

The bZIP Transcription Factor Fgap1 Mediates Oxidative Stress Response and Trichothecene Biosynthesis But Not Virulence in *Fusarium graminearum*

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Abstract

Redox sensing is of primary importance for fungi to cope with oxidant compounds found in their environment. Plant pathogens are particularly subject to the oxidative burst during the primary steps of infection. In the budding yeast *Saccharomyces cerevisiae*, it is the transcription factor Yap1 that mediates the response to oxidative stress via activation of genes coding for detoxification enzymes. In the cereal pathogen *Fusarium graminearum*, Fgap1 a homologue of Yap1 was identified and its role was investigated. During infection, this pathogen produces mycotoxins belonging to the trichothecenes family that accumulate in the grains. The global regulation of toxin biosynthesis is not completely understood. However, it is now clearly established that an oxidative stress activates the production of toxins by *F. graminearum*. The involvement of Fgap1 in this activation was investigated. A deleted mutant and a strain expressing a truncated constitutive form of Fgap1 were constructed. None of the mutants was affected in pathogenicity. The deleted mutant showed higher level of trichothecenes production associated with overexpression of *Tri* genes. Moreover activation of toxin accumulation in response to oxidative stress was no longer observed. Regarding the mutant with the truncated constitutive form of Fgap1, toxin production was strongly reduced. Expression of oxidative stress response genes was not activated in the deleted mutant and expression of the gene encoding the mitochondrial superoxide dismutase MnSOD1 was up-regulated in the mutant with the truncated constitutive form of Fgap1. Our results demonstrate that Fgap1 plays a key role in the link between oxidative stress response and *F. graminearum* secondary metabolism.

Citation: Montibus M, Ducos C, Bonnin-Verdal M-N, Bormann J, Ponts N, et al. (2013) The bZIP Transcription Factor Fgap1 Mediates Oxidative Stress Response and Trichothecene Biosynthesis But Not Virulence in *Fusarium graminearum*. PLoS ONE 8(12): e83377. doi:10.1371/journal.pone.0083377

Editor: Yin-Won Lee, Seoul National University, Korea, Republic Of

Received: September 23, 2013; **Accepted:** November 1, 2013; **Published:** December 12, 2013

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Funding: M. Montibus PhD fellowship is funded conjointly by the INRA and the Conseil Régional d'Aquitaine. This work is part of the ANR DON and Co project. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Fusarium graminearum (teleomorph *Gibberella zeae*) is the main causal agent of the Fusarium head blight (FHB), a disease that can affect wheat, barley, rice or maize, and potentially causes important economic losses. In addition, the occurrence of FHB is often associated with kernel contamination by mycotoxins that are produced in the field by *Fusarium*. Among the frequently encountered mycotoxins, type B trichothecenes (TCTB) are predominant [1]. Type B trichothecenes consist of deoxynivalenol (DON) and its acetylated C-3 and C-15 derivatives (3ADON and 15ADON, respectively), as well as nivalenol (NIV) and its C-4 acetylated derivative sometimes also called fusarenone X (4ANIV or FX)

[2]. The presence of these mycotoxins in edible grains represents an important problem of food safety [3]. In Europe, maximum acceptable DON levels in food destined for human consumption were established (EC No1126/2007). Accordingly, grains exceeding the established limits are not permitted for commercialization for human consumption. Trichothecenes are heat stable molecules and are not degraded during current food processing [4]. As a result, the best way to restrict trichothecenes content in food is to limit their biosynthesis by the fungus before harvest.

The genes involved in the type B trichothecenes biosynthesis pathway have been identified and named *Tri* genes [1,5,6]. Most *Tri* genes are clustered in the genome in a so-called *Tri5* cluster, named according to the gene coding for the first step of

the biosynthetic pathway. It includes two transcription factors Tri6 and Tri10 [7]. These two proteins activate expression of other *Tri* genes [5,8]. It is however now clearly established that control of TCTB production is largely exerted by various transcriptional regulators known to regulate basal metabolic functions in fungi [9,10]. The full range of mechanisms controlling the accumulation of type B trichothecenes is, nonetheless, far from being completely understood. Indeed, during plant-pathogen interactions, pathogen recognition by the host leads to a plant-released oxidative burst, that consists of the production of reactive oxygen species (ROS) such as H₂O₂, involved in processes related to plant defense mechanisms. In response to such an oxidative burst, pathogens induce oxidative stress response (OSR) mechanisms to scavenge the elevated ROS levels [11-13]. In addition, these ROS also play a role during fungal pathogenesis as demonstrated in the rice blast agent *Magnaporthe oryzae* [14]. The occurrence of an oxidative burst triggered after infection by *F. graminearum* has been demonstrated in barley or wheat [15,16]. Furthermore, previous works showed that treatment with H₂O₂ results in increased toxin accumulation and *Tri* genes expression by *F. graminearum* *in vitro* [17,18]. In that context, previous results suggested that the production of trichothecenes by *F. graminearum* could be a mechanism the fungus triggers to cope with ROS production by the plants [19].

The mechanisms underlying the regulation of TCTB production in the presence of H₂O₂ are largely unknown in *F. graminearum*. In yeasts, a large variety of transcription factors form a regulatory network that is activated by different oxidative stimuli such as peroxides and diamide [20,21]. Within this network, the key role of the well-known ap1 transcription factor Yap1, in response to oxidative stress has been largely documented in the yeast *Saccharomyces cerevisiae* [12,22]. Briefly, Yap1 localizes to the nucleus upon exposure to oxidative stress [23,24], where it activates the transcription of target detoxification genes, such as *cta1* and *ctt1*, coding respectively for peroxisomal and cytosolic catalase [25].

Yap1 homologues have been identified in several filamentous fungi. In the rice blast fungus *M. oryzae*, the homologue of Yap1, Moap1 mediates the response to oxidative stress and is essential for aerial growth, conidia formation and pathogenicity [14]. In *Aspergillus fumigatus*, a truncated version of the Yap1 homologue Afyap1 conferred resistance to oxidative stress [26]. In *Aspergillus parasiticus* and *Aspergillus ochraceus*, Apyap1 and Aoyap1 (the ap1 homologues in both species respectively) were not only involved in response to oxidative stress, but were also demonstrated to play a role in modulation of aflatoxin and ochratoxin A biosynthesis as well [27,28]. This last finding suggested a probable link between the response to oxidative stress and secondary metabolism.

Here, we characterize for the first time *Fgap1*, a homologue of *Yap1*, in *F. graminearum*.

We engineered genetically modified strains of *F. graminearum* lacking *Fgap1* ($\Delta Fgap1$ strain) or expressing a constitutively activated form of the protein (*Fgap1^c trunc* strain) and showed the key role played by *Fgap1* in response to oxidative stress, in relation with the *in vitro* and *in planta* production of TCTB.

