary Fig. S6). Previous results obtained for other fungi showed that HOG1 contributed to resistance against antibiotics. Strains defective in the HOG1 homologue of *Colletotrichum lagenarium*, Osl1, showed partial resistance toward fludioxonil (Kojima et al. 2004), and knock-out mutants in different isolates of *B. cinerea* showed distinct degrees of resistance toward fludioxonil and iprodione (Liu et al. 2008; Segmüller et al. 2007). Strains defective in FgRrg1 are also partially resistant toward fludioxonil (Jiang et al. 2011). The *F. graminearum* wild type and three independent FgOS-2 knock-out mutants were assayed on plates containing fludioxonil (phenylpyrrole), iprodione (dicarboximide), tebuconazole (triazole), and azoxystrobin (aromatic carbon hydrates) (Supplementary Fig. S7A). The absence of FgOS-2 led to a strongly increased resistance toward fludioxonil. Growth of the wild type completely ceased at a concentration of 0.5 mg liter⁻¹, while the mutant was able to grow almost as well as it did on CM control plates. Microscopic analysis of the germinating conidia on fludioxonil at 0.5 mg liter⁻¹ revealed an abnormal morphology of wild-type hyphae. Starting at the conidial compartments, the hyphae were swollen and frequently burst. In contrast, hyphae of the mutants ap-

peared to be unaffected. Expression analysis using quantitative real-time (qRT)-PCR revealed an upregulation in the transcript level of the glycerol-3-phosphate phosphatase (G3PP) gene in the wild type under fludioxonil stress, indicating an increased production of glycerol. In $\Delta FgOS-2$, G3PP expression remained unchanged on fludioxonil compared with CM. These in vitro experiments demonstrated that proper response to oxidative, osmotic, and fungicide stress in *F. graminearum* depends on FgOS-2 function. This applies for both hyphal growth and conidia germination.

FgOS-2 is involved in sexual but not asexual reproduction.

F. graminearum is able to complete its entire life cycle in axenic culture. Perithecia formation, representing sexual reproduction, can be induced on wheat nodes (Guenther and Trail 2005) or on carrot agar (Leslie and Summerell 2006). The wild type produces perithecia on both substrates after 3 to 4 weeks of incubation (Supplementary Fig. S8A). Inside the perithecia are numerous asci with eight four-celled ascospores. In two independent $\Delta FgOS-2$ -mutant strains, the formation of perithecia was completely abolished. This indicates that FgOS-2 plays a major role in sexual reproduction. Interestingly, the deletion mutants and the wild type produced nearly equal amounts of conidia.

The deletion of *FgOS-2* leads to reduced virulence toward wheat and maize.

Deletion mutants of HOG1 homologues in *M. oryzae* and *B. cinerea* show opposite phenotypes regarding their pathogenic potential. Whereas *M. oryzae* strains remain fully virulent (Dixon et al. 1999), *B. cinerea* mutants fail to colonize unwounded plant tissues (Segmüller et al. 2007). In order to address this question in $\Delta FgOS-2$ strains, we performed infection

assays on both wheat heads and maize cobs using three independent $\Delta FgOS-2$ strains, one ectopic strain, the wild type, and water as a negative control. Maize infection was evaluated 35 days postinoculation (dpi) according to the disease severity rating (dsr) proposed by Reid and associates (1996). The wild-type strain almost infected the entire maize cob (Fig. 4A; dsr = 7), whereas the cobs inoculated with the mutant strains showed no or only marginal disease symptoms (Fig. 4A; dsr = 1). The maize cobs inoculated with water remained completely symptomless (data not shown). In order to determine whether or not FgOS-2 is also involved in pathogenicity toward wheat, we performed point-inoculation infection assays on the highly susceptible wheat cultivar Nandu. For this experiment, the wild type, three independent $\Delta FgOS-2$ strains, and one ectopic strain were assayed on 30 wheat heads. All infected wheat ears showed typical FHB disease symptoms (bleaching and reduced kernel formation) when infected with the wild-type strain (Fig. 4A) and the ectopic strain (data not shown). The infection symptoms became visible approximately 5 dpi, starting in the inoculated spikelet and propagating throughout the entire wheat head within 21 dpi, when the infection assay was finally evaluated (Fig. 4A). Spikes infected with the $FgOS-2$ deletion strains showed a severe reduction in disease symptoms during the infection assay. The infection never spread to the spikelets adjacent to the point-infected ones (Fig. 4B). In order to further analyze the infection process in planta, we constructed strains of one deletion mutant and the wild type that constitutively expressed the red fluorescent protein dsRed in the cytosol. Using these strains, we again infected wheat spikelets. After 5 dpi, the mycelium of the strain derived from the wild type was observed in the infected spikelet and, after 7 dpi, it was also observed in the adjacent spikelet (Fig. 5), indicating that the hyphae successfully crossed the transition zone

Fig. 1. Colony morphology of wild type (WT:PH1), ectopic (ECT), and *FgOS-2* deletion strain after 3 days of growth on osmotic stress media. The basic medium used was potato dextrose agar (PDA) inoculated with mycelial plugs from 3-day-old cultures. In order to test growth behavior, this medium was supplemented with the osmotic agents sorbitol (0.6 M), NaCl (0.3 M), and KCl (0.3 M). Growth of the mutant strain was slightly retarded on the PDA with no supplement and nearly abolished on osmotic media. The WT and ECT strains showed no growth inhibition on all media tested.

between the rachis node and the rachis, which was previously shown to be critical for the spread of infection to occur (Jansen et al. 2005; Voigt et al. 2007). After 5 dpi, disease symptoms and dsRed fluorescence were hardly detectable in the spikelets inoculated with the strain derived from the $\Delta FgOS-2$ strain (Fig. 5). At 7 dpi, the inoculated spikelet was partially colonized by the $\Delta FgOS-2$ dsRed-strain (Fig. 5). However, no fluorescence and, thus, no growth of this strain were detectable within or beyond the rachis node (Supplementary Fig. S9).

Secondary metabolism is controlled by FgOS-2.

When grown on CM plates, the colonies of the mutant strains produced more red pigmentation compared with the wild type, indicating an upregulation of aurofusarin production (Supplementary Fig. S10A). This observation was already made by Ochiai and co-workers (2007a). This result was verified by qRT-PCR analysis on genes involved in the regulation of aurofusarin production (Supplementary Tables S1 and S2). The expression of *gip1* (putative laccase gene) (Kim et al. 2005), *gip2* (transcription factor) (Kim et al. 2006), and *pks12* (type 1 polyketide synthase) (Kim et al. 2005; Malz et al. 2005) was drastically upregulated in the mutants compared with the wild type both in vitro and during wheat infection. In contrast to aurofusarin, DON production is important for full virulence (Proctor et al. 1995) toward wheat. Mutants defective in the key enzyme of trichothecene biosynthesis, Tri5, failed to cross the transition zone between the rachis and the rachis node and, thus, failed to propagate from one spikelet to the next (Jansen et al. 2005). Because the pathogenicity assay using $\Delta FgOS-2$ deletion strains revealed the same phenotype, it is feasible that the mutants were impaired in DON production during infection and in the axenic culture. In the present study, spikes inoculated with the wild type and the $\Delta FgOS-2$

mutant strains were tested for toxin production using a highly sensitive enzyme-linked immunosorbent assay (ELISA) test (Table 1). Spikelets inoculated with the deletion strains only accumulated approximately 9 and 2% of wild-type level of

Fig. 3. Colony morphology of wild type (WT:PH1) and *FgOS-2* deletion strain. Plate assay (4 days postinoculation) of the WT and $\Delta FgOS-2$ mutant strain on complete medium (CM) and CM supplemented with 20 mM H_2O_2 . The mutant grew better on plates supplemented with H_2O_2 .

Fig. 2. Conidia germination assay. **A**, Approximately 1,000 conidia of wild-type (WT) and *FgOS-2* deletion strains were plated onto potato dextrose agar (PDA) and PDA supplemented with NaCl (0.8 M). The number of germinated conidia was counted. The germination rate of mutant conidia was significantly reduced ($P = <0.001$, *t* test). Error bars indicate the standard deviation ($n = 6$). Experiments were performed in triplicate. **B**, Bright-field microscopy of germinating conidia on the osmotic medium (0.8 M NaCl). WT conidia formed multiple normally shaped germ tubes. The $\Delta FgOS-2$ germ tubes generally emerged from one conidial compartment and were malformed. **C**, Fluorescence microscopy of germinating conidia on PDA and PDA containing 0.8 M NaCl. Nuclei were stained with 4',6-diamidino-2-phenylindole. Germ tubes emerged from WT conidia on the osmotic medium (c) and from WT and mutant conidia on nonosmotic media (a and b), normally containing two to three nuclei. Malformed germ tubes of the $\Delta FgOS-2$ mutant contained numerous nuclei (d). Scale bar: 10 μm .

