The ATF/CREB Transcription Factor Atf1 Is Essential for Full Virulence, Deoxynivalenol Production, and Stress Tolerance in the Cereal Pathogen *Fusarium graminearum*

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Fusarium graminearum is a necrotrophic plant pathogen of cereals that produces mycotoxins such as deoxynivalenol (DON) and zearalenone (ZEA) in grains. The stress-activated mitogen-activated protein kinase FgOS-2 is a central regulator in *F. graminearum* and controls, among others, virulence and DON and ZEA production. Here, we characterized the ATF/CREB-activating transcription factor FgAtf1, a regulator that functions downstream of FgOS-2. We created deletion and overexpression mutants of *Fgatf1*, the latter being also in an *FgOS-2* deletion mutant. FgAtf1 localizes to the nucleus and appears to interact with FgOS-2 under osmotic stress conditions. Deletion mutants in *Fgatf1* ($\Delta Fgatf1$) are more sensitive to osmotic stress and less sensitive to oxidative stress compared with the wild type. Furthermore, sexual reproduction is delayed. $\Delta Fgatf1$ strains produced higher amounts of DON under in vitro induction conditions than that of the wild type. However, during wheat infection, DON production by $\Delta Fgatf1$ is strongly reduced. The $\Delta Fgatf1$ strains displayed strongly reduced virulence to wheat and maize. Interestingly, constitutive expression of *Fgatf1* in the wild type led to hypervirulence on wheat, maize, and *Brachypodium distachyon*. Moreover, constitutive expression of *Fgatf1* in the $\Delta FgOS-2$ mutant background almost complements $\Delta FgOS-2$ -phenotypes. These data suggest that FgAtf1 may be the most important transcription factor regulated by FgOS-2.

Viability of every living entity depends on rapid adaptation to environmental changes. Sensing of external stimuli and subsequent cellular responses mainly rely on mitogen-activated protein (MAP) kinase phosphorelay systems such as the conserved stress-activated protein kinase (SAPK) pathway. In previous studies, we and others demonstrated the preeminent importance of SAPK signaling for stress adaptation, sexual reproduction, and virulence (Heller et al. 2012; Kawasaki et al. 2002; Nguyen et al. 2012; Segmüller et al. 2007). Disruption of *FgOS-2*, the *Fusarium graminearum* homolog of the *Saccharomyces cerevisiae* high osmolarity glycerol (HOG) kinase, impaired vegetative growth, sexual reproduction, osmotic stress adaptation, sec-

ondary metabolism, and, most strikingly, pathogenicity toward wheat and maize. We showed that most of these phenotypes could be connected to a disturbed reactive oxygen species (ROS) metabolism (Nguyen et al. 2012). In general, ROS are regarded as harmful by-products of numerous physiological processes (Apel and Hirt 2004) but they also facilitate an immediate response to pathogen attack. At the site of infection, localized ROS production by plant peroxidases leads to cell wall enforcement (Passardi et al. 2004). Furthermore, ROS exhibit antimicrobial activity toward the invading pathogen. In addition, ROS may act as a second messenger. It has been reported that ROS production regulates the production of numerous proteins involved in host defense reactions or apoptosis (Gadjev et al. 2008; Gechev et al. 2006; Orozco-Cárdenas et al. 2001). However, for successful penetration and host colonization, fungal pathogens evolved strategies to deal with oxidative stress (Heller and Tudzynski 2011). Biotrophic pathogens manage to detoxify plant-derived ROS by inducible catabolic enzymes (e.g., catalases and superoxide dismutases) (Molina and Kahmann 2007) or by secretion of ROS quencher (Voegelé et al. 2005). Pathogenic development of necrotrophic fungi may depend on the hypersensitive response of the plant. It is feasible that some necrotrophic fungi (i.e., *Botrytis cinerea*) (Govrin and Levine 2000) even actively contribute to plant-derived ROS by secretion of effectors (El Oirdi et al. 2011). Data obtained from studies in *Schizosaccharomyces pombe* involve SAPK-signaling cascades in the perception of oxidative stress and the regulation of ROS-responsive genes (Buck et al. 2001; Nguyen et al. 2000). These cascades consist of one or more sensor kinases, one or two response regulators, and the MAP kinase relay (three histidine kinases: MAP kinase-kinase-kinase, MAP kinase-kinase, and MAP kinase), which is conserved throughout all kingdoms. A putative downstream target of the SAPK-cascade is supposed to be the ATF/CREB transcription factor Atf1. In *S. pombe*, Atf1 is activated by Spc1/Sty1, the FgOS-2 homolog. After activation, it regulates the expression of a set of H₂O₂-responsive genes (Chen et al. 2003). Interestingly, the function of Atf1 homologs in phytopathogenic fungi is not conserved. Thus, their importance for disease development varies. Knock-out mutants of the *B. cinerea* Atf1 are hypervirulent toward different host plants. A comparative microarray approach in *B. cinerea* revealed a high number of genes regulated by Atf1. Among them are numerous genes involved in secondary metabolism (Temme et al. 2012). In the phytopathogenic fungus *Magnaporthe oryzae*, Atf1 mutants show a reduced virulence toward rice leaves, mainly because the plant defense was elevated compared with a wild-type infection (Guo et al. 2010). Furthermore, MoAtf1 plays a major role in the response to oxidative stress mediated by H₂O₂. Obviously, in

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the mutant strains, the capabilities for ROS degradation are compromised. In the biotrophic grass pathogen *Claviceps purpurea*, the Atf1 homolog TF1 is believed to act as a positive regulator of catalase genes and a repressor of *nox* genes (Nathues et al. 2004). In TF1 deletion mutants, virulence against rye is attenuated. In view of this diversity in function among phytopathogenic fungi, it is of special interest to investigate the Atf1 homolog in *F. graminearum*. The filamentous ascomycete *F. graminearum* is a highly organ-specific pathogen that resides on small grain cereals such as rice, wheat, and barley, entering its host through the flower at anthesis. Maize is infected through the silk channel or through wounds of the ears. Once the host plants are colonized, crops become unsuitable for food and feed production. This is mainly due to massive mycotoxin accumulation but also to reductions in grain development. The most devastating mycotoxins produced by *F. graminearum* are deoxynivalenol (DON) and zearalenone (ZEA). The trichothecene DON acts as a potent inhibitor of the eukaryotic translational machinery (Rocha et al. 2005). ZEA, a polyketide, shares some structural similarities to estrogen and thereby compromises the health of women and their unborn children (Kuiper-Goodman et al. 1987). The regulation of mycotoxin production is not yet fully understood. Several in vitro studies describe numerous factors that influence the regulation of mycotoxin biosynthesis. The production of DON was shown to be repressed under salt stress (Ochiai et al. 2007) and induced under oxidative stress conditions. Furthermore, the pH value (Gardiner et al. 2009c), nitrogen (Gardiner et al. 2009a), carbon source (Jiao et al. 2008), and even cobalt chloride (Tsuyuki et al. 2011) were found to influence DON production in vitro. However, there seem to be at least two distinct signaling cascades that regulate the production of DON. We could clearly demonstrate that *FgOS-2* deletion leads to an increased DON production under in vitro conditions and, on the other hand, to a strong decrease in DON production during wheat infection compared with the wild type. *Fgatf1* transcript levels are subject to the upstream kinase FgOS-2 (Nguyen et al. 2012). In the $\Delta FgOS-2$ deletion strains, *Fgatf1* is strongly downregulated during wheat infection and upregulated under oxidative stress conditions. In this study, we functionally characterize the *F. graminearum* Atf1 homolog in regard to its pivotal role in stress adaptation, secondary metabolism (including mycotoxin production), sexual reproduction, and virulence toward different hosts. Under stress conditions, FgAtf1 and FgOS-2 physically interact in the nucleus. Furthermore, results obtained from overexpression experiments indicate that FgAtf1 is the main downstream target of FgOS-2.

RESULTS

Generation of mutants.

In a genome-wide survey for regulatory motifs in the genome of *F. graminearum*, Kumar and co-workers describe an Atf1 ortholog (accession number FGSG_10142) according to its target regulatory motif ACGTCA. According to the Munich Information Center for Protein Sequences *Fusarium graminearum* genome database (Wong et al. 2010), the protein consists of 526 amino acids. Prosite database (Sigrist et al. 2010) analysis revealed that the putative FgAtf1 ortholog contains a basic-leucine zipper domain for DNA binding ranging from amino acid K407 to T489 (Supplementary Fig. S1).