Materials and Methods

Fungal strain and culture conditions

Fusarium graminearum INRA 605 (collection INRA from Bordeaux, France) of chemotype DON/15ADON was used throughout the study. In our culture conditions, 15ADON is predominantly detected. Wild-type strain and mutant strains were propagated on Potato Dextrose Agar (PDA, Difco-France) plates and maintained as frozen macroconidial suspensions at -80°C with 25% glycerol.

Growth assays were performed on complete medium [29] supplemented prior solidification with H₂O₂, cadmium, sorbitol or sodium chloride (Sigma Aldrich®, Saint Louis, USA).

Spore suspensions were generated by inoculating fifteen agar plugs in 150 mL of CMC medium (15 g/L carboxymethyl cellulose, 1 g/L yeast extract, 0.5 g/L MgSO₄·7H₂O, 1 g/L NH₄NO₃, 1 g/L KH₂PO₄) [30] and incubating at 150 rpm and 25°C for three days. After filtration through Sefar Nitex 03-100 (100 µm, SEFAR AG - Switzerland), spores were counted on a Thoma cell and used as inoculum for GYEP liquid cultures (50 g/L glucose, 1 g/L yeast extract, 1 g/L peptone).

Ten mL of GYEP in 25 mL Erlenmeyer flasks were inoculated with 10⁵ spores and incubated in the dark, at 25°C and 125 rpm for up to 14 days in the presence or absence of 0.5 mM H₂O₂ (Sigma Aldrich®, Saint-Louis, USA). Cultures were done in triplicate. Cultures were harvested by centrifugation during 15 minutes at 4500 g and 4°C. Supernatants were stored at -20°C until trichothecene levels analysis. Mycelia were desiccated for 48h at 65°C to determine fungal dry biomass. When harvested for total RNA extraction, mycelia were quickly collected by vacuum filtration, rinsed with sterile water, and flash frozen in liquid nitrogen before short-term storage at -80°C until further analysis.

Bacteria and yeast strains used for cloning

Escherichia coli XL1-blue strain (genotype *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacIqZΔM15 Tn10* (Tetr)]) and HST08 strain (genotype *F-*, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, $\Phi 80d$ *lacZΔ M15*, Δ (*lacZYA - argF*) *U169*, Δ (*mrr - hsdRMS - mcrBC*), Δ *mcrA*, λ -) (provided with the In-Fusion® HD Cloning Kit – Clontech) were used for plasmid propagation. *S. cerevisiae* FY1679 diploid strain (donated by François Doignon) of genotype MATa/MATα, *ura3-52/ura3-52*, *trp1Δ63/TRP1*, *leu2Δ/LEU2*, *his3Δ200/HIS3*, *GAL2/GAL2* was used to perform the construction of the truncated gene.

Type B trichothecenes analysis

Four ml of GYEP culture supernatant were extracted with 8 mL of ethyl acetate (Fisher Scientific, Waltham, USA). Five ml of the organic phase were then evaporated to dryness at 50°C under a nitrogen stream. For extraction from wheat samples, 5 volumes of acetonitrile/water (84%/16%) were added to finely grinded wheat kernels. After agitating for 1 hour, and centrifugation, 5 mL of the filtrate were purified using Trichothecene P columns (R-Biopharm, Darmstadt, Germany) before evaporation to dryness at 50°C under a nitrogen stream.

Dried samples were resuspended in 500 μ L of methanol/water (1:1, v/v) (Methanol Fisher Scientific, Waltham, USA) and cleaned up on 0.45 μ m filters (Phenomenex). Quantification analysis was performed on an Agilent Technologies 1100 series HPLC chain, equipped with an Agilent photodiode array detector (DAD) and the ChemStation chromatography manager software (Agilent, Waldbronn, Germany). Separation was achieved on a column kinetex 2.6U XB-C18 (4.6 \times 150mm) maintained at 45°C. The mobile phase consisted of water acidified with orthophosphoric acid to reach pH 2.6 (solvent A) and acetonitrile (solvent B). The flow was kept at 1 mL/min for a total run time of 27 min. The injection volume was set to 5 μ L. TCTB were separated in gradient elution as follows: 93-7% B, 70-30% B in 10 min, 10-90% B in 25 min, 93-7% B in 12 min. UV-VIS spectra were measured from 220 to 550 nm and peak areas were measured at 230 nm. Quantification was performed using external calibration with standard solutions prepared from pure powders (Sigma Aldrich®, Saint-Louis, USA).

In silico identification of Fgap1

The sequences of Yap1 from *S. cerevisiae* (NCBI accession number NP_013707.1) and its homologues in *Schizosaccharomyces pombe* (NCBI accession number NP_593662.1) and *A. fumigatus* (NCBI accession number XP_750882.1) were used as input for BLAST [31] against the *Fusarium* Comparative database from the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_group), to identify sequences with high similarities in the translated genome of *F. graminearum* (version 3). Multiple protein alignments were performed with ClustalW [32].

Engineering of Δ Fgap1, Δ Fgap1:ap1 and Fgap1^{c_{trunc}}

Δ Fgap1 was obtained by replacing the genomic copy of the Fgap1 coding sequence in the *F. graminearum* INRA 605 strain by a hygromycin resistance cassette. First, the flanking regions corresponding to a 988 bp fragment upstream the starting codon and a 800 bp fragment downstream the stop codon and the hygromycin resistance cassette were amplified using the Phusion High-Fidelity DNA polymerase (Finnzymes) as follows: the primers neoHY-1-fin F/neoHY-8-début R (see Table S1) were used to amplify a 2.1 kb fragment corresponding to the hygromycin resistance cassette, from the pBSCkneohygro plamid, containing *hph* gene under the control of the *Neurospora crassa* CPC1 promotor and the *Aspergillus nidulans* *trpC* terminator. The flanking regions were amplified with two pairs of primers, YapAmontlFC F/ YAPAmont-HY R and YAPAval-HY F/ YapAvallFC R respectively (see Table S1). PCR were performed using the following conditions: 98°C for 10 min, 35 \times [98°C for 30 s, 64°C for 30 s, 72°C for 30 s/kb], 72°C for 5 min. The amplified fragments were purified from a 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The In-Fusion® HD Cloning Kit (Clontech, Mountain View, USA) was used for recombination of the three PCR products *via* overlapping hygromycin sequences and cloning into the pBSCk+ (Stratagene), according to the manufacturer's instruction. The construction and sequence fidelity were confirmed by sequencing (Cogenics – Genome

Express Grenoble – France). The construction was amplified using primers YapamF and YapavR and was used to transform protoplasts of the *F. graminearum* strain. The deleted strain selected for further studies was complemented by co-transformation with the wild-type sequence of Fgap1, including 1000 bp of the promotor region and 800 bp of the terminator region, and the pSM334 plasmid [33] carrying the geneticin resistance marker.