DON after 3 and 7 days, respectively, normalized to the amount of fungal material (Supplementary Fig. S11a). Interestingly, DON production was also reduced by approximately 60% on inoculated wheat kernels in the mutant strains compared with the wild-type strain (Table 1). In a third assay, in which DON

production was induced in liquid induction medium (Harris et al. 2007), the deletion strains, surprisingly, showed a relatively higher DON production compared with the wild type. After an incubation period of 1 day, the supernatant of the wild-type culture contained approximately 30% less DON than the mu-

Fig. 4. Pathogenicity assay on wheat and maize. **A**, Maize cobs (left) and wheat heads (right) were infected with conidia of wild-type (WT) and *FgOS-2* deletion strains and incubated for 35 (maize) and 21 (wheat) days. Mutant strains caused nearly no disease symptoms on maize and **B**, the infection of wheat stopped in the inoculated spikelet (arrow heads). WT infection caused symptoms typical for maize cob rot and Fusarium head blight disease. Maize infections were repeated 6 times and wheat infections were performed 30 times.

tant culture. After 3 days, the deletion mutants accumulated approximately 50% more DON than the wild type (Table 1).

The third secondary metabolite under investigation is ZEA. Also, ZEA biosynthesis in vitro was different from that in

planta. The ELISA analyses revealed no significant difference in the ZEA contents between the deletion strains and the wild type (Table 1) when grown in YES medium (Gaffoor et al. 2005). However, the production of ZEA was found to be significantly reduced to approximately 20% of the wild-type level in wheat heads inoculated with the mutant strains (Table 1). All toxin measurements were performed on three independent mutants and in triplicate and normalized to the amount of fungal material in the sample determined by qPCR (Voigt et al. 2007). The ELISA measurements were substantiated by expression analyses of genes related to toxin production. Surprisingly, although DON production was greater in the mutant strains in axenic culture, trichodiene synthase (*tri5*) and cytochrome P450 monooxygenase (*tri4*) gene expression was reduced compared with the wild type. However, expression of the regulatory gene *tri10* and transcription factor *tri6* was up-regulated in the mutant strains compared with the wild type. During plant infection (7 dpi), the expression of *tri4*, *tri5*, *tri6*, and *tri10* was reduced in the mutant strains compared with the wild type. Accordingly, genes involved in the regulation of ZEA production were screened by qRT-PCR. Although ZEA production in planta was significantly lower in the $\Delta FgOS-2$ -mutants, expression of the regulatory transcription factor (*zeb2*) was higher in the mutant compared with the wild type. However, expression of the isoamyl alcohol oxidase *zeal* and of the nonreducing polyketide synthase *zeal* was reduced in planta compared with the wild type. Under in vitro conditions, the expression of all of the genes was almost the same in both strains. To the best of our knowledge, these results describe, for the first time, the signaling cascade of ZEA production and underline the key role of FgOS-2 in the regulation of mycotoxin production in *F. graminearum* during plant infection. Furthermore, these results emphasize the differences in the regulation of mycotoxin biosynthesis between in vitro and in planta growth conditions.

Osmotic stress leads to a strongly increased oxidative burst in *FgOS-2*-mutants.

Previous studies showed that oxidative stress modulates DON production under in vitro conditions (Ponts et al. 2006). In order to determine whether or not FgOS-2 is involved in ROS metabolism, we performed nitroblue tetrazolium (NBT) staining of wild-type and $\Delta FgOS-2$ -mutant colonies grown on agar plates containing a mild osmotic stress medium (0.2 M NaCl). These experiments demonstrated a massive release of ROS in the deletion strains (Fig. 6A). Quantification using an ROS-sensitive ELISA showed an approximate 1.6-fold increase in H₂O₂ production in $\Delta FgOS-2$ mutants compared with the wild type under osmotic stress conditions (Fig. 6B). The higher H₂O₂ production might be due to either an enhanced production or a decreased capability for decomposition. Therefore, we tested the gene expression of H₂O₂-producing enzymes (i.e., NADPH oxidases [NOX]), in a semiquantitative reverse-transcription PCR. Expression levels of *noxA*, *noxB*, and the regulator gene *noxR* are nearly the same between $\Delta FgOS-2$ and the wild type in all conditions tested (H₂O₂,

Fig. 5. Infection assay. Cross-sections of inoculated wheat spikelets. Spikelets were infected with strains that constitutively expressed dsRed in the cytosol and that were derived from the wild type (WT) and a $\Delta FgOS-2$ -strain. Water was used as the control. Overview cross-section, including the inoculated spikelet and adjacent tissues (rachis node, rachis, and adjacent spikelets), 5 and 7 days postinoculation (dpi), respectively. Inoculation points are indicated with white asterisks. The $\Delta FgOS-2$ strain weakly infected the inoculated spikelet, beginning at the stigmatic hairs (arrowheads). Bright-field microscopy revealed small necrotic lesions at the infection site at 5 dpi and complete colonization of the caryopse at 7 dpi. The dsRed-expressing WT derivative strain showed complete necrosis of the infected spikelet after 5 dpi. DsRed-fluorescence is evenly distributed throughout the spikelet. Scale bar = 1 mm.

Table 1. Enzyme-linked immunosorbent assay analysis of mycotoxin production under different growth conditions in the wild type (WT) and $\Delta FgOS-2$ deletion strains^a

Strain	DON (mg kg ⁻¹ mycelium)					ZEA (µg kg ⁻¹ mycelium)		
	In vitro		Wheat kernels	In planta		In vitro		In planta
	1 dpi	3 dpi	7 dpi	3 dpi	7 dpi	1 dpi	3 dpi	7 dpi
WT:PH1	1.1 (±0.026)	3.4 (±0.012)	0.33 (±0.008)	0.4 (±0.0028)	4.66 (±0.0113)	0.19 (±0.0057)	0.92 (±0.0047)	1.34 (±0.0057)
$\Delta FgOS-2$	1.5 (±0.012)	5.2 (±0.0042)	0.14 (±0.002)	0.04 (±0.0374)	0.09 (±0.0284)	0.33 (±0.053)	0.78 (±0.011)	0.25 (±0.053)

^a All values were normalized against the amount of fungal material in the sample using quantitative polymerase chain reaction. DON = deoxynivalenol, ZEA = zearalenone, and dpi = days postinoculation.

NaCl, in planta) (Supplementary Fig. S12). Only the expression of a putative calcium-dependent NOX (*noxC*) was drastically downregulated in planta in the deletion strains at 7 dpi but was like the wild type under in vitro conditions. qRT-PCR verified this result and revealed a 218-fold downregulation of *noxC* expression (Fig. 7B) during plant infection. These results suggest that, under in vitro conditions, the regulation of *nox* gene expression is independent of FgOS-2. However, regulation might occur through regulation of enzyme activity. In order to determine how FgOS-2 is involved in regulation of ROS

Fig. 6. Reactive oxygen species (ROS) production assay in the wild type (WT:PH1) and $\Delta FgOS-2$ mutant strains. **A**, Nitroblue tetrazolium (NBT) staining for ROS production in cultures of the WT and the $\Delta FgOS-2$ mutant strain on complete medium (CM) and CM supplemented with 0.2 M NaCl after 3 days of growth. The dark color indicates ROS production. The mutant strain showed a greater production of ROS upon osmotic stress compared with the WT. **B**, Quantitative analysis of H₂O₂ production in both strains using samples obtained from NaCl-supplemented, submersed cultures (0.2 M). Amplex red peroxide/peroxidase assay (Life Technologies, Germany) revealed a higher level of H₂O₂ in the mutant strain under osmotic stress conditions. Error bars indicate the standard deviation ($n = 6$).

decomposition, we performed expression analysis and activity assays on fungal catalases. Under oxidative stress conditions induced by exogenous H₂O₂ (10 mM), catalase gene expression (Fig. 8B), enzyme activity (Fig. 8D), and *atf1* (Fig. 7A) gene expression was greater in the *FgOS-2* mutant strains than in the wild type. This might explain the increased growth observed for the mutant on agar plates supplemented with H₂O₂ (Fig. 3). However, expression of all catalase genes tested (*cat1*, *cat2.1*, *cat2.2*, and *cat3*) and of the putative transcriptional regulator of catalase gene expression, *atf1*, was strongly downregulated in planta and in the osmotic stress medium (0.8 M NaCl) in the *FgOS-2* deletion strains (Figs. 7A and 8A and C). In order to determine whether or not this would lead to a decrease of individual catalase enzymes activity, we used 0.8 M NaCl-stressed mycelium and measured catalase activity in a native polyacrylamide gel electrophoresis. A complete loss of FgCat2.2 activity and a drastic downregulation of Cat1 are clearly shown in Figure 8E. Also, the highly sensitive fluorometric catalase activity assay certified the overall decrease in catalase activity in the $\Delta FgOS-2$ mutants compared with the wild type (Fig. 8D). Thus, FgOS-2 is involved in ROS catabolism under salt stress. Interestingly, supplementation of CM containing 0.2 M NaCl with purified catalase at up to 50 $\mu\text{g ml}^{-1}$ partially restores the usually observed growth defect of the $\Delta FgOS-2$ mutant on this medium (Supplementary Fig. S13).