In order to study the function of this protein in the life cycle of *F. graminearum*, the gene was deleted using a gene-replacement approach (Supplementary Fig. S2A). Fifty primary transformants were screened in a diagnostic polymerase chain reaction (PCR) (data not shown). Five independent transformants showed homologous integration of the replacement fragment (Supplementary Table S1). Single conidia of those mutants were

used to obtain homokaryotic deletion mutants. One additional mutant was chosen which showed amplification of the wild-type gene even after repetitive single-spore isolations, indicating an ectopic integration of the vector. All of the selected mutants and the wild type were analyzed in a Southern blot experiment. When probed for the integration of the hygromycin gene, all mutants but not the wild type showed a single integration of the fragment. When hybridized with a probe amplified from the *Fgatf1* gene, all mutants, except the one expected to have an ectopic integration of the replacement construct, showed no signal. In the wild type, a signal was visible. One $\Delta Fgatf1$ strain (number 13.2.) was subsequently selected for fluorescent labeling using the red fluorescent protein dsRed (discussed below). For localization studies, mutants carrying a C-terminal fusion of the open reading frame (ORF) of *Fgatf1* with the red fluorescent protein mCherry were generated. Additionally, mutants were generated that constitutively express *Fgatf1* under the control of the glyceraldehyde 3-phosphate dehydrogenase (*gpdA*) promoter from *Cochliobolus heterostrophus* (Salomon et al. 2012) (Supplementary Fig. S3A). Two independent transformants were obtained from this transformation (from now on referred to as *Fgatf1^{oe}* mutant strains). One of those mutants showed a 16-fold upregulation of *Fgatf1* transcript level compared with the wild type when raised for 4 days in liquid complete medium (CM) and a 15-fold upregulation during wheat head infection (10 days postinoculation [dpi]). In this particular *Fgatf1* overexpression mutant, *FgOS-2* was deleted using the methods described previously (Nguyen et al. 2012) (Supplementary Fig. S4A). Seven mutants with a disrupted FgOS-2 gene (named *Fgatf1^{oe}::ΔFgOS-2*) were obtained. In one of these mutants (1346.1.4), *Fgatf1* transcript level was determined to verify whether the *Fgatf1* transcript level is influenced by the deletion of FgOS-2. During growth in CM and during wheat infection, *Fgatf1* transcript level was elevated 12 and 14 times, respectively, compared with the wild type.

Functional FgAtf1 is necessary for proper vegetative growth and interacts with FgOS-2 under osmotic stress conditions.

To test the influence of FgAtf1 on vegetative growth, plate assays on different media were conducted using three independent $\Delta Fgatf1$ mutants, an ectopic mutant, and the wild type. When grown on CM agar plates, *Fgatf1* deletion mutants were slightly reduced in growth (Fig. 1A) compared with the wild type. On agar plates supplemented with osmotic agents (1.2 M sorbitol, 0.8 M KCl, and 0.8 M NaCl), the growth reduction was more pronounced. However, in contrast to *FgOS-2* deletion strains, growth was still possible on plates containing 0.8 M NaCl (Fig. 1A and B) (Nguyen et al. 2012). Also, other concentrations of NaCl and sorbitol (0.3 and 0.2 M) and other salts (such as MgCl₂, CaCl₂, LiCl, ZnCl₂, and FeCl₃) were tested in a plate assay. On the concentrations tested, the $\Delta FgOS-2$ showed a growth reduction on all media tested, whereas the *Fgatf1* deletion strain was most sensitive to sorbitol, LiCl, and ZnCl₃ (Supplementary Fig. S5).

Conidial germination of $\Delta Fgatf1$ mutants also differed from the wild type under salt stress conditions. In the presence of 0.8 M NaCl, germ tubes usually emerged at one apical compartment of $\Delta Fgatf1$ conidia. The wild type, in contrast, usually germinated out of two compartments. Under nonstress conditions, conidial germination was similar between the strains (Supplementary Fig. S6). Compared with the wild type, detection of ROS using nitro blue tetrazolium chloride (NBT) revealed no remarkable coloration of the colonies of all *Fgatf1* mutants on CM containing 0.2 and 0.8 M NaCl. In contrast, the *FgOS-2* deletion strain showed a strong coloration when grown on 0.2 M NaCl (Nguyen et al. 2012) (Supplementary

Fig. S7). Hence, there is no accumulation of ROS under osmotic stress conditions in the $\Delta Fgatf1$ strains. The $Fgatf1^{oe}$ mutant strains (two independent mutants were tested) also showed a slightly reduced growth on the osmotic stress medium (0.8 M NaCl) but grew better than the $Fgatf1$ deletion strains. Interestingly, the $Fgatf1^{oe}::\Delta FgOS-2$ mutants (three independent mutants were tested) grew much better than the $\Delta FgOS-2$ mutant strains on medium conferring osmotic stress (Fig. 1B). On medium containing 0.3 M NaCl, overexpression of $Fgatf1$ led to a nearly full restoration of growth in the $\Delta FgOS-2$ mutant background (Fig. 1B). The same applies for the growth on other metals. Accordingly, these mutants also accumulated less ROS compared with the $\Delta FgOS-2$ mutants: staining with NBT revealed wild-type like coloration at the growing edge of the $Fgatf1^{oe}::\Delta FgOS-2$ colonies on CM containing 0.2 M NaCl.

Unlike the $FgOS-2$ deletion strains, the $\Delta Fgatf1$ mutants exhibited no resistance to the phenylpyrrolic fungicide fludioxonil (Supplementary Fig. S8). The $Fgatf1^{oe}::\Delta FgOS-2$ mutant exhibited the highest resistance to fludioxonil. However, also on CM, the $Fgatf1^{oe}::\Delta FgOS-2$ mutants grew faster than the $\Delta FgOS-2$ mutants (Fig. 1B). These results indicate that FgAtf1 is a downstream target of FgOS-2 under osmotic stress and that activation of FgAtf1 is necessary for proper vegetative growth. Furthermore, fludioxonil seems not to act on FgAtf1.

The $Fgatf1$ deletion strains, not the overexpression mutants, grew much faster on medium supplemented with H_2O_2 compared with the wild type (Fig. 2). $FgOS-2$ deletion also leads to an increased resistance to oxidative stress (Nguyen et al. 2012). This phenotype is connected to a regulation of catalase gene expression and activity (discussed below). The increased resis-

Fig. 1. Colony morphology of **A**, the wild type (WT:PH1) and $\Delta Fgatf1$ and $Fgatf1^{oe}$ mutants and **B**, $\Delta FgOS-2$ and $Fgatf1^{oe}::\Delta FgOS-2$ mutants after 3 days of growth on complete medium (CM) and CM supplemented with 1.2 M sorbitol, 0.8 M KCl, 0.8 M NaCl, and 0.3 M NaCl. Agar plates were inoculated with mycelial plugs from 3-day-old cultures.

tance to H_2O_2 substantiates our assumption that the FgOS-2/FgAtf1-cascade is the central trigger of ROS metabolism in *F. graminearum*.

To further prove the interaction of FgOS-2 and FgAtf1 under stress conditions, we performed bimolecular fluorescence complementation (BiFC) (Hoff and Kück 2005). For this purpose, the ORF of *FgOS-2* was fused to the N terminus of the yellow fluorescent protein (YFP) and the ORF of *Fgatf1* was fused to the C terminus of YFP. A strain expressing a histone H1-mCherry fusion which facilitates detection of nuclei (A. L.

Martínez-Rocha and W. Schäfer, *unpublished results*) was used as recipient for both plasmids. Strains obtained from co-transformation of both plasmids were initially analyzed by PCR (data not shown). Mutants positive for both plasmids were subsequently analyzed by confocal laser-scanning microscopy (discussed below). Unstressed mutant mycelium as well as the wild type did not emit YFP-fluorescence when excited with a 514-nm laser line. Application of 1.2 M NaCl evoked fluorescence inside the nucleus of fungal hyphae of the mutants within 15 min after application (Fig. 3).

Fig. 2. Colony morphology of the wild type (WT:PH1) and the $\Delta Fgatf1$, $Fgatf1^{oe}$, $\Delta FgOS-2$, and $Fgatf1^{oe}::\Delta FgOS-2$ mutants after 4 days of growth on complete medium (CM) and CM supplemented with 10, 15, and 20 mM H_2O_2 . Agar plates were inoculated with mycelial plugs from 3-day-old cultures.

This result provides further evidence for an interaction of FgAtf1 and FgOS-2 under osmotic stress conditions. Zheng and coworkers (2012) recently reported that FgOS-2 translocates from the cytosol to the nucleus after treatment with NaCl. To elucidate whether FgAtf1 also translocates to the nucleus, we performed live-cell imaging using strains that expressed an FgAtf1-mCherry fusion protein. FgAtf1 constitutively resides in the nucleus. This localization pattern seems to be independent of FgOS-2, because it was also observed in $\Delta FgOS-2$ mutants. Furthermore, it is independent of osmotic and oxidative stress in both strains (Supplementary Fig. S9).

Constitutive expression of *Fgatf1* restores sexual reproduction in *FgOS-2* deletion strains.