Fgap1^{c_{trunc}} was generated in the yeast strain FY1679, maintained on solid YPD (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L). First, the *A. nidulans* promotor pGPD and the terminator TtrpC were amplified from the PAN8.1 plasmid [34] using the primer pairs 5fpGPDdeb F/pGPDyap R and TtrpC F/3rTtrpcfin R respectively (see Table S1). The Fgap1 truncated version (protein truncated after the Q residue at position 517, see Figure S1) of the Fgap1 coding sequence was amplified from the genomic DNA of the *F. graminearum* wild-type strain using the primer pair Yap-pGDP F/Yap-Ttrpc R (see Table S1). PCR was performed using the Phusion High-Fidelity DNA polymerase (Finnzymes) as follows: 98°C for 10 min, 35 \times [98°C for 30 s, 62°C for 30 s, 72°C for 30 s/kb], 72°C for 5 min. The final construction was obtained by transforming the yeast strain FY1679 with the three PCR products and the pRS426 plasmid digested by *Bam*HI and *Hind*III, according to Collopy et al [35], allowing multiple recombinations *via* the overlapping sequences. The full-length construct was finally amplified by PCR using the primers GPDdeb F and Ttrpcfin R and cloned in the pCB1004, carrying the hygromycin resistance cassette [36] before transforming *F. graminearum* wild-type strain. The construction and sequence fidelity were confirmed by sequencing (Cogenics – Genome Express Grenoble – France).

Fungal transformation

A total of 3 \times 10⁷ spores were incubated in 100 mL of GYEP liquid medium for 12h at 25°C and 150 rpm in the dark. Cultures were then filtered on vacuum system, washed with 20 mL of NH₄Cl 1M and treated with 16 mg of Driselase® (Sigma Aldrich®, Saint-Louis, USA) in a final volume of 20 mL of NH₄Cl 1M. After 2 h of incubation at 26°C and 60 rpm, protoplasts were filtered through Sefar Nitex 03-100 (100 μ m, SEFAR AG-Switzerland), collected by centrifugation at 950 g for 10 min and resuspended in 10 mL of 0.6 M Sorbitol in MOPS 10 mM, pH 6.3. The protoplast solution was then carefully layered onto 10 mL of a 1.2 M MgSO₄ pH5.8. After centrifugation at 950 g for 10 min, purified protoplasts were collected at the phases interface and resuspended in 20 mL of sorbitol 1M in MOPS 10 mM pH 6.3. After centrifugation at 950 g for 10 min, the pellet was finally resuspended in 200 μ L of sorbitol /CaCl₂ buffer (Sorbitol 1 M, MOPS 10 mM, CaCl₂ 40 mM, pH 6.3) and kept on ice until transformation.

2 \times 10⁷ protoplasts in 100 μ L of sorbitol /CaCl₂ buffer pH 6.3 were transformed with 5 μ g of target DNA in 60 μ L of Tris-EDTA-CaCl₂ buffer (Tris 10 mM, EDTA 1 mM, CaCl₂ 40 mM, pH7.5) and incubated for 20 min on ice. 160 μ L of PEG 60% in Sorbitol 0.6 M was then added and incubation was carried on for 15 min at room temperature. Protoplasts were finally spread on PDAS agar plates (39 g/L Potato Dextrose Agar and

Table 1. Primers used to amplify cDNA by real-time PCR.

Gene (accession No)	Forward primer (5' to 3')	Reverse primer (5' to 3')	Tm(°C)
<i>GAPDH</i> (FGSG_06257)	TCAAGGGTGTCTGGCCTAC	AGTAACCCCACTCGTTGTCTG	60
<i>Fgap1</i> (FGSG_08800)	AACAGAACGGTGGACAATTC	TGATTGCAGTTCATGTTCTGT	65
<i>Tri4</i> (FGSG_03535)	TATTGTTGGCTACCCCAAGG	TGTCAGATGCGCCTTACAAA	60
<i>Tri5</i> (FGSG_03537)	GACCCTAAGCGACTACAG	GTGCTACGGATAAGGTTC	60
<i>Tri6</i> (FGSG_03536)	AGCGCCTTGCCCTCTTTG	AGCCTTTGGTGCCGACTTCTTG	63
<i>Tri12</i> (FGSG_03541)	ATTCGGTACATGGGCT	GCAGTGTCTTCGTCA	60
<i>Tri101</i> (FGSG_07896)	AGGGCATTAGCGAGGGAAACACA	ACGGCACGCCTTGGAGAGTAGAC	61
<i>Catalase 1</i> (FGSG_06554)	GGAAAGCCAGAACCAACAA	CGTTGACGAGAGTGTAGGTGTTGA	58
<i>Catalase 2</i> (FGSG_02881)	TCTCTCCCTCCAACATGGTTC	CAGGGTGCCTAGACTTGGTT	62
<i>Catalase 3</i> (FGSG_06733)	TCAACCGTCCTGTCTCCTATCC	AGCCTTTTCTTGTGTCTGGTT	60
<i>MnSOD1</i> (FGSG_04454)	CTCTCCCGATCTTCCCTAC	CGGGTAAACATGGACAGAGT	62
<i>MnSOD2</i> (FGSG_02051)	TTGCGTATCGTTACCACCAA	TCAACATAGGCAGCCTTTCC	60
<i>Cu/ZnSOD1</i> (FGSG_08721)	CACACCTTCGGTGACAACAC	GGTCTCGTCAGAAGGAGCAC	62
<i>Cu/ZnSOD2</i> (FGSG_00576)	AGCTCTCGCAGCAGTCTTCTC	CAGGATCAACGTGAATGTGG	60

doi: 10.1371/journal.pone.0083377.t001

sucrose 20%) containing hygromycin B (60 µg/mL) or geneticin (100 µg/mL).

Transformants were purified by monoconidial isolation and screened for successful insertions of the desired constructs by PCR using the primers listed in Table S1. Genomic DNA was extracted from 50 mg of freeze dried mycelium as described previously [37]. Reactions were performed with the GoTaq DNA Polymerase (Promega, France) according to the manufacturer's instructions.

Southern blot analysis

Transformants that passed the PCR pre-screening were further checked by Southern blot analysis, using the DIG system (Roche, Germany) according to a previously described protocol [38]. Five µg of genomic DNA from $\Delta Fgap1$ or *Fgap1*^{trunc} was digested with Sall (Promega, France) or with PstI (Promega, France) respectively, and separated by 0.8% agarose gel electrophoresis. After transfer onto nylon membranes (Hybond N+, Amersham), DIG-labeled probes synthesized with a PCR DIG probe synthesis kit (Roche, Germany), were hybridized following manufacturer's instructions. The probe 1 matches the core sequence of *Fgap1* and was generated by PCR amplification using the primers 5'-AATTCCGGCAATGTCGATCTC-3' and 5'-TGGGACAAGCTTGGAGTTTC-3'. The probe 2 corresponding to the 3' end of *Fgap1*, was generated using the primers 5'-AACAGAACGGTGGACAATTC-3' and 5'-TGTGCCCCACTTGATGACTA-3'.