DISCUSSION

Functional characterization of the *S. cerevisiae* HOG1 orthologue in *F. graminearum*, FgOS-2, showed that this protein is a central regulator of all steps in the life cycle of this pathogen. It is involved in nearly all developmental processes, such as perithecia formation, oxidative and osmotic stress tolerance, fungicide resistance, virulence, and secondary metabolite production, including mycotoxins. In the following, we will develop the hypothesis that FgOS-2 regulates several of the diverse phenotypes by modulating ROS levels (Fig. 9).

Altered ROS metabolism in $\Delta FgOS-2$ influences spore development, germination, and mycelial growth under osmotic stress conditions.

The *FgOS-2* deletion strains grew better than the wild type on plates containing up to 20 mM H₂O₂ (Fig. 3). This was also observed in both *Epichloë festucae* SakA (Eaton et al. 2008) and *M. oryzae* *Osm1* deletion strains (Dixon et al. 1999). Lara-Rojas and co-workers (2011) found out that conidia but not mycelia of *A. nidulans* SakA deletion strains are sensitive toward oxidative stress. Analyses of catalase transcription levels and enzyme activities in *F. graminearum* cultures induced with H₂O₂ clearly showed a higher transcript level and enzyme activity of all catalases in the $\Delta FgOS-2$ mutants compared with the wild-type cultures (Fig. 8B and D). This upregulation might increase the ability of the mutants to decompose ROS and, thus, lead to the observed higher resistance to oxidative stress. On the other hand, catalase expression and activity was lower compared with the wild type in cultures induced with NaCl. The observed reduction in growth may be connected to the missing ROS-detoxification capabilities of the mutants due to the downregulated catalase genes. The NBT staining revealed a strong formation of ROS at the growing edges of the mutant colonies on agar containing NaCl (Fig. 6A) and the results of the ROS ELISA indicated an accumulation of ROS in the mutant strains under osmotic stress (Fig. 6B). Furthermore, addition of purified catalase to the medium partially restores the growth defect of the mutants under mild salt stress. However, because it was not possible to fully complement the growth defect, it seems likely that the growth defect is due to

pleiotropic effects of FgOS-2. Not only vegetative growth but also conidia formation is harmed under osmotic stress conditions. In the $\Delta FgOS-2$ mutant strains, we observed abnormal conidia germination under osmotic stress conditions, whereas

the wild type showed a normal germination pattern consisting of spore swelling, germination tube emergence and elongation from conidia, and hyphal branching (Seong et al. 2008). It is feasible that a defective phosphorelay system during conidia

Fig. 7. Gene expression analysis of **A**, the putative transcriptional regulator of reactive oxygen species (ROS) metabolic genes, *atf1*; and **B**, the putative calcium-responsive NADPH-oxidase *noxC* using cDNA obtained from inoculated wheat spikelets (7 days postinoculation), H_2O_2 -supplemented (10 mM), and NaCl-supplemented samples (0.8 M). Gene expression was assayed in the wild type (WT) and $\Delta FgOS-2$ mutant. The WT expression level was set at 1. The *atf1* and *noxC* gene expression in the $\Delta FgOS-2$ mutant strain was reduced during the infection of wheat and under salt stress and was massively induced in the samples supplemented with H_2O_2 . Error bars indicate the standard deviation. Quantitative real-time polymerase chain reaction was performed in triplicate.

Fig. 8. Catalase expression and activity assay. Quantitative real-time polymerase chain reaction (qRT-PCR) using cDNA obtained from **A**, inoculated wheat spikelets (7 dpi); **B**, H_2O_2 -supplemented samples (10 mM); and **C**, NaCl-supplemented samples (0.8 M). Expression of four different, putative catalase genes was assayed in the wild type (WT) and $\Delta FgOS-2$ mutant. The WT expression level was set at 1. Catalase expression in the $\Delta FgOS-2$ -mutant strain was reduced during the infection of wheat and in the medium supplemented with NaCl but not in the samples supplemented with H_2O_2 . Error bars indicate the standard deviation. qRT-PCR was performed in triplicate. **D**, Catalase activity assay using protein extract obtained from mycelium raised in complete medium (CM) with and without 2-h induction by 0.8 M NaCl and 10 mM H_2O_2 , respectively. Total catalase activity is reduced in the $\Delta FgOS-2$ mutant under no-stress and osmotic-stress conditions and elevated under oxidative stress conditions. The assay was performed using two biological and three technical replicates. **E**, Catalase activity staining using native protein extract from WT and mutant strain cultures supplemented with NaCl (0.8 M). Activity of all catalases was reduced in the mutant.

germination leads to this phenotype. In *Neurospora crassa* SakA, dephosphorylation is necessary in order to break spore dormancy and, thus, for the transition between non-growing and growing mycelia (Lara-Rojas et al. 2011). Furthermore, Egan and co-workers (2007) observed a significant accumulation of ROS in nongerminated conidia of *M. oryzae*. In germinating conidia, a tip-high gradient of ROS was observed. However, under normal growth conditions, *F. graminearum* genes involved in the oxidative stress response are slightly down-regulated during spore swelling, germination, and germ-tube formation (data obtained from supplemental material of Seong et al. 2008), which indicates that the germinating spore does not face imminent ROS stress. Our ROS measurements indicated increased H_2O_2 production under osmotic stress conditions in the deletion strains. This increase could interfere with physiological processes, leading to spore germination and germ tube formation. Thus, it is likely that the defective spore germination in the $\Delta FgOS-2$ strains is due to a disturbed ROS metabolism. In response to fludioxonil, conidial germination of the wild type is defective. However, the question whether this phenotype is connected to an abnormal ROS homeostasis remains open. It is assumed that fludioxonil triggers an abnor-

mal accumulation of glycerol via a fungicide-mediated deregulation of the osmoregulatory pathway (Pillonel and Meyer 1997). Furthermore, an *A. nidulans* microarray study published by Hagiwara and associates (2009) revealed a large number of genes simultaneously regulated by HogA and its putative downstream target, Atf1, after fludioxonil treatment. Among them was a gene encoding glycerol-3-phosphate dehydrogenase, an enzyme that is also involved in glycerol biosynthesis. We observed a massive upregulation of the gene encoding a G3PP in the wild type when exposed to fludioxonil; this enzyme catalyses the dephosphorylation of glycerol-3-phosphate to glycerol and phosphate. This hyper-accumulation of glycerol increases cellular turgor and leads to cell rupture. The $\Delta FgOS-2$ strains exhibit no upregulation of the G3PP gene, avoiding the hyper-accumulation of glycerol and allowing normal growth.

Expression of a putative calcium-responsive NOX is regulated by FgOS-2.

In *B. cinerea* (Segmüller et al. 2007) and *E. festucae* (Eaton et al. 2008), *nox* genes seem not to be transcriptionally regulated by the respective HOG1 homologues. However, in *A.*

Fig. 9. Proposed model: FgOS-2 orchestrates numerous physiological functions such as sexual reproduction, secondary metabolism, virulence, and stress response in *Fusarium graminearum*. FgOS-2 controls reactive oxygen species (ROS) production and degradation, presumably by regulating ROS metabolic enzymes such as catalases and NADPH oxidases.