Functional FgOS-2 is necessary for sexual reproduction in *F. graminearum* (Nguyen et al. 2012). To test the importance of FgAtf1 in this process, we analyzed the *Fgatf1* mutant strains and the $\Delta FgOS-2$ for perithecia formation on both carrot agar and detached wheat nodes (Fig. 4). On both substrates, perithecia formation was delayed in the $\Delta Fgatf1$ -strains (three independent mutants were tested). Even after prolonged incubation, approximately 50% of all perithecia remained immature (Fig. 4B and G). Although the wild type usually produces mature

perithecia containing numerous asci and ascospores after 3 weeks (Fig. 4A and F), it took 7 and 8 weeks (wheat nodes and carrot agar, respectively) for the $\Delta Fgatf1$ -strains. However, those perithecia that finally matured also contained asci and ascospores (Fig. 4B). Ascospores discharged from the perithecia on wheat nodes were viable and gave rise to new colonies (Supplementary Fig. S10). Interestingly, overexpression of *Fgatf1* restored the ability of an *FgOS-2* deletion strain to produce perithecia. Although the $\Delta FgOS-2$ strains completely failed to reproduce sexually (Fig. 4D and I), three independent *Fgatf1^{oe}::* $\Delta FgOS-2$ mutants produced mature asci and ascospores after 7 and 8 weeks (wheat nodes and carrot agar, respectively) (Fig. 4E and J). However, the clusters of perithecia appeared much smaller and not much aggregated to clusters compared with the wild type. Over expression of *Fgatf1* in the wild type did not affect perithecia formation (Fig. 4C and H).

Deletion of *Fgatf1* attenuates virulence toward wheat and maize.

Along with others, we implicated the FgOS-2 signaling cascade in virulence toward two important hosts of *F. graminearum*, wheat and maize (Jiang et al. 2011; Nguyen et al. 2012). Surprisingly, deletion of *Fgatf1* resembles neither the apatho-

Fig. 3. Bimolecular fluorescence (BF) complementation assay. FgOS-2 was fused to the N terminus and FgAtf1 to the C terminus of yellow fluorescent protein (YFP). Both constructs were co-transformed into a *Fusarium graminearum* strain expressing a histone-1-mCherry fusion protein facilitating visualization of nuclei. At 24 h after germination of conidia, osmotic stress mediated by 1.2 M NaCl was applied and hyphae were subsequently imaged using confocal laser-scanning microscopy. Scale bar: 10 μ m.

genic phenotype of the *FgOS-2* deletion strains on maize nor the reduced virulence on wheat. The pathogenic potential of $\Delta Fgatl$ strains (tested with three independent mutants) was reduced compared with the wild type but clearly increased

compared with the $\Delta FgOS-2$ strains (Fig. 5). On wheat ('Nandu'), generally, only the point-inoculated spikelet got colonized by the $\Delta Fgatl$ strains within 21 dpi. However, the colonization occurred faster compared with the $\Delta FgOS-2$ mutant. The

Fig. 4. Perithecia and ascospore development on **A** to **E**, detached wheat nodes and **F** to **J**, carrot agar. Mycelia of **A** and **F**, the wild type (WT:PH1) and the mutants **B** and **G**, $\Delta Fgatl$; **C** and **H**, $Fgatl$; **D** and **I**, $\Delta FgOS-2$; and **E** and **J**, $Fgatl::\Delta FgOS-2$ were assayed. **A** to **E**, Clusters of perithecia are indicated by the dotted circles in **A** (a), **B** (d), **C** (a), and **E** (c). Squeezing these clusters released the asci in **A** (b), **B** (b and d), **C** (b), and **E** (b and d). The $\Delta Fgatl$ (arrows in **B** [a]) and the $Fgatl::\Delta FgOS-2$ (arrows in **E** [a]) mutants produced immature perithecia clusters, which mature after 56 days of incubation (**B** [c] and **E** [c]). **F**, The wild type and **H**, the $Fgatl$ mutants started to produce perithecia after 21 days postinoculation (dpi) on carrot agar. **G**, After 56 dpi, the $Fgatl$ deletion strains also produced mature perithecia (immature after 21 dpi; arrowheads). **J**, On plates inoculated with the $Fgatl::\Delta FgOS-2$, tiny clusters of perithecia became visible after 56 dpi. Deletion of *FgOS-2* in the wild type led to a complete loss of perithecia formation.

Fig. 5. Pathogenicity assay on wheat and maize. Wheat heads (upper row) and maize cobs (lower row) were inoculated with conidia of the wild type (WT:PH1) and the $\Delta Fgatl$, $Fgatl$, $\Delta FgOS-2$, and $Fgatl::\Delta FgOS-2$ mutants and incubated for 35 (maize) and 21 (wheat) days. Inoculation with water served as a control. The $\Delta Fgatl$ mutant strain was reduced in virulence toward both hosts. On wheat, the infection of wheat stopped in the inoculated spikelet. The wild-type and the $Fgatl$ and $Fgatl::\Delta FgOS-2$ strains caused symptoms typical for maize cob rot and Fusarium head blight disease. Maize infections were repeated 6 times and the wheat infections performed 30 times.

$\Delta Fgatl1$ mutant was unable to bridge the transition zone (rachis node) between the inoculated spikelet and the transmitting tissue. This finding was verified by histological observations on longitudinal sections of single spikelets and entire spikes inoculated with a $\Delta Fgatl1$ strain and the wild type, respectively, that were both transformed with vector pII99::dsRed (Ilgen et al. 2009) that facilitates the expression of the red-fluorescent protein dsRed (Fig. 6). After 7 dpi, the $\Delta Fgatl1$ -dsRed strain colonized the inoculated spikelet but growth toward the rachis node was attenuated. At this timepoint, the wild type already passed the rachis node at the bottom of the inoculated spikelets and grew inside the adjacent one (Fig. 6). Within 21 dpi, the wild type colonized the complete spike. Hyphal growth can be seen in each spikelet below and above the point-inoculated one. Growth of the $\Delta Fgatl1$ mutants in contrast ceased in the inoculated spikelet (Fig. 6).

The $\Delta Fgatl1$ were also reduced in virulence toward maize. All maize cobs inoculated with the $Fgatl1$ deletion strains were only superficially colonized. The wild type colonized the entire maize cob within the 35 dpi (Fig. 5).

Constitutive expression of $Fgatl1$ increased the virulence. On susceptible 'Nandu' wheat, infection proceeded faster compared with the wild type (two independent mutants were tested) (Fig.

5). This accelerated infection became more obvious on the more resistant wheat 'Amaretto'. The average infection of the $Fgatl1^{oe}$ mutants proceeded more quickly and spread further compared with the wild-type infection (Supplementary Fig. S11A). The same applied to the infection on the close relative to cereal grain species, *Brachypodium distachyon*. When inoculated with the wild type, the infection usually stopped in the point-inoculated spikelet. However, the average infection of the $Fgatl1$ -over-expressing mutant proceeded beyond the inoculated spikelet. Intriguingly, the constitutive expression of $Fgatl1$ partially complements the reduced pathogenicity toward wheat and maize resulting from the deletion of $FgOS-2$ (three independent mutants were tested) (Fig. 5). The analysis of DON production in the $Fgatl1^{oe}::\Delta FgOS-2$ mutants revealed a massive increase in DON production compared with the $\Delta FgOS-2$ mutants (discussed below) during wheat head colonization. This increase might contribute to the reconstitution of pathogenicity.

Taken together, our results suggest that FgAtf1 plays a pivotal role in proper pathogenic development of *F. graminearum* on different hosts. Furthermore, we can now hypothesize that FgOS-2 regulates DON biosynthesis not only through FgAtf1 but also through at least one more transcriptional regulator that is as yet unknown.

Fig. 6. Infection assay. Cross-sections of inoculated wheat spikelets (upper row) and entire spikes (lower row). Spikelets were inoculated with strains that were derived from the wild type (WT:PH1) and the $\Delta Fgatl1$ -strain. They express dsRed constitutively in the cytosol. Inoculation with water served as a control. Inoculated spikelets: overview cross-section, including the inoculated spikelet and the rachis node at 7 days postinoculation (dpi). Inoculation points are indicated with white asterisks. The $\Delta Fgatl1$ mutant infection was attenuated compared with the wild-type-derived mutant. Bright-field microscopy revealed disease symptoms in the inoculated spikelet but not in the adjacent one. The wild-type-derived strain completely colonized the inoculated and the adjacent spikelet. Entire spikes (composite picture): infection of the $\Delta Fgatl1$ mutant stopped in the inoculated spikelet, while the wild-type derived strain successfully colonized the complete spike at 21 dpi. Scale bar = 1 mm.