RNA extraction and cDNA preparation

Fifty mg of freeze-dried mycelium was grinded with the TissueLyser® (Qiagen, Venlo, The Netherlands) for 2 min at 30 Hz, and total RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, Venlo, The Netherlands) following the manufacturer's instructions. Eventual contaminations with gDNA were removed after treatment with the TURBO DNA-free™ Kit (Ambion) following the manufacturer's instructions.

The samples were quantified with the Nanodrop ND 1000 spectrophotometer (Labtech International, UK) and their quality was assessed by agarose gel electrophoresis. 1 µg of total RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies, Invitrogen) with oligo dT priming, according to the manufacturer's instructions and kept at -20°C until further analysis.

Gene expression analysis

Analyses were performed using 1 µL of each cDNA preparation (corresponding to 10 ng of total RNA input in the reverse transcription) mixed in a 10 µL-reaction volume, using the QuantiFast™ SYBR® Green PCR kit (Qiagen, Venlo, The Netherlands). Reactions were carried out on a LightCycler® LC2.0 system equipped with the LightCycler software 3.5.3 (Roche, Germany). For each gene, efficiency was evaluated with serial dilutions of the pooled cDNA samples and data analyses were performed as previously described [17]. Expression levels of the studied genes (see Table 1), normalized to the expression of the housekeeping gene *GAPDH* were performed using REST® software (Relative Expression Software Tool) [39,40].

Plant infection assays

The susceptible spring wheat cultivar Nandu (Lochow-Petkus, Bergen-Wohlde, Germany) was used for virulence assays. Plants were cultivated in a growth chamber at 20°C with a photoperiod of 16 h and 60% relative humidity, and then transferred to infection chambers with optimized conditions. For each strain tested, a suspension of 10³ 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 according to a previously described protocol [41]. The inoculated spikes were enclosed in small plastic bags misted with water for the first 3 days and then monitored for up to three weeks in the infection chambers. Wheat spikes inoculated with 10 µl of pure water were used as the negative control. After three weeks, the

ears were separated from the whole plant; index values were determined by evaluating the proportion of infected spikelets for each spike and is given as the mean \pm SD for all spikes. Spikes were then lyophilized, grinded and conserved at -20°C until toxin analysis.

Expression of results and statistical analysis

All results for trichothecene production were expressed as the arithmetic mean values \pm SD of experiments performed in three independent biological repeats. Results for disease index were expressed as the arithmetic mean values \pm SD of all spikes. Values were compared by performing the Student's *t*-test. The value $p = 0.05$ was chosen as the point of statistical significance. Differential gene expression was analyzed with REST [39,40] using the gene encoding GAPDH as internal reference (5000 permutations, $p \leq 0.05$). Results are reported in the \log_2 value of the ratio mutant vs wild-type strain or treated condition vs control.

Results

Identification of Fgap1, a Yap1 homologue in *F. graminearum*

A genome-wide search of *F. graminearum* for the presence of a putative ap1 protein homologous to the *S. cerevisiae* Yap1, the *Schizosaccharomyces pombe* Pap1, and the *A. fumigatus* Afyap1 was performed using BLAST (see Materials and Methods). We identified the gene FGSG_08800 (Fusarium Comparative Database, accession number), coding for a 588 amino acids-long protein with significant homology with the above ap1 factors, referred to as *Fgap1* from now on. *Fgap1* is 2456 bp-long and contains two introns. Alignment of the *Fgap1* protein sequence to those of Yap1, Pap1 and Afyap1 shows two conserved domains: an N-terminal b-zip domain (basic leucine zipper domain), and a C-terminal nuclear export signal (NES) embedded in a characteristic cysteine-rich domain, or c-CRD [42] (Figure S1). Yap1 and Pap1 classically contains another CRD, the n-CRD (Figure S1), that is absent from *Fgap1*. Previous data showed however that this n-CRD is optional for nuclear localization in *S. cerevisiae* [24] and the function of *Fgap1* is likely unaffected by its absence. *Fgap1* contains 3 others cysteines in the C-terminal part of the protein (Figure S1), whose function remains to be clarified. In conclusion, *Fgap1* contains the bZip domain and the c-CRD domain containing the NES, essential for ap1-like full functions, and the corresponding nucleic sequence can be used for functional analysis of *Fgap1*.

Stress tolerance and *Fgap1*

We analyzed the role of *Fgap1* in tolerance to stress, using reverse genetics approaches. Three different *F. graminearum* mutant strains were engineered. First, we deleted the genomic copy of *Fgap1* in *F. graminearum*, to create a loss-of-function mutant ($\Delta Fgap1$) (Figure S2). We verified using Southern blot that *Fgap1* was properly deleted and mono-copy (Figure S2). An intact copy of *Fgap1* including the promotor and the terminator regions, was subsequently re-introduced in the

$\Delta Fgap1$ strain ($\Delta Fgap1:ap1$) to restore its full functions. Finally, a truncated form of *Fgap1* lacking NES was constructed, therefore expected to be constitutively active (*Fgap1*^{c_{trunc}}), and placed under the control of the constitutive *A. nidulans* promotor GPD and terminator TtrpC (see Figure S2) [36]. This form was introduced ectopically in *F. graminearum* wild-type strain. This construct was inspired by a previous study where a truncated version of *Yap1* was introduced in yeast [43] (Figure S2). We verified by Q-RT-PCR that *Fgap1* was not detected in the deleted mutant, overexpressed in the truncated mutant *Fgap1*^{c_{trunc}}, and expressed as wild-type levels in $\Delta Fgap1:ap1$ mutant. In *M. oryzae*, such a deletion was shown to affect conidiation and radial growth [14]. Conidiation in liquid CMC medium of our mutants as well as growth of mycelia on CM plates after 4 days of culture were not affected (data not shown). Further, we tested $\Delta Fgap1$, $\Delta Fgap1:ap1$, and *Fgap1*^{c_{trunc}} mutants for tolerance to oxidative stress by H₂O₂ or cadmium, and NaCl or sorbitol-induced osmotic stresses (Figure 1). We compared the ability of the wild-type strain and the mutants to grow on agar complete medium supplemented with H₂O₂ 15 mM, NaCl 1 M, sorbitol 1 M, or cadmium 150 μM . Such concentrations did not affect substantially the growth of the wild-type strain after 7 days of growth. The growth of the deleted mutant $\Delta Fgap1$ was however significantly affected. Even after seven days of growth the thallus was severely hampered and never reached the margin of the plate (Figure 1). Complementation restored the wild-type phenotype as shown with the $\Delta Fgap1:ap1$ add-back strain. The growth of the *Fgap1*^{c_{trunc}} mutant was similar to that of the wild-type strain, except for sorbitol or NaCl-induced stresses where the *Fgap1*^{c_{trunc}} mutant exhibited a better adaptation. These results demonstrate that *Fgap1* is involved in the regulation of stress response in *F. graminearum*.