Fig. 10. Hypothetical regulation network of reactive oxygen species (ROS) metabolic genes. The results highlight the central role of FgOS-2 and the putative downstream transcription factor Atf1 in the regulation of ROS metabolism. Conclusions: in the deletion mutant background under oxidative stress conditions, *atf1*, catalase, and *noxC* gene expressions are drastically upregulated. Under osmotic stress conditions, *atf1*, *noxC*, and catalase gene expression decreases. Finally, during in planta growth, the expression of catalases, *noxC*, and *atf1* is massively downregulated. Intriguingly, the regulation of other *nox* genes seemed to be independent of FgOS-2/Atf1 and regulated by an unknown factor. Bold arrows indicate the regulation pattern and narrow arrows indicate a putative signaling connection.

nidulans, the deletion of Sak1 led to the upregulation of *nox* genes (Lara-Ortiz et al. 2003). Our study also showed no drastic differences in the transcription of *noxA*, *noxB*, or the putative Nox-regulator p67phox (*noxR*) in the mutant background. Only a putative *nox* gene (*noxC*) with high homology to the calcium-responsive NOX protein Nox5 (Lambeth 2004) was strongly regulated in a FgOS-2-dependent manner (Fig. 7B). During in planta growth, *noxC* expression is almost abolished in the Δ FgOS-2 mutant but upregulated under oxidative stress conditions (Fig. 7B). The physiological function of NoxC in *F. graminearum* and its possible contribution to the in planta ROS level remains elusive because deletion of this gene leads to no obvious phenotype (T. V. Nguyen, W. Schäfer, and J. Bormann, unpublished data). In *F. graminearum*, the transcription level of the major *nox* genes (*noxA* and *B*) was not changed in the Δ FgOS-2 mutant strains compared with wild type; therefore, it is feasible that post-transcriptional regulation contributes to altered ROS production. This agrees with the finding in *E. festucae* that indicates a post-transcriptional regulation of Nox activity (Eaton et al. 2008).

FgOS-2 regulates catalase expression and activity through its putative downstream target Atf1.

In *A. nidulans*, the central transcriptional regulator Atf1/AtfA downstream of HOG1/SakA orchestrates the fungal response to oxidative stress, mainly by the positive regulation of catalase genes (Lara-Rojas et al. 2011). The Atf1 homologue of the biotrophic grass pathogen *Claviceps purpurea*, Tfl, is believed to act as a positive regulator of catalase genes and a repressor of *nox* genes (Nathues et al. 2004). Our results also indicate a FgOS-2-dependent regulation of catalase but not *noxA/B* gene expression through Atf1 (Figs. 6 and 9). However, as mentioned above, expression of the dispensable gene encoding NoxC (discussed above) is regulated by FgOS-2/Atf1 (Fig. 7B). An overview of the putative regulatory pathways is shown in Figure 10. Under oxidative stress conditions, deletion of *FgOS-2* leads to a massive upregulation of *atf1* expression. In consequence, catalase (and *noxC*) gene expression was higher in Δ FgOS-2 compared with the wild type. Thus, FgOS-2 seems to act as a repressor of *atf1* expression under oxidative stress conditions. Under osmotic stress conditions, catalase (and *noxC*) gene expression and activity was lower in Δ FgOS-2 compared with the wild type, although gene expression of *atf1* was only slightly reduced in the deletion strains (Fig. 7A). During the infection of wheat florets, the interplay between FgOS-2 and Atf1 seems to be most important: *atf1* gene expression is drastically reduced in the Δ FgOS-2 mutant strains in the course of infection. Accordingly, catalase (and *noxC*) gene expression was also reduced in the mutant strains but not in the wild type. Taken together, these results suggest that, in *F. graminearum*, Atf1 acts downstream of FgOS-2 and is mainly involved in regulation of ROS catabolism. Furthermore, Fgatf1 seems not to be a repressor of *noxA/B* gene expression.

Pathogenic development and perithecia formation of *F. graminearum* depends on tight regulation of ROS metabolism by FgOS-2.

Pathogenicity assays on wheat and maize revealed a drastically reduced virulence of FgOS-2-deficient strains compared with the wild type (Fig. 4). The HOG1 homologues in phytopathogenic fungi contribute to virulence at different degrees. Whereas the *M. oryzae* homologue Osm1 is dispensable for appressorium formation and pathogenicity toward rice, *B. cinerea* BcSak1 is necessary for the colonization of unwounded plant tissue (Dixon et al. 1999; Segmüller et al. 2007). *F. graminearum* OS-2 seems to be an “intermediate” regarding its im-

portance for pathogenic development on wheat: the protein is not needed for cuticle penetration or initial colonization of the wheat plant. However, infection is always restricted to the inoculated spikelet because it never proceeds beyond the rachis node. Deletion mutants of FgRrg1 also show reduced virulence on wheat (Jiang et al. 2011). The severely reduced virulence may also be caused by a disturbed ROS balance. Desmond and co-workers (2008) could show that DON provokes a release of ROS in wheat leaves which leads to programmed cell death (PCD). In general, ROS-induced PCD might facilitate necrotrophic fungal growth. Because the mutant produces less DON during wheat infection, this deficiency might prevent PCD and, thereby, further infection. The reduced virulence toward maize cannot be explained by this model because DON is no virulence factor for maize infection. Regarding the drastically reduced virulence of FgOS-2 deletion strains toward maize, the colonization process through the silk channel of maize cobs needs further histological and molecular evaluation. The tight regulation of the equilibrium between ROS generation and degradation facilitates normal host–pathogen interactions (Kim et al. 2009; Nathues et al. 2004; Tanaka et al. 2006). *E. festucae* mutants defective in ROS producing NOX 1 (NoxA) showed a drastic switch in lifestyle: instead of mutualistic symbiosis (wild type), the NoxA-deletion strains strongly affected the viability of colonized plants (Tanaka et al. 2006). The NoxA-deletion strains of *C. purpurea* showed severely reduced virulence toward rye (Giesbert et al. 2008). Gene expression analysis on infected wheat spikes revealed a downregulation of all catalase genes in the FgOS-2 deletion strains. This might indicate an altered ROS balance in the interaction between wheat and the mutant strains. In addition, the total loss of perithecia development in the FgOS2 mutants is in agreement with previous findings linking sexual development to the local formation of ROS. The deletion of the H₂O₂-producing NOX NoxA in *A. nidulans* led to reduced ROS production and the complete loss of fruiting body formation (Lara-Ortiz et al. 2003). NoxA deletion in other filamentous fungi such as *Podospira anserina* (Malagnac et al. 2004) and *N. crassa* (Cano-Dominguez et al. 2008) also blocked fruiting body development.

FgOS-2 is a central regulator of secondary metabolism in *F. graminearum*.

The reduced virulence phenotype of Δ FgOS-2 mutants very much resembles the pathogenic potential of DON-deficient mutants. The production of DON is a prerequisite for the progression of infection beyond a point-inoculated spikelet (Desjardins et al. 1996; Maier et al. 2006; Proctor et al. 1995; Seong et al. 2009). Because we observed a regulation of DON production by the HOG-signaling cascade and a severe downregulation of DON biosynthesis in Δ FgOS-2 mutants in planta, it is feasible that further infection might be blocked due to this observed downregulation of DON production in the mutant strains. Our study indicated an ambivalent role of FgOS-2 in the regulation of mycotoxin production. The production of DON in planta and on wheat kernels was drastically reduced. However, under in vitro liquid culture induction conditions (Harris et al. 2007), DON accumulation was higher than in the wild type. This latter result disagrees with a previous analysis of DON production in OS-2-deficient strains of *F. graminearum* (Ochiai et al. 2007a). The authors described a lack of DON production in aerial hyphae of mutants after being cultivated on a medium containing glucose and yeast extract. The DON quantification was performed using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). It is likely that the different experimental setup contributed to this disagreement. In our analysis, we took ad-

vantage of the highly sensitive DON ELISA technique instead of TLC. For the measurement of DON in a rice medium, which seems to be equivalent to the wheat kernel assay we used, Ochiai and co-workers (2007a) used an HPLC method. The results obtained in this experiment fit nicely with our findings of reduced DON contents in wheat kernels inoculated with OS-2-deficient strains. A reduced DON content in sterilized wheat kernels was also shown in mutants deficient in FgRrg1 (Jiang et al. 2011). We have to assume that DON production is regulated via different and independent signaling pathways. The activation of either pathway and the specific role of the HOG cascade in this regulatory network seem to depend on culture conditions. In our experimental conditions, FgOS-2 represses DON production in vitro, whereas it activates DON production in planta. Also, ZEA production is regulated by FgOS-2 in a growth-condition-dependent manner. Interestingly, however, only during wheat infection does FgOS-2 seem to be necessary for proper regulation of ZEA production. In vitro ZEA levels are nearly wild-type like. Demonstrating that the in vitro induction of DON and ZEA is different from in planta induction, our results highlight the necessity of searching for conditions that induce DON production during plant infection. There are good indications for a pivotal role of ROS in this regulatory process. H_2O_2 is a potent inducer of DON production in vitro (Ponts et al. 2006) and we could demonstrate that fungal secretion of H_2O_2 is altered in the $\Delta FgOS-2$ mutant depending on the growth conditions (Fig. 6). Of course, the situation during plant infection might be more sophisticated. As mentioned above, DON elicits PCD as a result of ROS production when injected into wheat leaves (Desmond et al. 2008). Because the *FgOS-2* mutant produces less DON during wheat infection, it is possible that the decrease in PCD-related ROS release leads to the missing DON induction in the mutant strain. Further microscopic analysis (e.g., by expression of a redox-sensitive green fluorescent protein variant) (Heller and Tudzynski 2011) will elucidate the ROS status in the interaction between *F. graminearum* and its hosts.