***F. graminearum* Atf1 orchestrates secondary metabolite production.**

The ability to produce the trichothecene mycotoxin DON and the pathogenic potential toward wheat correlate (Maier et al. 2006; Proctor et al. 1995). Mutants deficient in the gene encoding a trichodiene synthase are unable to grow through the rachis node. Deletion of *FgOS-2* leads to a reduced DON production in planta and, thus, to a reduced virulence (Nguyen et al. 2012). To address the question of whether FgAtf1 regulates DON-production, highly sensitive enzyme-linked immunosorbent assay (ELISA) measurements were conducted using infected wheat spikelets and samples obtained from liquid toxin-induction cultures. The assay was conducted using the strains $\Delta Fgatf1$, $Fgatf1^{oe}$, and $Fgatf1^{oe}::\Delta FgOS-2$ (two independent mutants each). The results are summarized in Supplementary Table S2. Interestingly, in planta, the $\Delta Fgatf1$ DON level was between the wild-type and $\Delta FgOS-2$ DON levels. The $Fgatf1$ deletion strains produced approximately 3 times less DON than the wild type and approximately 20 times more than the $\Delta FgOS-2$ mutants 7 dpi (Fig. 7) (Nguyen et al. 2012). As mentioned above, constitutive expression of *Fgatf1* increased DON production in planta and led to a higher aggressiveness of *F. graminearum* toward different hosts. This upregulation even overrules the strong decrease in DON production in the mutants in which $\Delta FgOS-2$ is deleted. This finding suggests that FgAtf1 contributes to the regulation of DON biosynthesis during plant infection. Intriguingly, under in vitro conditions, the $\Delta Fgatf1$ mutants produced much more DON than the wild type (and the $\Delta FgOS-2$ mutant). Compared with the wild type, the $\Delta Fgatf1$ mutant produced approximately 3.5 and 1.7 times more DON after 1 and 3 dpi, respectively. All measurements are normalized to the amount of fungal material in the sample using quantitative (q)PCR (Voigt et al. 2007). Expression of four genes involved in DON-production (*tri4*, encoding a P450 monooxygenase; *tri5*, trichodiene synthase; and *tri6* and *tri10*,

Fig. 7. Deoxynivalenol (DON) concentrations in wheat heads infected with the wild type (WT:PH1), the *Fgatf1* mutants ($\Delta Fgatf1$, $Fgatf1^{oe}$, and $Fgatf1^{oe}::\Delta FgOS-2$), and the $\Delta FgOS-2$ mutant. The DON concentration was determined by enzyme-linked immunosorbent assay at 7 days postinoculation (dpi). The DON contents were lower in the *Fgatf1* deletion strains but higher in $Fgatf1^{oe}$ mutants compared with the wild type. As described previously (Nguyen et al. 2012), the *FgOS-2* deletion strains produce less DON during wheat infection. This lack in DON production is partially complemented by the overexpression of *Fgatf1*. Toxin measurements were repeated twice with four replicates each. Error bars indicate the standard deviation.

both transcription regulators) was regulated accordingly (Supplementary Table S3). This substantiates our previous finding that FgOS-2, in concert with the downstream transcriptional regulator FgAtf1, is the central trigger for growth-condition-dependent DON production. Furthermore, our results indicate a dominant inhibitory effect of FgAtf1 on the production of DON under in vitro conditions. Whether this is an effect of direct regulation or due to pleiotropic effects demands further investigation.

A second important mycotoxin, ZEA, accumulates in infected grains and is also produced in axenic culture (Gaffoor et al. 2005). After 7 dpi on wheat spikes, the $\Delta Fgatf1$ mutant synthesized significantly less ZEA compared with the wild type. As already observed for the $\Delta FgOS-2$ mutant (Nguyen et al. 2012), the transcript level of the putative regulatory transcription factor (*zeb2*) was higher in the *Fgatf1* deletion mutants compared with the wild type at 7 dpi. Expression of genes encoding an isoamyl alcohol oxidase (*zeb1*) and a non-reducing polyketide synthase (*zeal*) was reduced in planta compared with the wild type. Under in vitro induction conditions, there is no difference in ZEA production (and the regulation of gene expression) between $\Delta Fgatf1$ and the wild type.

A third prominent secondary metabolite of *F. graminearum* is the red pigment aurofusarin. Regulation of aurofusarin production is regulated in an FgOS-2-dependent manner (Nguyen et al. 2012). This regulation partially takes place via FgAtf1-signaling, because pigment production and expression of genes involved in aurofusarin biosynthesis (*gip1*, encoding a putative laccase; *gip2*, transcription factor; and *pks12*, polyketide synthase) were upregulated in $\Delta Fgatf1$ mutants. This led to an increased red coloration of the mutant colonies (Supplementary Fig. S12).

Taken together, these results underline the importance of the FgOS-2/FgAtf1-signaling cascade in the regulation of secondary metabolite production.

***F. graminearum* Atf1 acts as a repressor of catalase gene expression and activity under oxidative stress conditions.**

We assume that the global ROS metabolism in *F. graminearum* is modulated by the FgOS-2/FgAtf1 signaling machinery mainly by regulation of catalase gene expression and activity. *FgOS-2* deficiency leads to a massive induction of catalase gene expression and enzyme activity after exposure to H_2O_2 (Nguyen et al. 2012). In this regard, we tested gene expression and enzyme activity of four putative catalase genes under different conditions (osmotic, oxidative stress, and during wheat infection) in the different *Fgatf1* mutants (two independent $\Delta Fgatf1$, $Fgatf1^{oe}$, and $Fgatf1^{oe}::\Delta FgOS-2$ mutants in each experiment) and the $\Delta FgOS-2$ mutants (two mutants tested) (Fig. 8; Supplementary Table S4A to C). Intriguingly, gene expression of all catalase genes was drastically elevated under oxidative stress conditions in the $\Delta Fgatf1$ mutants compared with the wild type (Fig. 8B). A catalase activity assay confirmed that oxidative stress also increased the overall activity of catalases in the $\Delta Fgatf1$ mutants compared with the reference condition (only CM), and the wild type (stressed with H_2O_2) (Fig. 8D). Deletion of *Fgatf1* did not affect the expression of catalases 1 and 3 (*cat1* and *cat3*) during wheat infection. However, in both mutants with constitutive expression of *Fgatf1* ($Fgatf1^{oe}$ and $Fgatf1^{oe}::\Delta FgOS-2$), expression of these two catalase genes was much lower than in the wild type but approximately six and five times higher, respectively, compared with *FgOS-2* deletion strains (Fig. 8A). Moreover, expression level of *cat2.1* and *cat2.2* was elevated in the $Fgatf1^{oe}$ mutants compared with the $\Delta FgOS-2$ mutant and the wild type during wheat infection. As mentioned earlier, constitutive expression

of *Fgatf1* increases virulence and complements the corresponding phenotype of $\Delta FgOS-2$ mutants. It seems feasible that the elevated expression of two catalase genes contributes to an enhanced immunity against a putative hypersensitive plant defense reaction. Exposure to osmotic stress evoked an increase in transcript level of the *cat1* gene in the $\Delta Fgatf1$ mutants: it was upregulated approximately three times compared with the wild type (Fig. 8C). However, this upregulation did not lead to an increase in overall catalase activity (Fig. 8D). Interestingly, constitutive expression of *Fgatf1* in the $\Delta FgOS-2$ mutant background led to a significant increase in *cat1* expression compared with $\Delta FgOS-2$ mutants. This elevation might contribute to the partial reversion of the hypersensitivity phenotype of the $\Delta FgOS-2$ mutant toward osmotic stress. The expression levels of the other catalases (*cat2.1*, *cat2.2*, and *cat3*) remained more or less unchanged compared with the wild type. Taking these results, we assigned to FgAtf1 a function as repressor of catalase gene expression in response to oxidative stress. Whether this is a direct regulation or not and which protein, in turn, acts as a positive regulator of catalase expression remains open (Fig. 9).

DISCUSSION

ROS are unavoidable byproducts in all aerobic organisms. Along with their often harmful effects on cell walls, membranes, lipids, and DNA (Finkel 2003), ROS are also recognized as important signaling molecules in the host–pathogen interaction

(Apel and Hirt 2004; Heller and Tudzynski 2011). In a previous study, we characterized the stress-activated protein kinase FgOS-2 as a central regulator in the life cycle of *F. graminearum* (Nguyen et al. 2012). Our results led to the conclusion that many of the observed phenotypes were related to a disturbed ROS equilibrium. Well-balanced ROS equilibria are crucial for proper development in plants (Foreman et al. 2003), mammals (Ježek and Hlavatá 2005; Thannickal and Fanburg 2000), and fungi (Scott and Eaton 2008). In the present study, we analyzed the putative downstream target of FgOS-2, the basic leucine zipper transcription factor FgAtf1, in regard to its importance for pathogenicity, secondary metabolite production, and sexual reproduction. In some aspects, we found additional evidence for our hypothesis of a causal connection between a disruption of the ROS homeostasis and the observed phenotypes. However, because some phenotypes observed in the $\Delta Fgatf1$ mutants only partially resemble the phenotypes observed upon *FgOS-2* deletion, we postulated additional, as-yet-unknown downstream targets of FgOS-2 (Fig. 9). Interestingly, the constitutive overexpression of *Fgatf1* increases virulence and partially complements deficiencies in this and other regards in $\Delta FgOS-2$ mutants. Whether this effect is solely explainable by the relatively higher abundance of *Fgatf1* transcript in these mutants or whether it benefits from an FgOS-2-independent posttranslational activation remains to be tested. Furthermore, we demonstrated that FgAtf1 constitutively localizes to the nucleus regardless of the genetic background (i.e., $\Delta FgOS-2$) or the growth conditions.