Trichothecene production and *Fgap1*

In order to determine whether or not *Fgap1* is involved in DON and 15ADON production, the wild-type strain, the $\Delta Fgap1$ and the *Fgap1*^{c_{trunc}} strains were cultured for up to 14 days and compared to control in terms of trichothecene production and *Tri* genes levels of expression (Figure 2). Toxins were detectable after 5 days of growth (Figure 2-A). The deleted mutant produced significantly more toxins than the wild-type strain at 5 days and at 14 days whereas the *Fgap1*^{c_{trunc}} mutant produced only traces amounts of trichothecenes, even after 14 days of growth. *Tri* genes expression was measured after 5 days of growth (Figure 2-B). Expression of *Tri 4* and *Tri 101* was slightly up-regulated in the $\Delta Fgap1$ mutant compared to the wild-type strain and all the *Tri* genes were strongly down-regulated in the *Fgap1*^{c_{trunc}} mutant (Figure 2-B). These results strongly imply a role of *Fgap1* in the regulation of trichothecene accumulation and *Tri* genes expression.

In order to confirm the stimulating effect of the deletion of *Fgap1* on trichothecene accumulation, in another experiment, we compared the production of the $\Delta Fgap1:ap1$ add-back strain to that of the *F. graminearum* wild-type strain and the $\Delta Fgap1$ strain. The strains were then cultured for 4 days in GYEP liquid medium. Trichothecene (DON+15ADON) and *Tri5* gene expression levels were measured (Table 2). Again, toxin

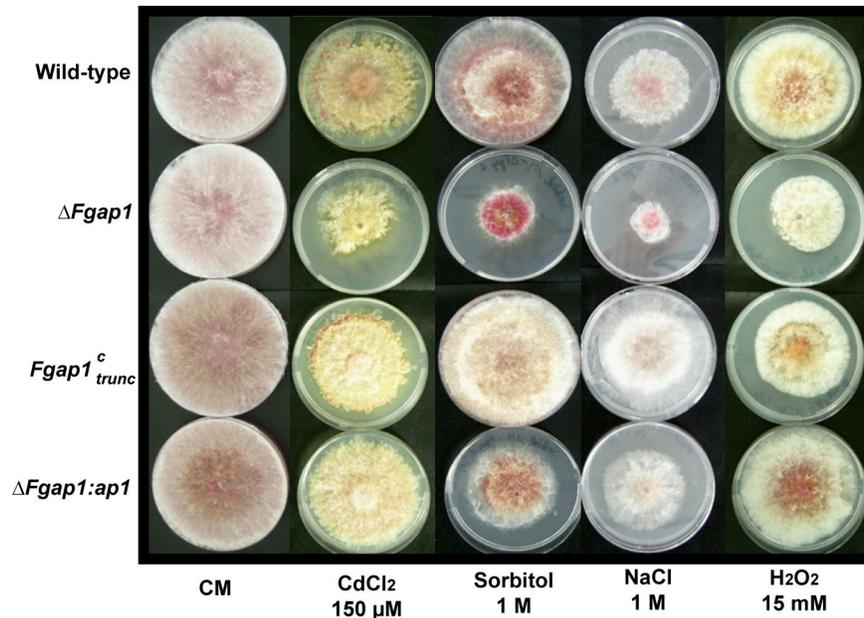


Figure 1. Radial growth assay to evaluate stress tolerance in *F. graminearum* wild-type strain and mutants. *F. graminearum* wild-type strain, $\Delta Fgap1$, $\Delta Fgap1:ap1$, and $Fgap1^c_{trunc}$ were grown 7 days under oxidative stress with cadmium chloride 150 μM (CdCl_2) or H_2O_2 (15 mM), osmotic stress with sorbitol (1 M) or NaCl (1 M).

doi: 10.1371/journal.pone.0083377.g001

accumulation was strongly enhanced in the strain lacking *Fgap1*, with levels 20-fold higher than the wild-type strain. The complementation suppressed this strong increase, restoring a level of production in the same order of magnitude as the wild-type phenotype (Table 2). Concurrently, *Tri5* expression was up-regulated about 5-fold (log₂ ratio of 2.3) in *F. graminearum* $\Delta Fgap1$ strain compared to wild-type, consistent with toxin levels. A level of expression similar to wild-type was restored in the add-back strain $\Delta Fgap1:ap1$ (Table 2). This result reinforces the role of *Fgap1* in the regulation of trichothecenes accumulation in *F. graminearum*.

Pathogenicity of *Fgap1* mutants

Both pathogenicity, i.e. the capacity to infect the inoculated spikelet, and virulence, i.e. the capacity to progress from one spikelet to the other ones below the inoculated point of the *Fgap1* mutant strains, were assessed using point inoculation on wheat.

The wild-type strain, the $\Delta Fgap1$ mutant and the add-back strain were pathogenic, caused serious bleaching, and presented similar disease index value (Figure 3). The $Fgap1^c_{trunc}$ mutant showed a slightly reduced virulence, the spikelets at the bottom of the ears were not bleached and the disease index was slightly lower (Figure 3). However, due to high variability of the values measured for each spike, this difference was not significant.

We further measured the levels of DON and 15ADON produced by each strain tested on the infected ears (Table 3). The wild-type strain and the $\Delta Fgap1:ap1$ add-back strain produced similar levels of TCTB, meanwhile the deleted strain

showed only a slight decrease in trichothecene accumulation. Moreover, the strain expressing the truncated *Fgap1* produced about three times less trichothecenes than the wild-type strain, consistent with the lesser virulence observed for this strain. Altogether, our results demonstrate that *Fgap1* is not essential for pathogenicity in *F. graminearum*.

Fgap1 and trichothecenes B production in response to H_2O_2 mediated oxidative stress

The wild-type strain, the deleted strain $\Delta Fgap1$ and the strain expressing the truncated $Fgap1^c_{trunc}$ were inoculated in liquid cultures supplemented or not with 0.5mM H_2O_2 . Toxin accumulation was measured after 3, 5 or 14 days of growth and *Tri* genes expression were recorded after 5 days of culture. For each trial, treatment with H_2O_2 did not affect the accumulation of fungal biomass. In addition, supplementation with H_2O_2 did not affect pH compared to the not-supplemented control (data not shown).