Jahn and co-workers (1997, 2000) showed that the loss of melanin production in different *Aspergillus* spp. led to an increased sensitivity to oxidative stress. The deletion of OS-2 in *F. graminearum* led to an increase in aurofusarin production compared with the wild type. We found a gene encoding a putative laccase involved in the biosynthesis of aurofusarin (*gip1*) (Frandsen et al. 2006) drastically upregulated in the mutant strain both in vitro and in planta. This laccase may exhibit anti-oxidant potential (Giardina et al. 2010; Thurston 1994). This might lead to a higher resistance to ROS and, thereby, facilitate better growth. Maybe acting antagonistically, aurofusarin was shown to inhibit the growth of fungal hyphae (Malz et al. 2005). Surprisingly, the expression of genes involved in aurofusarin production was also upregulated during in planta growth of the mutant when compared with the wild type; hence, the reduced growth rate of mutant strains on agar plates and in planta might also be due to this increased aurofusarin production.

Taken together, these results highlight the possibility that the diverse physiological functions controlled by FgOS-2 are executed through the regulation of ROS; these include perithecia formation, the production of secondary metabolites, and virulence.

MATERIALS AND METHODS

Fungal strains and culture conditions.

F. graminearum wild-type strain PH1 was used for this study. Conidia water suspensions were stored at -70°C . Co-

nidiation was induced on synthetic low-nutrient agar plates incubated for 2 weeks at 18°C under near-UV light and white light (both TL 40W-33 RS; Philips, Eindhoven, The Netherlands) with a 12-h photoperiod. Sexual reproduction was induced on carrot agar plates and detached wheat nodes on water agar plates (double-autoclaved wheat parts with a nodium in the middle). Conidia of the wild type and $\Delta FgOS-2$ strains were inoculated on carrot agar plates at 28°C in the dark. After 3 days, the aerial mycelia were knocked down with 1 ml of sterile 2.5% Tween 60 solution, using a sterile glass rod. The carrot agar plates and wheat nodes on water agar plates were further incubated at 18°C under near-UV light and white light with a 12-h photoperiod for 7 days. In order to examine whether or not the perithecia that developed contained asci with spores, the perithecia were excised using a knife and placed on objective slides. They were then crushed between the slide and a cover slip and analyzed for asci and ascospores under a microscope. The growth rates of the wild type and several independent *FgOS-2* mutant strains were determined by inoculating 5-mm mycelial plugs taken from the edge of a 3-day-old colony on CM (1 liter of CM contained 10 ml of solution A [$\text{Ca}(\text{NO}_3)_2 \times 4\text{H}_2\text{O}$ at 100 g liter^{-1}], 10 ml of solution B [KH_2PO_4 at 20 g liter^{-1} , $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ at 25 g liter^{-1} , and NaCl at 10 g liter^{-1} , sterilized by filtration], 10 g of glucose, 1 ml of suspension D [H_3BO_3 at 60 g liter^{-1} , $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ at $390 \text{ mg liter}^{-1}$, KI at 13 mg liter^{-1} , $\text{MnSO}_4 \times \text{H}_2\text{O}$ at 60 mg liter^{-1} , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \times 4\text{H}_2\text{O}$ at 51 mg liter^{-1} , $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ at $5.48 \text{ g liter}^{-1}$, and $\text{FeCl}_3 \times 6 \text{H}_2\text{O}$ at $932 \text{ mg liter}^{-1}$], 1 g of yeast extract, 0.5 g of enzymatically hydrolyzed casein, and 0.5 g of acid-hydrolyzed casein) or PDA plates. In order to test the effect of several chemical substances on the growth performance of the wild-type and mutant strains, the plates were supplemented with the agents D-sorbitol, NaCl , KCl , H_2O_2 , fludioxonil, iprodione, and azoxystrobin at the concentrations indicated in the figure legends and Results section. The plates were inoculated at 28°C for at least 3 days in the dark. For temperature stress conditions, the plates were incubated at 18, 30, and 32°C . The diameter of the mycelial colonies was measured using a technical ruler or scale. The analyses were performed on at least three independent mutants with at least five replicates. For RNA isolation, the strains were cultured in liquid CM for 3 days. Mycelia were harvested using a sieve ($200 \mu\text{m}$) and washed with distilled water several times. Equal portions of mycelia were shifted in new liquid media containing NaCl , H_2O_2 , or fludioxonil in the concentrations shown in figure legends and in the Results section. Mycelia harvested from the toxin induction media (discussed below) were also used for the RNA isolation analysis.

Vector construction.

Standard recombinant DNA methods were performed according to Ausubel and associates (2002) and Sambrook and associates (1989). A double homologous recombination construct was generated for the disruption of *FgOS-2* in the *F. graminearum* strain PH1. As flanking regions, 1.34-kb upstream and 1.32-kb downstream fragments of *FgOS-2* were cloned by PCR from genomic DNA. The PCR was initiated by denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 60 s. The PCR included a final extension step at 72°C for 10 min and a cooling step at 4°C . The replacement vector was constructed using fusion PCR. The PCR amplification was performed using the following parameters: initial denaturation at 94°C for 4 min; followed by 20 cycles of 94°C for 45 s, 58°C for 2 min, and 72°C for 4 min; and a final extension step at 72°C for 10 min. The upstream and downstream fragments (200 ng each), together with the hygromycin (*HPH*) cassette (1.8 kb) released from

the pGEM-HPH vector (A. Lösch, University of Hamburg, Germany) by digestion (400 ng), were used in a 25- μ l PCR without the addition of primers because the fragments acted as primers and templates for the elongation. The PCR product was ligated into the pGEM-T vector (Promega Corp., Madison, WI) to generate pGEM-FgOS-2-HPH; this was then transformed into XL1-blue *Escherichia coli*-competent cells (DNA Cloning Services; Biozentrum Klein Flottbek, Hamburg, Germany). Bacteria were cultivated in sterile Luria-Bertani medium (Sambrook et al. 1989) either as a liquid culture or on agar plates. Vector p99II::dsRed was used for the generation of dsRed-fluorescent strains (Ilgen et al. 2009). This vector facilitates the constitutive expression of dsRed under the control of the *gpdA*-promoter of *A. nidulans* and confers geneticin resistance.

Transformation of *F. graminearum*.

For the fungal transformation, pGEM-FgOS-2-HPH was digested with *Xba*I to release the disruption construct. A solution of 30 to 50 μ l containing 10 to 30 μ g of DNA was used for the transformation. The protoplast transformation method was performed as previously described (Jansen et al. 2005; Proctor et al. 1995), where 100 ml of YEPD medium (0.3% yeast extract, 1% bacto peptone, and 2% D-glucose) was inoculated with 1×10^6 conidia and incubated overnight at 28°C and 150 rpm. The mycelia were collected by filtering with a 200- μ m-diameter sieve or Miracloth and washed by rinsing three times with double-distilled water. Then, 0.5 to 2 g of mycelia were resuspended in a 20-ml mixture of driselase and lysing enzymes (2.5%: 0.5% in 0.6 M KCl) (Life Technologies, Darmstadt, Germany) and digested for 2 to 3 h at 30°C and 80 rpm. Undigested hyphal material was removed from the protoplast suspension by subsequent filtration, first through a 100- μ m Wilson sieve and then through a 40- μ m Wilson sieve or three layers of cheesecloth. The protoplasts were pelleted at room temperature by centrifugation for 10 min at 2,000 rpm, washed once with 10 ml STC (20% sucrose, 10 mM Tris-HCl [pH 8.0], and 50 mM CaCl_2), centrifuged again, and then resuspended and adjusted in STC at 1×10^8 protoplasts/ml. For transformation, 200 μ l of the protoplast suspension was supplemented with 10 μ g of plasmid DNA. The samples were mixed and incubated at room temperature for 20 min. Following this, 1 ml of PEG (40% polyethylene glycol 4000 and 60% STC) was added and incubated at room temperature for 20 min. The protoplast suspension was added to 5 ml of TB3 medium (100 g of sucrose, 0.3% yeast extract, and 0.3% casamino acids) and shaken overnight at room temperature and 100 rpm for cell wall regeneration. After 12 to 24 h, the regenerated protoplasts were collected by centrifugation at 5,000 rpm for 10 min at room temperature, and 5 ml of TB3 was removed. The regenerated protoplasts were mixed in 1.5% TB3 agar at 50°C with hygromycin B (250 μ g/ml) and plated out in 596-mm petri dishes (10 ml/plate). After 12 to 24 h, the plates were overlaid with the same amount of 1.5% TB3 agar and double the amount of hygromycin B as used the day before. Putative transformants were obtained after 2 days posttransformation. They were transferred to fresh plates of CM medium supplemented with hygromycin B at 250 μ g/ml and incubated at 28°C. Several transformants were purified by single-spore isolation and subsequently checked by diagnostic PCR.