Fig. 8. Catalase expression and activity assay. **A** to **C**, Quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) using cDNA obtained from **A**, inoculated wheat spikelets (7 days postinoculation [dpi]); **B**, H₂O₂-supplemented (10 mM); and **C**, NaCl-supplemented samples (0.8 M). Expression of four different, putative catalase genes was assayed in the wild type (WT:PH1), the *Fgatf1* mutants ($\Delta Fgatf1$, *Fgatf1*^{oe}, and *Fgatf1*^{oe}:: $\Delta FgOS-2$), and the $\Delta FgOS-2$ mutant. The wild-type expression level was set to 1. Catalase expression in the $\Delta Fgatf1$ mutant strain was not drastically changed during the infection of wheat. Except catalase 1, which was upregulated approximately three times, all catalases were only slightly upregulated in the $\Delta Fgatf1$ mutant in response to osmotic stress (NaCl). In the presence of oxidative stress (H₂O₂), all catalases were drastically upregulated in the $\Delta Fgatf1$ mutant compared with the wild type. qRT-PCR was performed in triplicate. **D**, Catalase activity measurement using native protein extract from the wild type and the $\Delta Fgatf1$ mutant strains. Cultures were supplemented with 0.8 M NaCl and 10 mM H₂O₂, respectively. Under oxidative stress condition, catalase activity was massively upregulated in the $\Delta Fgatf1$ mutant compared with the wild type. Error bars indicate the standard deviation.

F. graminearum Atf1 plays an ambivalent role in stress response.

We drew a connection between fungal growth, the alteration of ROS level in response to environmental stresses, and a regulation of catalases in *F. graminearum* (Nguyen et al. 2012). A pivotal role in this response regulation is assigned to FgAtf1. Here, we show that the contribution of FgAtf1 varies depending on the type of stress and suggest that FgAtf1 is less important for mycelia growth under salt stress conditions compared with FgOS-2. Thus, it is feasible that FgOS-2 directly regulates the osmotic stress response or that it does not activate FgAtf1 exclusively but also other transcription factors. In *Saccharomyces cerevisiae*, the Hog1 MAP kinase regulates at least four transcription factors: namely, Msn2, Msn4, Hot1, and Sko1 (Hohmann 2002). Growth of *Fgatf1* deletion strains was still possible on medium containing 0.8 M NaCl. At this concentration, mycelia growth of the $\Delta FgOS-2$ completely ceased, most likely because the mutant produces plenty of ROS and is no longer able to induce catalase gene expression in order to detoxify them (Nguyen et al. 2012). Unlike the *FgOS-2* deletion strains, $\Delta Fgatf1$ mutants did not evoke an increase in ROS accumulation when exposed to osmotic stress. Furthermore, catalase gene expression and activity were not much elevated in $\Delta Fgatf1$ mutants under osmotic stress compared with the wild type (Fig. 8C). In this regard, however, it is noteworthy that we observed an unusual germination pattern in $\Delta Fgatf1$ mutants under osmotic stress conditions. $\Delta Fgatf1$ germ tubes did not swell, were not polynuclear, and did not stop growing after a short period of growth; however, they emerged only at the apex of the conidium, as they did in the $\Delta FgOS-2$ mutants (Nguyen et al. 2012). In this context, the regulation pattern of a putative spore-specific catalase (Navarro et al. 1996; Paris et al. 2003) (*cat1* in *F. graminearum*) is remarkable. In $\Delta FgOS-2$ mutants, the transcript level of this catalase is very low (Nguyen et al. 2012), whereas it is increased three times in the $\Delta Fgatf1$ under osmotic stress conditions compared with the wild type. This upregulation might contribute to a higher stress tolerance of conidia on osmotic stress medium. Consequently, upregulation

of all four catalase genes in the *Fgatf1^{oe}::ΔFgOS-2* mutants may contribute to the full restoration of growth on mild osmotic stress medium (Fig. 1B) by the restoration of ROS detoxification capabilities. BiFC experiments demonstrated that FgOS-2 and FgAtf1 physically interact under salt stress conditions (Fig. 3). This interaction is also documented for *Aspergillus nidulans* (Lara-Rojas et al. 2011). However, in this fungus, the interaction occurs in response to oxidative stress. In *B. cinerea*, it is feasible that the physical interaction takes place under osmotic stress condition, because Heller and associates (2012) localized the *B. cinerea* HOG1 homolog BcSak1 in the nucleus after exposure to osmotic stress. In *F. graminearum*, Atf1 permanently resides in the nucleus whereas FgOS-2 translocates into the nucleus upon osmotic stress, presumably after getting phosphorylated (Zheng et al. 2012). Intriguingly, FgAtf1 localization is not affected by osmotic or oxidative stress and the presence or absence of FgOS-2. Hence, it is feasible that the physical interaction of both proteins inside the nucleus is necessary for the proper response to osmotic stress. This would explain the sensitivity of both the $\Delta FgOS-2$ and the $\Delta Fgatf1$ mutant toward osmotic stress and would fit the situation in *S. pombe*, where the stress-induced gene regulation relies on the interaction of the OS-2 ortholog Sty1 and Atf1 (Reiter et al. 2008).

In regard to oxidative stress response, FgAtf1 seem to be the major regulator. $\Delta Fgatf1$ mutants grew much better than the wild type (and $\Delta FgOS-2$) on medium containing up to 20 mM H₂O₂ (Fig. 2). This increase in oxidative stress resistance is likely due to the drastic upregulation of catalase gene expression and catalase activity under oxidative stress conditions. This finding highlights again the idea that functions of the Atf1 homologs within the polyphyletic group of necrotrophic plant pathogens evolved differently, because deletion of the *M. oryzae* Atf1 ortholog (*Moatf1*) leads to hypersensitivity against oxidative stress (Guo et al. 2010). The authors connect this phenotype to the mutant's disability to detoxify ROS. The *B. cinerea* Atf1 ortholog is reported to act as a regulator of catalase B gene expression upon exposure to oxidative stress (Temme et al.

Fig. 9. Proposed model. How FgOS-2 and FgAtf1 influence numerous physiological functions like vegetative growth, sexual reproduction, germination, and pathogenicity in *Fusarium graminearum*. FgAtf1 is activated by FgOS-2 and environmental cues such as a host plant or osmotic or oxidative stress conditions. Among others, FgAtf1 contributes to reactive oxygen species (ROS) production and degradation, presumably by regulating ROS metabolic enzymes such as catalases. In this regulatory system, we have to assume a substantial contribution by additional, as-yet-unknown factors. Heavy arrows indicate the relatively higher importance of a particular connection under certain conditions.

2012). Catalase B expression is diminished as a result of the deletion of *Bcatf1*. Nonetheless, $\Delta bcatf1$ mutants show no increased sensitivity toward oxidative stress mediated by H₂O₂. Thus, in regard to viability under oxidative stress conditions, the importance of Atf1 varies in necrotrophic fungi from dispensable (*B. cinerea*) over essential (*M. oryzae*) to inhibitory (*F. graminearum*). In *F. graminearum*, both FgOS-2 and FgAtf1 are not essential for vegetative growth under oxidative stress conditions and might even act as a repressor of catalase gene expression. Contrary to expectations, constitutive expression of *Fgatf1* neither increases nor decreases sensitivity toward oxidative or osmotic stress but, interestingly, caused an increased aggressiveness toward several host plants (Fig. 5) (discussed below). The *Fgatf1* deletion mutants were not resistant against fludioxonil as was observed in *FgOS-2* deletion strains. Application of fludioxonil leads to an upregulation of a glycerol-3-phosphate phosphatase (Nguyen et al. 2012), which dephosphorylates glycerol-3-phosphate (Albertyn et al. 1994; Pillonel and Meyer 1997). In consequence, glycerol accumulates and the cells swell and frequently burst. The fact that *Fgatf1* deletion mutants are as sensitive to fludioxonil as the wild type underscores that FgOS-2 possesses unique functions that are independent of FgAtf1. In *Neurospora crassa*, Atf1 and OS-2 share some functions in response to fludioxonil, although $\Delta Fgatf1$ strains were as susceptible to this fungicide as the wild type (Yamashita et al. 2008).

Secondary metabolite production depends on functional FgAtf1.