DON and 15ADON were first detected after 5 days of culture in all cases (Figure 4-A). After 5 days of wild-type strain growth, TCTB accumulate seven times more in treated cultures than in control ones. After 14 days of growth, accumulation is still significantly enhanced in H_2O_2 -treated cultures of *F. graminearum* wild-type (Figure 4-A). The tested *Tri* genes were broadly overexpressed in H_2O_2 -treated wild-type cultures (Figure 4-B), including the gene *Tri101* localized outside the cluster *FgTri5*, consistent with higher toxin accumulation. The regulators *Tri6* and *Tri10* were significantly overexpressed in the presence of H_2O_2 . These results are consistent with our previously published work [17]. In the absence of *Fgap1*

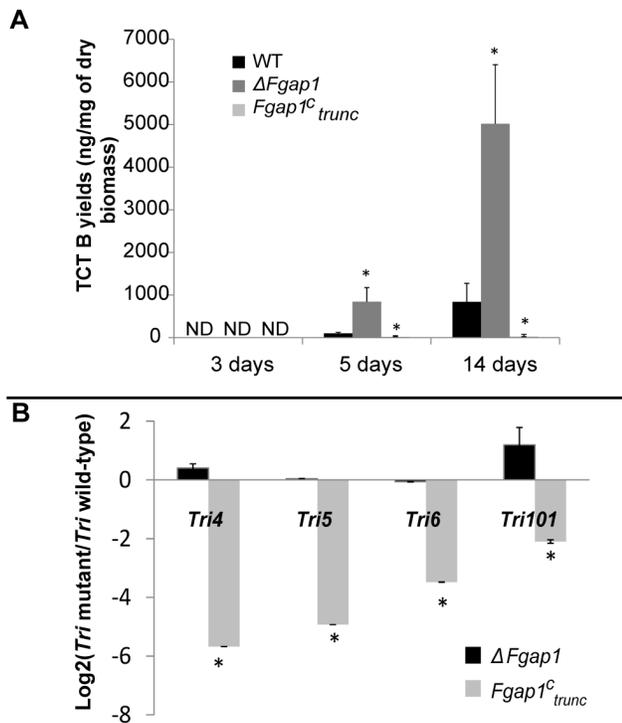


Figure 2. *Fgap1* plays a role in toxin accumulation and *Tri* genes expression. (A) DON + 15ADON yields (in ng of toxins produced per mg of dry fungal biomass) in the wild-type and the mutant strains after 3, 5 and 14 days of growth, ND stands for not detected. Error bars represent the standard deviation of three biological replicates. (B) Expression ratio mutant/wild-type (in log₂ ratio scale) for *Tri* genes after 5 days of culture. The star indicates a significant difference compared to the wild-type ($p < 0.05$).

doi: 10.1371/journal.pone.0083377.g002

Table 2. Toxin analysis and genes expression in the wild-type strain, the $\Delta Fgap1$ and the $\Delta Fgap1:ap1$ after 4 days of growth.

Analysis	Wild-type	$\Delta Fgap1$	$\Delta Fgap1:ap1$
DON+15ADON yield (ng/mg of dry biomass)	10.4 ± 1.3	208.7 ± 90.5 ^a	3.3 ± 2.1 ^a
<i>Tri5</i> expression (log ₂ (Mutant/Wild-type)) ^b	—	2.3 ± 0.2 ^a	0.9 ± 0.08

^a means significantly different from wild-type ; $p \leq 0.05$

^b Expression ratio mutant/wild-type in log₂ scale

doi: 10.1371/journal.pone.0083377.t002

($\Delta Fgap1$), the toxin producing-activating effect of H₂O₂ previously observed for the wild-type strain was no longer visible (Figure 4-A), and *Tri* genes expression does not vary significantly upon treatment (Figure 4-B). On the contrary, the $Fgap1^C_{trunc}$ strain, in which *Fgap1* is truncated and which produced only traces amounts of toxins in control condition,

responded to H₂O₂ only with a slight increase in toxin accumulation (Figure 4-A) and no significant change in *Tri* genes expression was observed (Figure 4-B). All at once, these results demonstrate the strong role played by *Fgap1* in the regulation of toxin accumulation and *Tri* genes expression in response to oxidative stress, in *F. graminearum*.

Fgap1 and genes encoding antioxidant activities

Our initial hypothesis postulates that *Fgap1* is a *Yap1* homologue, able to activate the expression of several genes encoding antioxidant activities required for detoxification upon oxidative stress. The level of expression of *Fgap1* in 5 days-old cultured mycelia was not affected by the treatment with 0.5 mM H₂O₂ (Figure 5-A). These observations suggest, that as in budding yeast, *Fgap1* may be regulated by stress-dependent nuclear localization rather than induced by oxidative stress [23]. We further considered the expression of seven genes (Table 1) encoding catalase or SOD activities (Figure 5), which are potentially under the control of *ap1*. These activities are involved in response to oxidative stress [12] and their expression was recently demonstrated to be *Yap1*-dependent in *A. ochraceus* [27]. The genes encoding the catalases considered in this study refer to the classification established in 2006 for fungal catalases. Catalase 1 corresponds to the spore-specific catalase, catalase 2 to a peroxisomal one, and catalase 3 to a secreted catalase [44]. Among SOD activities, two genes encoding putative MnSOD and two genes encoding putative Cu/ZnSOD were selected.

Three of the seven tested genes were significantly up-regulated under H₂O₂-induced oxidative stress in the wild-type strain (Figure 5-A): *Catalase1*, *Catalase3*, and *MnSOD1*, which showed the highest induction. These data indicate a direct involvement of these three genes in response to oxidative stress.

The expression of the same seven genes encoding antioxidant activities was then analyzed in the two $\Delta Fgap1$ and $Fgap1^C_{trunc}$ mutants and compared to the wild-type after 5 days of growth (Figure 5-B). In the deleted $\Delta Fgap1$ mutant strain, the down-regulation of the three catalases and the two Cu/ZnSOD was consistent with an *Fgap1*-dependant expression (Figure 5-B, black bars). In the strain overexpressing the truncated *Fgap1*, a more than 10 time overexpression of *Fgap1* was observed compared to the wild-type as expected from an expression driven by the constitutive pGDP promoter (Figure 5-B, leftmost grey bar). Considering antioxidant genes, *MnSOD1* was the only one for which a clear overexpression was observed (Figure 5-B). These observations strongly support the role of *Fgap1* in the transcriptional regulation of genes encoding antioxidant activities.