Southern blot analysis.

Genomic DNA of the wild-type and Δ FgOS-2 strains was isolated and restricted overnight using the restriction enzyme *Hind*III. The digested DNA was then separated on 0.8% agarose gel by electrophoresis at 70 V for 6 to 7 h. Then, the DNA was transferred by capillary blotting onto a Hybond NX mem-

brane (Amersham Biosciences, Little Chalfont, U.K.) and then hybridized with a digoxigenin-labeled (Roche, Grenzach-Wyhlen, Germany) DNA internal probe, which was specifically amplified by PCR. Detection and visualization procedures were carried out following the manufacturer's manual (Roche). In order to confirm single-copy integration of the hygromycin cassette, rehybridization with a hygromycin probe was performed.

Virulence assays on wheat and maize.

The susceptible spring wheat cultivar Nandu (Lochow-Petkus, Bergen-Wohlde, Germany) was cultivated in a greenhouse at 18 to 20°C, 60% relative humidity, and a photoperiod of 16 h, then transferred to infection chambers with optimized conditions. A suspension of 200 conidia was inoculated into each of two central spikelets at the early stages of anthesis (Jenczmionka and Schäfer 2005). The inoculated spikes were enclosed in small plastic bags misted with water for the first 3 days and then they were monitored for up to 3 weeks in the infection chambers. Inoculation with 20 μ l of pure water was used as the negative control. The wheat infection assay was performed using the wild type, three independent deletion strains, and one ectopic strain with 10 replicates each. The maize inbred line A188 (Green and Phillips 1975) was grown in the greenhouse (temperature, 26 to 30°C; humidity, 70 to 85%; natural daily photoperiod with additional artificial light when required). Before inoculation, the silks were manually pollinated to ensure optimal pollination. Each maize cob was inoculated by injecting conidia suspensions into the silk channel of primary ears using a syringe and cannula (Reid et al. 1995). The infection procedure was performed with 4 ml of conidial suspension at a concentration of 2×10^4 conidia/ml of the wild type and three independent deletion strains, respectively, and with two replicates each. Maize cobs inoculated with 4 ml of pure water were used for the negative control. The inoculated cobs were enclosed in plastic bags for the first 3 days. Infection was finally evaluated after 5 weeks of incubation.

DON and ZEA production analysis.

In vitro DON production was measured using 5×10^3 conidia of the wild type and three independent Δ FgOS-2 strains cultured in yeast-peptone-dextrose medium for 3 days. The mycelia were collected by filtering with a 200- μ m-diameter sieve or Miracloth, were washed by rinsing at least three times with double-distilled water, and were then dried on sterile Whatman paper. Then, 1 g of semi-dry mycelia were further incubated for 24 and 72 h in minimal medium (CM but without solution A, yeast extract, and casein) supplemented with 5 mM $(\text{NH}_4)_2\text{SO}_4$. Each supernatant (50 μ l) was taken for DON measurement using a highly sensitive DON ELISA technique (RIDAscreen DON kits; R-Biopharm AG, Darmstadt, Germany). For the in planta analysis, four wheat spikes were each inoculated with wild-type and Δ FgOS-2 strains, respectively. The spikes were inoculated with pure water as a negative control. The inoculated samples were collected after 3 or 7 dpi and 200 mg of the infected spikelets were dried in a vacuum and ground to a powder under liquid nitrogen. Then, 50 mg of ground material was suspended in 500 μ l of distilled water. The extract was manually mixed by hand or vortex and centrifuged. The DON quantification assay used 50 μ l of the supernatant. The production of DON on the wheat kernels was analyzed as follows. First, 15 g of kernels and 10 ml of water were autoclaved in a 300-ml Erlenmeyer flask. Autoclaved kernels were inoculated with 2×10^4 conidia of the wild-type strain and the Δ FgOS-2 mutant. Water-inoculated samples served as the negative control. The samples were incubated out at 28°C

in the dark for 7 days. After 7 days, the inoculated wheat kernels were treated as described earlier for the in planta DON assay. The production of DON was measured following the manufacturer's instructions. All measurements were subsequently normalized to the amount of mycelium in the sample using qPCR, as previously described (Voigt et al. 2007). The sample preparation procedure for the in planta and in vitro ZEA measurements was similar to that for DON measurements, except that YES medium (2% yeast extract, 1.5% sucrose, 0.05% $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 1 ml of trace metal solution, and 885 ml of Milli Q water [pH 6.5]) was used for in vitro ZEA induction. Quantification was performed according to the manual contained in the RIDAscreen ZEA kit (R-Biopharm AG).

ROS measurement.

The production of ROS was determined via two independent methods. First, O_2^- production in whole fungal colonies was detected by staining with NBT (Sigma, Seelze, Germany). Wild-type and three independent $\Delta FgOS-2$ strains were cultured on CM agar plates, which were supplemented with 0.2 M NaCl at 28°C in the dark for 3 days. Then, each plate was stained with 20 ml of 0.2% (wt/vol) NBT solution (NBT diluted in water) and incubated in the dark at 28°C for 30 to 45 min. The supernatant was drained and the plates were washed with 99% ethanol. The plates were reincubated for 30 to 45 min in the dark at 28°C before images of whole colonies were taken.

The production of ROS was also measured using a sensitive ELISA-based assay. In order to detect the release of H_2O_2 , 40 mg of dry mycelia were incubated in a reaction mixture (50 μM Amplex red reagent and HRP at 0.1 U/ml in Krebs-Ringer phosphate buffer [KRPB] consisting of 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl_2 , 1.22 mM MgSO_4 , and 5.5 mM glucose [pH 7.35]). In order to detect the release of H_2O_2 in the osmotic medium, KRPB was supplemented with 0.2 M NaCl. The in vitro levels of H_2O_2 were measured using the Amplex red peroxide/peroxidase assay kit (Life Technologies), according to the manufacturer's instructions. Extinction at 530 to 560 nm was measured using the Fluoroskan II fluorescence 96-well microplate reader (Synergy HT; Biotek, Bad Friedrichshall, Germany).

Protein extraction from ground mycelia of the wild-type and mutant strains was performed using potassium phosphate buffer. Catalase activity staining was performed after native isoelectric focusing of the protein extracts following the protocol of Robertson and associates (1987): 7 ml of water, 2 ml of acrylamide mixture (30% [wt/vol] acrylamide and 1% [wt/vol] bis-acrylamide), 2.4 ml of 50% (vol/vol) glycerol, and 0.6 ml of ampholyte (pH 3 to 10). The components were mixed and degassed. Then, 50 μl of 10% (wt/vol) ammonium persulphate and 20 μl of tetramethylethylenediamine (TEMED) were added. The cathode solution was 25 mM NaOH and the anode solution was 20 mM acetic acid. Up to 40 μg of each sample was loaded and electrophoresed for 2 h at 100 V, 10 to 13 h at 200 V, and 2 h at 500 V. The gels were stained for catalase activity using diaminobenzidine (DAB) as follows (Clare et al. 1984). Each gel was incubated in horseradish peroxidase (50 pg ml^{-1}) in phosphate buffer (50 mM potassium phosphate, pH 7.0) for 45 min. Then, H_2O_2 was added at a concentration of 5.0 mM and the mixture was incubated for a further 10 min. The gel was rinsed twice with water and placed in DAB (in phosphate buffer) at 0.5 mg ml^{-1} until the staining became visible. Total catalase activity was measured using the Amplex red catalase assay kit (Life Technologies) according to the manufacturer's instructions. Catalase activity was normalized to the amount of total protein content determined by Bradford Reagent (Sigma).