Proper regulation of secondary metabolite production depends on the FgOS-2 signaling cascade and culture conditions. Production of prominent secondary metabolites such as DON, ZEA, and aurofusarin is regulated by FgOS-2 in a growth-condition-dependent manner (Nguyen et al. 2012). Although the $\Delta FgOS-2$ mutants produced more DON under in vitro induction conditions, biosynthesis was reduced during wheat infection. Aurofusarin synthesis, however, was deregulated and occurred also (unusually) during wheat head infection. Deletion of *Fgatf1* also elevates aurofusarin production. Aurofusarin exhibits antimicrobial potential and inhibits fungal growth (Malz et al. 2005; Medentsev et al. 1993). Thus, it is feasible that, due to an upregulation of aurofusarin biosynthesis, vegetative growth of the $\Delta Fgatf1$ strains is reduced. Atf1 was assigned a function in the regulation of secondary metabolism before. Deletion of *B. cinerea* Atf1 resulted in an upregulation of phytotoxin production (Temme et al. 2012). Also, FgAtf1 is of major importance for the regulation of mycotoxins and, again, the regulation pattern turned out to depend on the transcript level of *Fgatf1* and the culture conditions. Absence of *Fgatf1* led to a drastic increase in DON production under in vitro conditions compared with the wild type but a strong reduction during wheat infection. Intriguingly, constitutive expression of *Fgatf1* resulted in an increased DON production during wheat infection. As shown by Ponts and associates (2006), DON production in vitro is induced by H₂O₂. Whether this is the main inducer of DON biosynthesis during wheat infection is still under investigation. During wheat infection, catalase gene expression is much higher in the $\Delta Fgatf1$ mutants compared with the $\Delta FgOS-2$ mutants but not much changed compared with the wild type (Fig. 8A). Hence, ROS might not accumulate in tissues infected with the $\Delta Fgatf1$ mutants. This might partially explain why DON production is much higher in the $\Delta Fgatf1$ mutants compared with the $\Delta FgOS-2$ mutants, although it is still lower compared with the wild type. These results underline the idea that FgOS-2 regulates DON production not only by the regulation of FgAtf1 but also through other, as-yet-unknown factors. Also, proper ZEA production relies on FgAtf1. In vitro, ZEA levels are

nearly wild type like, whereas ZEA production is diminished in planta in the $\Delta Fgatf1$ mutants. We observed the same regulation pattern in $\Delta FgOS-2$ mutants (Nguyen et al. 2012). Secondary metabolite production is generally influenced by different culture conditions (Calvo et al. 2002; Keller and Hohn 1997; Kim et al. 2005; Linnemannstons et al. 2002). ZEA biosynthesis is mainly induced by grain-specific factors (Gaffoor et al. 2005). Hence, as already noted for DON production, FgAtf1 interferes specifically with plant-derived cues. In vitro, however, the regulatory pathways are fundamentally different. This underscores the belief that it is inevitable to determine the conditions that induce secondary metabolite production during wheat infection.

Balanced *Fgatf1* transcript levels are a prerequisite for sexual reproduction.

FgOS-2 deficiency leads to a complete loss of sexual reproduction (Nguyen et al. 2012). Interestingly, deletion of *atf1* in *F. graminearum* also negatively influenced formation of reproductive structures. Development of perithecia was delayed in $\Delta Fgatf1$ mutants compared with the wild type on carrot agar and on detached wheat nodes but ascospores forcibly discharged from those perithecia were viable. Deletion of the *N. crassa atf1* ortholog, *asl-1*, in contrast, led to nonfunctional ascospores (Colot et al. 2006). The inability of the $\Delta FgOS-2$ mutants to produce perithecia and ascospores can be restored by a constitutive expression of *Fgatf1*. This attributes to FgAtf1 a prominent role in the regulation of sexual reproduction. Because the deletion of *Fgatf1* does not lead to full sterility, other factors may also interfere. Perithecia formation might be regulated through distinct ROS levels. There are examples from other fungi such as *A. nidulans* (Lara-Ortiz et al. 2003), *Podospira anserina* (Malagnac et al. 2004), and *N. crassa* (Cano-Domínguez et al. 2008), in which disturbance in ROS balance by deletion of NADPH oxidases lead to disability to reproduce sexually. Hence, it is feasible that the misregulation of catalases in *F. graminearum* interferes with mating processes and, thereby, interrupts sexual development.

Virulence of *F. graminearum* relies on *Fgatf1* transcript level.

Our results suggest a close relationship between *Fgatf1* transcript level and pathogenicity toward wheat and maize. Furthermore, *Fgatf1* expression is regulated by FgOS-2. In *FgOS-2* deletion mutants, *Fgatf1* transcription is very low and so is virulence toward both hosts (Nguyen et al. 2012). In the present study we showed that deletion of *Fgatf1* from the genome led to a severe reduction in virulence against wheat and maize. In contrast, an overexpression of *Fgatf1* results in a greater aggressiveness against these hosts (and *Brachypodium distachyon*). Strikingly, constitutive expression of *Fgatf1* partially restored virulence of *FgOS-2* deletion strains (Fig. 5). This strongly suggests that FgAtf1 is a superior regulator of virulence in *F. graminearum*. However, the question remains open as to whether the high abundance of *Fgatf1* transcript is already sufficient to increase the virulence or FgAtf1 function requires a posttranslational modification (i.e., through phosphorylation). Atf1 orthologs are described to be activated by phosphorylation through an upstream (HOG1) MAP kinase (Moye-Rowley 2003). Because the *Fgatf1*^{oc}:: $\Delta FgOS-2$ mutants lack this kinase, we have to assume that FgAtf1 either gets autophosphorylated or is the target of a different, as-yet-unknown MAP kinase that translocates to the nucleus under certain conditions. The Sko1 of *Saccharomyces cerevisiae* and Atf1 of *Schizosaccharomyces pombe* are known to be negatively regulated by the cAMP-dependent protein kinase A and positively regulated by a MAP kinase (Davidson et al. 2004; Pascual-Ahuir et al. 2001). In the

case of *S. pombe*, this kinase is PMK1 (Takada et al. 2007), which is well described in *F. graminearum* (Jenczmionka and Schäfer 2005; Jenczmionka et al. 2003; Urban et al. 2003) but, thus far, was not involved in SAPK signaling cascades, although there are overlapping phenotypes in the deletion mutants of FgOS-2 and Gpmk1, especially regarding sexual reproduction and virulence. Recent results published by Zheng and associates (2012) indicate that—under salt-stress conditions—there is a crosstalk between FgOS-2 and the MAP kinases Gpmk1 and Mgv1. We hypothesize that, upon deletion of FgOS-2, FgAtf1 gets phosphorylated by different MAP kinases. Thus far, to our knowledge, the overruling regulatory function of an Atf1 homolog has not been described. This function has consequences on the transcript level of target genes involved in ROS metabolism. Lamb and associates (2012) found out that, in *N. crassa*, *asl-1* and catalase 1 gene expression was increased under salt stress conditions. In contrast to *F. graminearum*, this increase in catalase expression in *N. crassa* is ASL-1-dependent.

Gene expression analysis on infected wheat spikes revealed a downregulation of all catalase genes in the *FgOS-2* deletion strains compared with the wild type and an upregulation in *Fgatf1^{oe}::ΔFgOS-2* mutants compared with the *ΔFgOS-2* mutants. Regulation of balanced ROS levels is important for a proper host–microbe interaction (Scott and Eaton 2008). The drastically reduced virulence of *FgOS-2*-deficient strains and partially restored virulence of the *Fgatf1^{oe}::ΔFgOS-2* mutant might be due to an altered ROS balance in the interaction between wheat and the mutant strains.