Discussion

In this study, we provide for the first time the functional characterization of *Fgap1* in *F. graminearum*. This protein is likely orthologous to the previously characterized *ap1* fungal proteins, such as the one identified in *S. cerevisiae* [22] or in *Aspergillus* species [27,28]. These *ap1* fungal proteins were shown to play a key role in the plant oxidative stress response



Figure 3. Wheat ears inoculated with *F. graminearum* wild-type strain, $\Delta Fgap1$, $\Delta Fgap1:ap1$, and $Fgap1^c_{trunc}$. Wheat ears 21 days after point inoculation of two central spikelets. Bleaching indicates successful infection. Infection assays were performed with 10 replicates for each strain. For each strain, calculated disease index (% of infected spikelets) was: WT = 90.2 ± 25.6 , $\Delta Fgap1$ = 94.2 ± 11.1 , $\Delta Fgap1:ap1$ = 90.0 ± 22.4 , $Fgap1^c_{trunc}$ = 77.9 ± 25.2 .

doi: 10.1371/journal.pone.0083377.g003

launched during a fungal pathogen infection process. Indeed, the generation of ROS, which is one of the earliest responses of the plant, is a defense mechanism in response of the recognition of the pathogen by the plant. Fungal pathogens have to induce an OSR mechanism to scavenge the elevated ROS levels and to be able to overcome the oxidative burst [11,13].

It is known in several fungi, that *ap1* is one of the major regulators involved in activating OSR. In *S. pombe*, activation of genes encoding antioxidants activities, such as catalase, is mediated by *ap1* [45]. In *Botrytis cinerea* and *A. ochraceus*, several antioxidant activities, such as catalase, superoxide dismutase or thioredoxin, are activated by *ap1* [27,46]. Furthermore, the *ap1* factor was demonstrated to be involved in the pathogenicity of *M. oryzae* or *Alternaria alternata* [14,47]. On the contrary, the factor has no impact on pathogenicity in *B. cinerea* [46] and in the foliar pathogen of maize *Cochliobolus heterostrophus* [48]. This demonstrates that *ap1* proteins are required for full response to oxidative stress, but their role in virulence is divergent and suggests that other factors are redundant in this response to oxidative burst [49].

Here, we have, for the first time, identified *ap1* in *F. graminearum* by sequences homology with the *ap1* proteins of *Aspergillus* spp. and yeasts. This gene, called *Fgap1*, was deleted and a truncated form was created to study the role of *Fgap1* in term of response to stress, trichothecene accumulation and pathogenicity in *F. graminearum*.

We first compared the ability of the wild-type strain and the mutants to grow on complete mediums supplemented with H_2O_2 , NaCl, sorbitol, or cadmium, to mimic several stresses. Our results suggested that in *F. graminearum*, *Fgap1* plays a role in response to these different stresses. Strikingly, the deleted mutant for *Fgap1* was only slightly sensitive to high levels of H_2O_2 . This is different from the results observed for *Candida albicans* or *B. cinerea*, where the mutants were strongly affected in growth [43,46]. This suggests that in *F.*

Table 3. DON + 15ADON levels in wheat ears after 21 days after point inoculation of two central spikelets.

Strain	Toxin levels ($\mu\text{g}/\text{mg}$ dry wheat)
Wild-type	153.4 ± 2.0
$\Delta Fgap1$	113.9 ± 8.1^a
$\Delta Fgap1:ap1$	156.1 ± 1.3
$Fgap1^c_{trunc}$	49.6 ± 10.7^a

^a means significantly different from wild-type ; $p \leq 0.05$

doi: 10.1371/journal.pone.0083377.t003

graminearum, other pathways of response to oxidative stress may compensate the lack of *ap1* in response to H_2O_2 . However, the deleted mutant for *Fgap1* was more sensitive to cadmium, NaCl and sorbitol. Concerning cadmium stress, our results are in full accordance with the observation that in *S. pombe* and *C. albicans*, deletion of *ap1* resulted in an increased sensitivity to cadmium [43,45]. Studies in *S. pombe* have demonstrated that overexpression of *ap1* results in an increased expression of genes encoding ABC-type transporters. These proteins, when overexpressed, result in resistance to drugs [45,50]. These proteins could also be regulated by *ap1* in *F. graminearum*. This could explain the phenotype of our deleted strain in response to cadmium. Furthermore, studies have demonstrated that in *S. pombe*, *ap1* and *Sty1* are part of the same signal transduction pathway. *Sty1* is activated by MAPK pathways in response to numerous stresses [45]. This could explain the phenotype of the *F. graminearum* deleted *Fgap1* strain in response to osmotic stress.

We previously showed that oxidative stress stimulated the accumulation of type B trichothecenes and *Tri* genes expression [17]. Here we clearly showed that *Fgap1* was involved in the regulation of toxin accumulation through

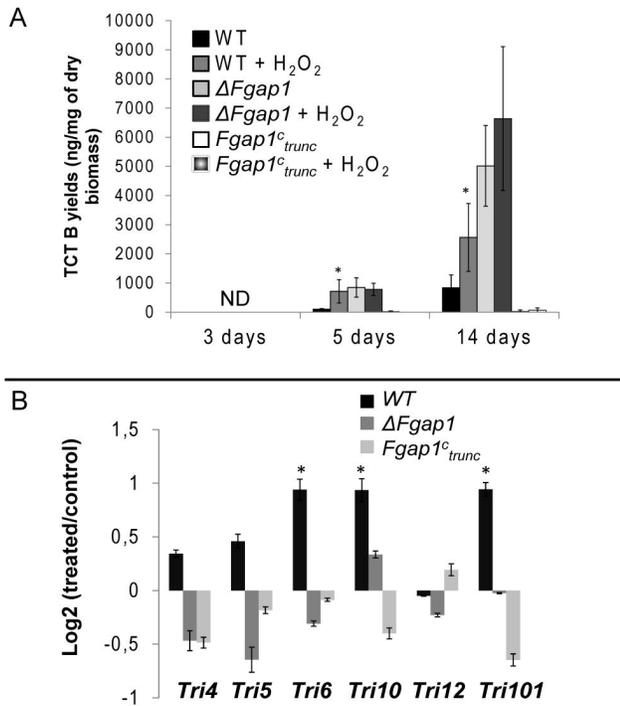


Figure 4. Toxin production and *Tri* genes expression in the wild-type strain and in the $\Delta Fgap1$ and $Fgap1^{c_{trunc}}$ mutants after exposure to oxidative stress by H₂O₂. *F. graminearum* wild-type or mutants were cultured up to 14 days in liquid medium supplemented or not supplemented with 0.5 mM H₂O₂. (A) DON + 15ADON yield (ng of toxins per mg of dry fungal biomass) after 3, 5 or 14 days of growth. Error bars represent the standard deviation of three biological replicates. The star indicates a significant difference for the treated condition compared to the control condition ($p < 0.05$). (B) *Tri* genes expression (expression ratios treated/not treated in log₂ scale) in 5 day-old cultures. *Tri4*, *Tri5*, *Tri6*, *Tri10*, *Tri12*, and *Tri101* were considered. The star indicates a significant difference of expression between treated vs. not treated ($p < 0.05$).

doi: 10.1371/journal.pone.0083377.g004

modulation of *Tri* genes expression. The fact that the absence of ap1 resulted in an increase in toxin accumulation and *Tri* genes expression argue, however, in favor of an indirect effect. This is corroborated by the fact that the overexpression of the truncated form of *Fgap1* resulted in a strong down-regulation of *Tri* genes expression and toxin accumulation *in vitro*. These results correlate with those observed in *A. parasiticus* and in *A. ochraceus*, where a deleted strain for ap1 produced more toxins than the wild-type strain [27,28]. H₂O₂ was demonstrated to accumulate in the media during the culture of *F. graminearum* [51]. We can make the hypothesis that the ap1 deleted mutant is unable to scavenge properly this excess of oxidant. Then, an increase of trichothecenes production, which consumes oxygen species, could represent an alternative way to reduce this excess [11].