DAPI nuclei staining.

To stain the nuclei, wild-type and $\Delta FgOS-2$ strains were cultured in a liquid potato dextrose (PD) medium and a PD medium supplemented with 0.8 M NaCl for 16 h, then centrifuged for 15 min at 13,000 rpm. The pellets were rinsed and diluted again with distilled water prior to staining. Samples were placed on glass slides and stained with DAPI at 1 $\mu\text{g ml}^{-1}$ for 10 min before being analyzed under a Zeiss microscope Axio Imager Z1 (Carl Zeiss, Germany) equipped with filter sets for excitation at 538 to 562 nm and emission wavelength detection from 570 to 640 nm.

Expression analysis by qRT-PCR.

In order to study the expression of possible targets of OS-2 in *F. graminearum* in vitro and in planta, RNA was isolated using a Nucleospin RNA II Kit (Macherey-Nagel, Düren, Germany). Samples were collected from in vitro submersed cultures and from wheat spikes at 3 and 7 dpi, respectively, and frozen in liquid nitrogen for subsequent RNA isolation. For RT-PCR, SuperScript II RNase H⁻ Reverse Transcriptase (Life Technologies) was used, according to the manufacturer's instructions. The resulting single-stranded cDNA was later used as a template for qRT-PCR tests. Transcript levels of the target genes were normalized against tubulin gene expression. The qRT-PCR tests were carried out using gene-specific primers, with SYBR Green qPCR SuperMix-UDG (Fermentas, St. Leon-Rot, Germany) in a volume of 20 μl in a light Cycler 480 (Roche). The PCR program was as follows: incubation for 2 min at 50°C, then 2 min at 95°C, followed by up to 40 cycles of denaturation at 94°C for 30 s, annealing at 55 to 58°C for 30 s, and extension at 72°C for 15 s, followed by a melting curve analysis in order to check the specificity of fragment amplification. All the measurements were repeated twice, each with three replicates and using at least two independent deletion strains and the wild type. Relative changes in gene expression were calculated using the comparative cycle threshold method (using LC 480 software for the light Cycler 480).

ACKNOWLEDGMENTS

We thank B. Haderl and C. Kröger for technical assistance, M. Boenisch for assistance with the microscopy, B. Doormann for critical reading of the manuscript, and Hong Duc University, Vietnam, for their support and cooperation.

LITERATURE CITED

- Ádám, A. L., Kohut, G., and Hornok, L. 2008. Fphog1, a HOG-type MAP kinase gene, is involved in multistress response in *Fusarium proliferatum*. J. Basic Microbiol. 48:151-159.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 2002. Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology. Wiley, New York.
- Cano-Dominguez, N., Alvarez-Delfin, K., Hansberg, W., and Aguirre, J. 2008. NADPH oxidases NOX-1 and NOX-2 require the regulatory subunit NOR-1 to control cell differentiation and growth in *Neurospora crassa*. Eukaryot. Cell 7:1352-1361.
- Clare, D. A., Minh Ngoc, D., Darr, D., Archibald, F., and Fridovich, I. 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. 140:532-537.
- Desjardins, A. E., Proctor, R. H., Bai, G., McCormick, S. P., Shaner, G., Buechley, G., and Hohn, T. M. 1996. Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Mol. Plant-Microbe Interact. 9:775-781.
- Desmond, O. J., Manners, J. M., Stephens, A. E., Maclean, D. J., Schenk, P. M., Gardiner, D. M., Munn, A. L., and Kazan, K. 2008. The *Fusarium* mycotoxin deoxynivalenol elicits hydrogen peroxide production, programmed cell death and defence responses in wheat. Mol. Plant Pathol. 9:435-445.