The deletion of the ROS-producing NADPH oxidase 1 in *Epichloë festucae* leads to a dramatic switch in the life style of this symbiotic fungus: the mutants harmed the viability of their host plants (Tanaka et al. 2006). Deletion of this enzyme in the hemibiotrophic pathogen *Claviceps purpurea* leads to a decreased virulence toward rye (Giesbert et al. 2008). The importance of Atf1 orthologs for parasitic development varies among the limited number of phytopathogenic fungi in which this gene was functionally characterized thus far. Gene expression of *M. oryzae atf1* (*Moatf1*) is elevated during germination of conidia and initial penetration of plant surface (Guo et al. 2010). *Moatf1* deletion mutants are severely attenuated in virulence on rice. The mutant is able to cause small restricted lesions on spray-inoculated rice leaves. Staining experiments using 3,3'-diaminobenzidine revealed an enhanced ROS production upon penetration of the plant surface by the mutant, indicating a disturbed ROS balance. Because we could not observe an altered in planta catalase gene expression level upon *Fgatf1* deletion, misbalance in the ROS level might not be that drastic in the *ΔFgatf1*–wheat interaction. Although the Atf1 homologs of *M. oryzae* and *F. graminearum* obviously both preserved importance for disease development, the *B. cinerea* Atf1 seems to have an even inhibitory effect on virulence, because deletion strains were more aggressive against different hosts (Temme et al. 2012). On inoculated host plants, vigorous hyphal growth is accompanied by a disability to produce conidia. However, we also observed a greater aggressiveness in parasitic growth only upon constitutive expression of *Fgatf1*. This indicates that, during evolution, *Fgatf1* orthologs in different organisms gain divergent gene-repressing and -activating capacities, respectively. In *F. graminearum*, the production of DON is prerequisite for successful colonization of wheat. Wheat head infection of mutants deficient (Maier et al. 2006; Proctor et al. 1995) or markedly reduced (Nguyen et al. 2012) in DON production is always restricted to the point-inoculated spikelet. In this study, we observed a significant reduction in DON production by the *ΔFgatf1* mutant strains that was, however, still higher than in the *ΔFgOS-2* strains. Hence, it is feasible that the reduced virulence toward wheat is due to the reduced DON level. In this

regard, it seems likely that the increased DON production observed in the *Fgatf1^{oe}* strains as compared with the wild type (Fig. 7) has a positive impact on the pathogenic potential. Gardiner and co-workers (2009b) reported that the deletion of a putative cytochrome P450 monooxygenase and a hypothetical protein with homology to a terpene cyclase leads to an increased DON level and, thus, to an increased virulence toward wheat. Interestingly, both genes are not part of the *tri*-gene cluster but their expression is under the control of the positive transcriptional regulator Tri6. Whether or not *Fgatf1* is also directly involved in this regulatory machinery remains to be elucidated. The increased production of DON in the *Fgatf1^{oe}* mutants also elevates the DON accumulation in the *Fgatf1^{oe}::ΔFgOS-2* strains and, thereby, may contribute to the reversion of the apathogenicity phenotype of the *FgOS-2* deletion. Yet, this link between DON production and *Fgatf1* transcript level does not explain the virulence phenotypes on maize. We observe virulence patterns in the maize infection assay similar to the wheat infection: reduced infection rate in the *ΔFgatf1* strain and increased infection in the overexpression mutants (both compared with wild type) (Fig. 5). Although DON is not a pathogenicity factor on maize (Maier et al. 2006), its production is reported to increase the aggressiveness toward this host (Harris et al. 1999). Thus, an increase in DON accumulation might facilitate colonization of maize cobs. In addition, other factors, such as an altered ROS level, may contribute to the increased virulence toward maize.

Altogether, this study highlights the fact that, in *F. graminearum*, Atf1 acts as one downstream target of FgOS-2. It shares some functions with FgOS-2 but also has important independent features. Most importantly, we found that FgAtf1 comprises physiological functions without A phosphorylation by its putative upstream MAP kinase FgOS-2 (i.e., response to osmotic stress, sexual reproduction, and virulence). In this regard, we have to assume additional, as-yet-unknown factors for activation of FgAtf1. It remains to be elucidated to what extent those factors contribute to the variety of phenotypes we observed. Autophosphorylation is also feasible. To what extent FgOS-2 contributes to the phenotypes in the *Fgatf1^{oe}* mutant (in the wild-type background) is subject to ongoing research.

MATERIALS AND METHODS

Fungal strains and culture conditions.

All mutants described in this study derived from the *F. graminearum* wild-type strain PH1. Conidiation was induced in liquid wheat medium incubated for 4 days at 28°C. Sexual reproduction was monitored on carrot agar plates and detached wheat nodes placed on water agar plates (double autoclaved wheat parts with a nodule in the middle). Conidia of the wild type, *ΔFgOS-2*, and *Fgatf1* mutant strains (*ΔFgatf1*, *Fgatf1^{oe}*, and *Fgatf1^{oe}::ΔFgOS-2*) were inoculated on carrot agar plates and incubated 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 inoculated 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 up to 8 weeks. To observe asci and ascospores in the microscope, the perithecia were excised using a knife, placed on objective slides, and then crushed between the slide and a cover slip. Viability of ascospores was checked in an agar lid assay. In this, the lid of a petri dish was covered with a thin layer of CM agar and placed on a plate that contained inoculated wheat nodes. Forcibly discharged ascospores landed on the agar cover, where new colonies were growing. Growth assays were performed using the wild type and three independent *ΔFgOS-2* and *Fgatf1* mutant strains. 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 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 g liter⁻¹, and $\text{FeCl}_3 \times 6 \text{ H}_2\text{O}$ at 932 mg liter⁻¹], 1 g of yeast extract, 0.5 g of enzymatically hydrolyzed casein, and 0.5 g of acid-hydrolyzed casein) and placed in the middle of the assay plates. The growth assay plates were supplemented with the following agents: D-sorbitol, NaCl , KCl , H_2O_2 , and fludioxonil at concentrations as indicated in the figures and captions. The plates were inoculated at 28°C for at least 3 days in the dark. The diameter of the colonies was measured using a technical ruler. The analyses were performed with at least five replicates. For RNA isolation, the strains were precultured in liquid yeast-peptone-dextrose (YPD) medium for 3 days. Mycelia were harvested and washed with distilled water several times. Equal portions of semidried mycelia were then shifted in new liquid CM containing no additional supplement, NaCl , or H_2O_2 at the concentrations indicated in the figure captions. Mycelia raised in the toxin induction media (discussed below) were also used for the RNA isolation.

Vector construction

for deletion and overexpression of *Fgatf1*.

Standard recombinant DNA methods were performed according to Ausubel and associates (2002) and Sambrook and associates (1989). The complete ORF of *Fgatf1* was deleted using a double homologous recombination approach. For vector construction, flanking regions (1.054 kb upstream and 1.056 kb downstream of *Fgatf1*) were cloned by PCR from genomic DNA (gDNA) using the primers listed in Supplementary Table S5. 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 HPH cassette (hygromycin 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- μl PCR reaction 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, U.S.A.) to generate pGEM-*Fgatf1*-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 lysogeny broth medium (Sambrook et al. 1989) either as a liquid culture or on agar plates. For the fungal transformation (discussed below), pGEM-*Fgatf1*-HPH was digested with *XbaI* to release the disruption construct. The vector pII99::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.

For constitutive expression of *Fgatf1* controlled by the *gpdA* from *Cochliobolus heterostrophus*, the ORF of *Fgatf1* was amplified by PCR from gDNA using the *Pfu* proofreading polymerase (Promega Corp., Mannheim, Germany) primers 11 and 12. The PCR product was ligated into pJET1.2 vector (Fisher Scientific, Schwerte, Germany) and sequenced. The ORF

(1,898 bp) was excised from pJET1.2 by restriction enzymes *SacI* and *XbaI* and cloned into pII99 vector, which contains the *gpdA* promoter from *C. heterostrophus*, and a geneticin-resistance cassette. The resulting plasmid, pII99-*Fgatf1*, was linearized at the unique *ApaI* restriction site which cuts the *Fgatf1* sequence at position 1,050 to facilitate single crossover events. The linearized construct was used for fungal transformation (discussed below).

For generation of *FgOS-2* deletion mutants in an *Fgatf1* overexpression mutant, *Fgatf1*^{oe} mutant number 1.2 was chosen for *FgOS-2* deletion. The construct and method for *FgOS-2* deletion is described by Nguyen and associates (2012).

BiFC experiments,

For BiFC experiments, *FgOS-2* and *Fgatf1* were tagged with separated halves of the YFP. To fuse the N-terminal half of YFP to *FgOS-2*, a 1.074-kb *FgOS-2* PCR product was amplified using primers 13 (which introduces an *NcoI* restriction site before the starting ATG) and 14 (which introduces an *NorI* restriction site after the stop codon). To fuse the C-terminal part of YFP to *Fgatf1*, primers 15 (which introduces an *NcoI* restriction site before the starting ATG) and 16 (which introduces a *NorI* restriction site after the stop codon) were used to generate a 1.581-kb PCR product. Both products were cloned into pJET1.2 plasmids and subsequently released by restriction using enzymes *NcoI* and *NorI*. The fragments were ligated into the vectors YFP-N and YFP-C (Hoff and Kück 2005) to generate plasmid YFP-N::*FgOS-2* and YFP-C::*Fgatf1*, respectively. Both plasmids were linearized by *EcoRI* (YFP-N::*FgOS-2*) and *XmnI* (YFP-C::*Fgatf1*) and subsequently co-transformed into *F. graminearum* (discussed below).

Transformation of *F. graminearum*.