- Dixon, K. P., Xu, J. R., Smirnov, N., and Talbot, N. J. 1999. Independent signaling pathways regulate cellular turgor during hyperosmotic stress and appressorium-mediated plant infection by *Magnaporthe grisea*. *Plant Cell* 11:2045-2058.
- Eaton, C., Jourdain, I., Foster, S., Hyams, J., and Scott, B. 2008. Functional analysis of a fungal endophyte stress-activated MAP kinase. *Curr. Genet.* 53:163-174.
- Egan, M. J., Wang, Z. Y., Jones, M. A., Smirnov, N., and Talbot, N. J. 2007. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. *Proc. Natl. Acad. Sci. U.S.A.* 104:11772-11777.
- Frandsen, R. J. N., Nielsen, N. J., Maolanon, N., Sørensen, J. C., Olsson, S., Nielsen, J., and Giese, H. 2006. The biosynthetic pathway for aurofusarin in *Fusarium graminearum* reveals a close link between the naphthoquinones and naphthopyrones. *Mol. Microbiol.* 61:1069-1080.
- Gaffoor, I., Brown, D. W., Plattner, R., Proctor, R. H., Qi, W., and Trail, F. 2005. Functional analysis of the polyketide synthase genes in the filamentous fungus *Gibberella zeae* (anamorph *Fusarium graminearum*). *Eukaryot. Cell* 4:1926-1933.
- Gardiner, D. M., Kazan, K., and Manners, J. M. 2009a. Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genet. Biol.* 46:604-613.
- Gardiner, D. M., Osborne, S., Kazan, K., and Manners, J. M. 2009b. Low pH regulates the production of deoxynivalenol by *Fusarium graminearum*. *Microbiology* 155:3149-3156.
- Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S., and Sannia, G. 2010. Laccases: A never-ending story. *Cell. Mol. Life Sci.* 67:369-385.
- Giesbert, S., Schürg, T., Scheele, S., and Tudzynski, P. 2008. The NADPH oxidase Cpx1 is required for full pathogenicity of the ergot fungus *Claviceps purpurea*. *Mol. Plant Pathol.* 9:317-327.
- Goswami, R. S., and Kistler, H. C. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Mol. Plant Pathol.* 5:515-525.
- Green, C. E., and Phillips, R. L. 1975. Plant regeneration from tissue cultures of maize. *Crop Sci.* 15:417-421.
- Guenther, J. C., and Trail, F. 2005. The development and differentiation of *Gibberella zeae* (anamorph: *Fusarium graminearum*) during colonization of wheat. *Mycologia* 97:229-237.
- Hagiwara, D., Asano, Y., Marui, J., Yoshimi, A., Mizuno, T., and Abe, K. 2009. Transcriptional profiling for *Aspergillus nidulans* HogA MAPK signaling pathway in response to fludioxonil and osmotic stress. *Fungal Genet. Biol.* 46:868-878.
- Harris, L. J., Alexander, N. J., Saparno, A., Blackwell, B., McCormick, S. P., Desjardins, A. E., Robert, L. S., Tinker, N., Hattori, J., and Piche, C. 2007. A novel gene cluster in *Fusarium graminearum* contains a gene that contributes to butenolide synthesis. *Fungal Genet. Biol.* 44:293-306.
- Heller, J., and Tudzynski, P. 2011. Reactive oxygen species in phytopathogenic fungi: Signaling, development, and disease. *Annu. Rev. Phytopathol.* 49:369-390.
- Ilgen, P., Hader, B., Maier, F. J., and Schafer, W. 2009. Developing kernel and rachis node induce the trichothecene pathway of *Fusarium graminearum* during wheat head infection. *Mol. Plant-Microbe Interact.* 22:899-908.
- Jahn, B., Koch, A., Schmidt, A., Wanner, G., Gehring, H., Bhakdi, S., and Brakhage, A. A. 1997. Isolation and characterization of a pigmentless-conidium mutant of *Aspergillus fumigatus* with altered conidial surface and reduced virulence. *Infect. Immun.* 65:5110.
- Jahn, B., Boukhallouk, F., Lotz, J., Langfelder, K., Wanner, G., and Brakhage, A. A. 2000. Interaction of human phagocytes with pigmentless *Aspergillus* conidia. *Infect. Immun.* 68:3736.
- Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K. H., Felk, A., and Maier, F. J. 2005. Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl. Acad. Sci. U.S.A.* 102:16892-16897.
- Jenczmionka, N. J., and Schäfer, W. 2005. The Gpmk1 MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. *Curr. Genet.* 47:29-36.
- Jiang, J., Yun, Y., Fu, J., Shim, W.-B., and Ma, Z. 2011. Involvement of a putative response regulator FgRrg-1 in osmotic stress response, fungicide resistance and virulence in *Fusarium graminearum*. *Mol. Plant Pathol.* 12:425-436.
- Jiao, F., Kawakami, A., and Nakajima, T. 2008. Effects of different carbon sources on trichothecene production and Tri gene expression by *Fusarium graminearum* in liquid culture. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 285:212-219.
- Kawasaki, L., Sánchez, O., Shiozaki, K., and Aguirre, J. 2002. SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. *Mol. Microbiol.* 45:1153-1163.
- Kim, J. E., Han, K. H., Jin, J., Kim, H., Kim, J. C., Yun, S. H., and Lee, Y. W. 2005. Putative polyketide synthase and laccase genes for biosynthesis of aurofusarin in *Gibberella zeae*. *Appl. Environ. Microbiol.* 71:1701-1708.
- 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.
- Kim, K.-H., Willger, S. D., Park, S.-W., Puttikamonkul, S., Grahl, N., Cho, Y., Mukhopadhyay, B., Cramer, R. A., Jr., and Lawrence, C. B. 2009. TmpL, a transmembrane protein required for intracellular redox homeostasis and virulence in a plant and an animal fungal pathogen. *PLoS Pathog.* 5:e1000653. Published online.
- Kojima, K., Takano, Y., Yoshimi, A., Tanaka, C., Kikuchi, T., and Okuno, T. 2004. Fungicide activity through activation of a fungal signalling pathway. *Mol. Microbiol.* 53:1785-1796.
- Lambeth, J. D. 2004. NOX enzymes and the biology of reactive oxygen. *Nat. Rev. Immunol.* 4:181-189.
- Lara-Ortiz, T., Riveros-Rosas, H., and Aguirre, J. 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. *Mol. Microbiol.* 50:1241-1255.
- Lara-Rojas, F., Sánchez, O., Kawasaki, L., and Aguirre, J. 2011. *Aspergillus nidulans* transcription factor AtfA interacts with the MAPK SakA to regulate general stress responses, development and spore functions. *Mol. Microbiol.* 80:436-454.
- Leslie, J. F., and Summerell, B. A. 2006. The *Fusarium* Laboratory Manual. Blackwell Publishing, Ames, IA, U.S.A.
- Li, S., Ault, A., Malone, C. L., Raitt, D., Dean, S., Johnston, L. H., Deschenes, R. J., and Fassler, J. S. 1998. The yeast histidine protein kinase, Sln1p, mediates phosphotransfer to two response regulators, Ssk1p and Skn7p. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:6952-6962.
- Liu, W., Leroux, P., and Fillinger, S. 2008. The HOG1-like MAP kinase Sak1 of *Botrytis cinerea* is negatively regulated by the upstream histidine kinase Bos1 and is not involved in dicarboximide- and phenylpyrrole-resistance. *Fungal Genet. Biol.* 45:1062-1074.
- Maier, F. J., Miedaner, T., Hader, B., Felk, A., Salomon, S., Lemmens, M., Kassner, H., and Schafer, W. 2006. Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. *Mol. Plant Pathol.* 7:449-461.
- Malagnac, F., Lalucque, H., Lepere, G., and Silar, P. 2004. Two NADPH oxidase isoforms are required for sexual reproduction and ascospore germination in the filamentous fungus *Podospora anserina*. *Fungal Genet. Biol.* 41:982-997.
- Malz, S., Grell, M. N., Thrane, C., Maier, F. J., Rosager, P., Felk, A., Albertsen, K. S., Salomon, S., Bohn, L., Schäfer, W., and Giese, H. 2005. Identification of a gene cluster responsible for the biosynthesis of aurofusarin in the *Fusarium graminearum* species complex. *Fungal Genet. Biol.* 42:420-433.
- McMullen, M., Jones, R., and Gallenberg, D. 1997. Scab of wheat and barley: A re-emerging disease of devastating impact. *Plant Dis.* 81:1340-1348.
- Nathues, E., Joshi, S., Tenberge, K. B., von den Driesch, M., Oeser, B., Baumer, N., Mihan, M., and Tudzynski, P. 2004. CPTF1, a CREB-like transcription factor, is involved in the oxidative stress response in the phytopathogen *Claviceps purpurea* and modulates ROS level in its host *Secale cereale*. *Mol. Plant-Microbe Interact.* 17:383-393.
- Ochiai, N., Tokai, T., Nishiuchi, T., Takahashi-Ando, N., Fujimura, M., and Kimura, M. 2007a. Involvement of the osmosensor histidine kinase and osmotic stress-activated protein kinases in the regulation of secondary metabolism in *Fusarium graminearum*. *Biochem. Biophys. Res. Commun.* 363:639-644.
- Ochiai, N., Tokai, T., Takahashi-Ando, N., Fujimura, M., and Kimura, M. 2007b. Genetically engineered *Fusarium* as a tool to evaluate the effects of environmental factors on initiation of trichothecene biosynthesis. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 275:53-61.
- O'Rourke, S. M., Herskowitz, I., and O'Shea, E. K. 2002. Yeast go the whole HOG for the hyperosmotic response. *Trends Genet.* 18:405-412.
- Pillonel, C., and Meyer, T. 1997. Effect of phenylpyrroles on glycerol accumulation and protein kinase activity of *Neurospora crassa*. *Pestic. Sci.* 49:229-236.
- Ponts, N., Pinson Gadais, L., Verdal Bonnin, M. N., Barreau, C., and Richard-Forget, F. 2006. Accumulation of deoxynivalenol and its 15 acetylated form is significantly modulated by oxidative stress in liquid cultures of *Fusarium graminearum*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 258:102-107.
- Posas, F., Wurgler-Murphy, S. M., Maeda, T., Witten, E. A., Thai, T. C., and Saito, H. 1996. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell* 86:865-875.
- 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.
- Reid, L. M., Hamilton, R. I., and Mather, D. E. 1995. Effect of macroconidial suspension volume and concentration on expression of resistance to *Fusarium graminearum* in maize. *Plant Dis.* 79:461-466.
- Reid, L. M., Canada, A., and Agri-Food Canada, Research Branch. 1996. Screening Maize for Resistance to *Gibberella* Ear Rot. Agriculture and Agri-Food Canada, Research Branch, Ottawa, Canada.
- Robertson, E. F., Dannelly, H. K., Malloy, P. J., and Reeves, H. C. 1987. Rapid isoelectric focusing in a vertical polyacrylamide minigel system. *Anal. Biochem.* 167:290-294.
- Sambrook, J., Russell, D. W., Janssen, K., and Argentine, J. 1989. Molecular Cloning: A Laboratory Manual on the Web. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Segmüller, N., Ellendorf, U., Tudzynski, B., and Tudzynski, P. 2007. BcSAK1, a stress-activated mitogen-activated protein kinase, is involved in vegetative differentiation and pathogenicity in *Botrytis cinerea*. *Eukaryot. Cell* 6:211-221.
- Seong, K. Y., Zhao, X., Xu, J. R., Guldener, U., and Kistler, H. C. 2008. Conidial germination in the filamentous fungus *Fusarium graminearum*. *Fungal Genet. Biol.* 45:389-399.
- Seong, K. Y., Pasquali, M., Zhou, X., Song, J., Hilburn, K., McCormick, S., Dong, Y., Xu, J. R., and Kistler, H. C. 2009. Global gene regulation by *Fusarium* transcription factors Tri6 and Tri10 reveals adaptations for toxin biosynthesis. *Mol. Microbiol.* 72:354-367.
- Sutton, J. C. 1982. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Can. J. Plant Pathol.* 4:195-209.
- Tanaka, A., Christensen, M. J., Takemoto, D., Park, P., and Scott, B. 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell* 18:1052-1066.
- Thurston, C. F. 1994. The structure and function of fungal laccases. *Microbiology* 140:19-26.
- Tsuyuki, R., Yoshinari, T., Sakamoto, N., Nagasawa, H., and Sakuda, S. 2011. Enhancement of trichothecene production in *Fusarium graminearum* by cobalt chloride. *J. Agric. Food Chem.* 59:1760-1766.
- Voigt, C., von Scheidt, B., Gácsér, A., Kassner, H., Lieberei, R., Schäfer, W., and Salomon, S. 2007. Enhanced mycotoxin production of a lipase-deficient *Fusarium graminearum* mutant correlates to toxin-related gene expression. *Eur. J. Plant Pathol.* 117:1-12.
- Xu, J. R. 2000. MAP kinases in fungal pathogens. *Fungal Genet. Biol.* 31:137-152.