DNA (10 to 30 μg dissolved in 30 to 50 μl of distilled H_2O) was used for protoplast transformation of *F. graminearum*. The protoplast transformation method was performed as previously described (Jansen et al. 2005; Proctor et al. 1995). Yeast-peptone-glucose medium (100 ml; 0.3% yeast extract, 1% Bacto peptone, and 2% D-glucose) was inoculated with 1×10^6 conidia and incubated overnight on a shaker (New Brunswick Innova 44, Eppendorf, Wesseling-Berzdorf, Germany) at 28°C and 150 rpm. The mycelia were collected by filtering with a 200- μm -diameter sieve and washed with double-distilled water. Mycelia were resuspended in a 20-ml mixture of driselase and lysing enzymes (2.5%; 0.5% in 0.6 M or 1.2 M KCl) (Life Technologies, Darmstadt, Germany) and incubated for 2 to 3 h on a shaker (Eppendorf) at 30°C and 80 rpm. Undigested hyphal material was removed from the protoplast suspension by subsequent filtration. The protoplasts were pelleted by centrifugation at $670 \times g$, washed once with 10 ml of 20% sucrose, 10 mM Tris-HCl (pH 8.0), and 50 mM CaCl_2 (STC), centrifuged again, and then resuspended and adjusted in STC at 1×10^8 protoplasts/ml. For transformation, 200 μl of the protoplast suspension was mixed with DNA. The samples were incubated at room temperature for 20 min. Subsequently, 1 ml of 40% polyethylene glycol 4000 and 60% STC was added and again incubated at room temperature for 20 min. The protoplast suspension was added to 5 ml of 100 g of sucrose, 0.3% yeast extract, and 0.3% casamino acid (TB3) medium and shaken overnight on a biological shaker (Eppendorf) at room temperature and 100 rpm for cell wall regeneration. The regenerated protoplasts were pelleted by centrifugation at $4,200 \times g$ and, then, mixed with TB3 agar (1.5%) at 50°C with hygromycin B (250 $\mu\text{g ml}^{-1}$) or geneticin (100 $\mu\text{g ml}^{-1}$). The mixture was then plated out on petri dishes (10 ml/plate). After 24 h, an overlay comprising of TB3 agar (1.5%) and hygromycin B at 500 $\mu\text{g ml}^{-1}$ or geneticin (100 $\mu\text{g ml}^{-1}$) was

added to the plates. Putative transformants were obtained after 2 dpi at 28°C. They were transferred to fresh plates of CM supplemented with hygromycin B at 250 µg ml⁻¹ or geneticin (200 µg ml⁻¹) and incubated at 28°C. The transformants were purified by single-spore isolation and subsequently checked by diagnostic PCR.

Southern blot analysis.

For Southern hybridization analysis, approximately 3 µg of gDNA of the wild type and *Fgatf1* mutant strains was restricted with appropriate restriction enzymes overnight. 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 membrane (GE Healthcare, Munich) and hybridized with a digoxigenin-labeled (Roche, Penzberg, Germany) DNA internal probe, which was generated with the gene-specific primers 7 and 8. 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, maize, and *B. distachyon*.

The susceptible spring wheat 'Nandu' (Lochow-Petkus, Bergen-Wohlde, Germany) and the partially resistant wheat 'Amarretto' (Berthold Bauer, Niedertraubling, Germany) were used for wheat virulence assay. Plants were cultivated in a growth room at 20°C with a photoperiod of 16 h and 60% relative humidity, then transferred to infection chambers with optimized conditions. A suspension of 500 conidia in 10 µl of the wild type and all mutants 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 monitored for up to 3 weeks in the infection chambers. Wheat spikes inoculated with 10 µl of pure water were used as the negative control. The wheat infection assay was performed using three independent knock-out strains, two independent overexpression mutant strains, and 10 replicates in parallel with the wild type. The maize inbred line A188 (Green and Phillips, 1975) was grown in the greenhouse (temperature, 26 to 30°C; humidity, 70 to 85%; and 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 the wild type and the mutant strains, each at 2×10^4 conidia/ml. 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 and the inoculation lasted for 5 weeks.

Brachypodium distachyon (diploid inbred line Bd21) was cultivated in a growth room at 22°C with a 20-h photoperiod and then transferred to infection chambers at 22°C, 65% humidity, and a photoperiod of 8 h. A suspension of 50 conidia (1 µl) was inoculated into the central spikelet of each spike at the early stages of anthesis. The inoculated spikes were enclosed in small plastic bags for the first 2 days and then monitored for up to 2 weeks in the infection chambers.

DON and ZEA production analysis.

For in vitro DON production measurement, 5×10^3 conidia of the wild type and the *Fgatf1* mutant strains were precultured in YPD for 3 days. The mycelia were harvested by filtering with a 200-µm-diameter sieve, washed by rinsing at

least three times with distilled water, and then dried on sterile Whatman paper (GE Healthcare). Then, 1 g of semidried mycelia was further incubated for 24 and 72 h in minimal medium supplemented with 5 mM (NH₄)₂SO₄ for DON induction. Each supernatant (50 µl) 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 the wild type and the mutant strains. The spikes were inoculated with pure water as the negative control. The inoculated samples were collected after 7 dpi, immediately frozen in liquid nitrogen, and dried in vacuum. Each sample (200 mg) was ground to a powder under liquid nitrogen. Then, 50 mg of ground material was suspended in 500 µl of distilled water. The extract was manually mixed by hand or vortex and centrifuged. For the DON quantification assay, 50 µl of the supernatant was used. 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 quantitative polymerase chain reaction (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 medium consisting of 2% yeast extract, 1.5% sucrose, 0.05% MgSO₄ × 7H₂O, 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).

Catalase activity measurement and ROS detection assay.

For the catalase activity measurement, the wild type, $\Delta FgOS-2$, and $\Delta Fgatf1$ were precultured in YPD and then shifted to liquid CM with and without addition of 0.8 M NaCl and 10 mM H₂O₂. After 2 h of exposure, mycelia were harvested and then mixed with extraction buffer (150 mM KCl, 200 mM EDTA, and 50 mM K₂PO₄, pH 7.4). The resulting homogenate was centrifuged at 16,100 × g for 20 min at 4°C, and the supernatant was used for enzyme assay. 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, Taufkirchen, Germany). The superoxide production in whole fungal colonies was detected by staining with NBT (Sigma). The wild type and $\Delta FgOS-2$ and *Fgatf1* mutant strains were cultured on CM and CM agar plates, which were supplemented with 0.2 M NaCl or 0.8 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.

Expression analysis by semiquantitative reverse-transcriptase PCR and real-time qPCR.

In order to study the expression of possible targets of *FgOS-2* and *Fgatf1* 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 submerged cultures and from wheat spikes at 3 and 7 dpi, respectively, and frozen in liquid nitrogen for subsequent RNA isolation. For reverse transcription (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 real-time qRT-PCR reactions. Transcript levels of the target genes were normalized

against β -tubulin gene expression. The qRT-PCR reactions were carried out using gene-specific primers, with SYBR Green qPCR SuperMix-UDG (Thermo Scientific, Dreieich, Germany) in a volume of 20 μ l in a LightCycler 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 of the measurements were repeated twice, each with three replicates. Relative changes in gene expression were calculated using the comparative cycle threshold method (using LC 480 software for the LightCycler 480). The PCR reactions were conducted with specific primers. The level of constitutively expressed β -tubulin mRNA served as reference.

Microscopic analysis of the germinating conidia, BiFC assays, and FgAtf1 localization studies.

For microscopic analysis of the germinating conidia, the wild type and $\Delta Fgaf1$ strains were precultured in a liquid CM and a CM supplemented with 0.8 M NaCl for 16 h and then centrifuged for 15 min at $16,100 \times g$. The pellets were rinsed and diluted again with double-distilled water. Samples were placed on glass slides and analyzed under a Zeiss microscope Axio Imager Z1 (Carl Zeiss, Jena, Germany). For BiFC assays, conidia of the wild type and BiFC strains were precultured in liquid CM for 16 h and then centrifuged and washed with double-distilled water to remove liquid CM. Mycelia were treated with 1.2 M NaCl and examined by confocal laser-scanning microscopy with filter sets for excitation at 514 nm (YFP) and 594 nm (dsRed) and emission wavelength detection from 519 to 573 nm (YFP) and 588 to 740 nm (dsRed). Untreated samples served as the negative control. For microscopy of the infection progress in wheat, inoculated wheat heads were manually harvested after 7 and 21 dpi and cut longitudinally using a razor blade. Red fluorescence of fungal mycelium was visualized using a Leica MZ FLIII (Leica Microsystems, Heerbrugg, Switzerland) equipped with a 545- to 530-nm excitation and a 620- to 660-nm BP emission filter set. To stain the nuclei, strains were cultured in a liquid CM for 16 h, then centrifuged for 15 min at $16,100 \times g$. The pellets were rinsed and diluted again with distilled water prior to staining. Samples were placed on glass slides and stained with 4',6-diamidino-2-phenylindole at $1 \mu\text{g ml}^{-1}$ for 10 min before being analyzed under a Zeiss microscope Axio Imager Z1 equipped with filter sets for excitation at 538 to 562 nm and emission wavelength detection from 570 to 640 nm.

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