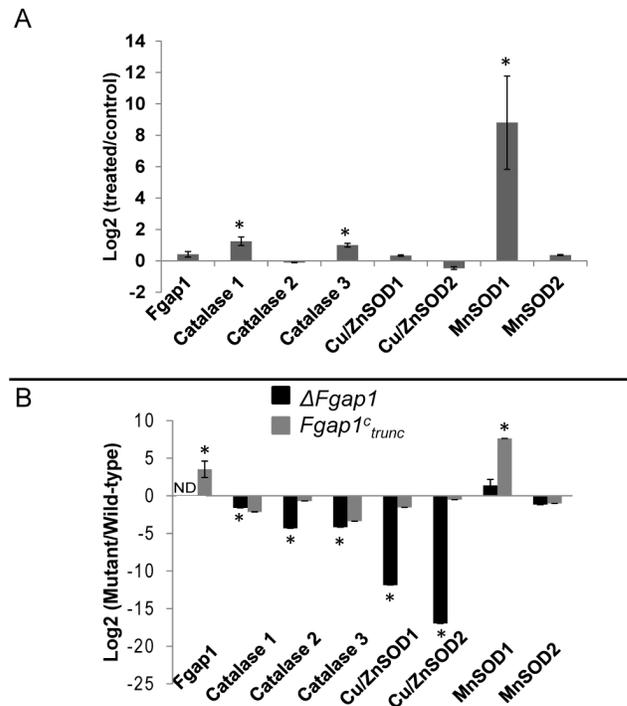


Figure 5. Expression of genes encoding antioxidant activities in the wild-type strain and in the $\Delta Fgap1$ and $Fgap1^{c_{trunc}}$ mutants. (A) Antioxidant genes expression (expression ratio treated/not treated in log₂ scale) in the wild-type strain in 5-day old cultures. The star indicates a significant difference between treated vs. not treated ($p < 0.05$). ND stands for not detected. (B) Antioxidant genes expression in the mutants strain compared to wild-type strain (expression ratios mutant/wild-type strain in log₂ scale) in 5-day old cultures. The star indicates a significant difference for mutant compared to wild-type ($p < 0.05$).

doi: 10.1371/journal.pone.0083377.g005

In our results, the wild-type strain showed an overexpression of *Tri* genes and an increased level of trichothecene accumulation in response to oxidative stress. This response was lost in the deleted mutant. Expression of the truncated form of *Fgap1* led to a strong down regulation of *Tri* genes and very few amounts of toxins even when an oxidative stress was applied. These results clearly demonstrated that *Fgap1* is involved in the regulation of trichothecene production in response to oxidative stress. The fact that overexpression of the truncated form strongly decreased the accumulation of trichothecenes suggests that the regulation by *Fgap1* is indirect. The truncation deletes the NES and is supposed to target *Fgap1* in the nucleus. If *Fgap1* was directly involved in *Tri* genes regulation, one could expect an increase of *Tri* genes expression. We can then raise the hypothesis that this *Tri* genes regulation operates via the control of the expression of one or various genes of which expression is controlled by ap1.

It has already been demonstrated that ap1 was involved in the activation of OSR genes in several fungi [27,45]. *Catalase* expression and *sod* expression were especially reduced in an

A. ochraceus $\Delta Aoyap1$ mutant. As expected, *Fgap1* seems also strongly involved in the modulation of genes encoding antioxidant activities in *F. graminearum* as the absence of the gene resulted in a significant down regulation of the genes encoding catalase or sod activities. Surprisingly, for the truncated mutant, only one of these genes was strongly overexpressed. This gene encodes a putative MnSOD1. Others studies demonstrated that in *S. cerevisiae*, both *ap1* and *skn7* are necessary to activate genes involved in response to oxidative stress, via the NES of *ap1*. Here, the absence of the NES could impair the activation of OSR genes as the cooperation with *skn7* become impossible [52]. This could explain why OSR genes are strongly down regulated when *Fgap1* is absent but not up-regulated when *Fgap1* is truncated. In order to explain the fact that the truncated version of *Fgap1* results in a strong overexpression of MnSOD1, we raise the hypothesis that this gene only requires *ap1* for its activation and does not need the interaction with *skn7*. Blastp with this MnSOD1 showed that this sequence is homologous to the mitochondrial SOD of *S. cerevisiae*. Mitochondria are particularly sensitive to oxidative stress and play a central role in fungal cells [53]. This raises the hypothesis that regulation of the trichothecene pathway may be interconnected with the regulation of the mitochondrial metabolism of oxidative species. This observation is in agreement with the already described connection of secondary metabolism with mitochondrial metabolism [54,55].

Our results demonstrate that even if the *ap1* deleted strain produced more toxins than the wild-type strain *in vitro*, the deletion had no impact on pathogenicity and virulence *in planta*. This last result is consistent with results observed with *B. cinerea* or *C. heterostrophus* where the deletion of the *ap1* factor showed no effect on virulence [46,48] but is opposite to the central role in virulence reported for *M. oryzae* [14]. This difference could come from the fact that the function of *ap1* as pathogenicity factor is limited to biotroph pathogens [46].

To our knowledge, this is the first time that such a truncated form of *ap1* was tested for pathogenicity. This mutant appears slightly less virulent and seems to produce lower levels of trichothecene *in planta* than the wild-type strain. It was demonstrated that trichothecene production in *F. graminearum* acts as an aggressiveness factor [56]. Therefore, the slight reduction in virulence we observed could result from the decrease in trichothecene production. Our result *in planta* differs slightly from the results obtained *in vitro* where the strain with the truncated *ap1* consistently produced very low level of toxins. The difference observed between *in vitro* and *in planta* data could result from the occurrence of other compounds *in planta* such as agmatine, for example, that can stimulate toxin production and mask the effect of *ap1* modification [57]. Trichothecene induction by plant compounds could then greatly hide the negative effect of the truncated *ap1*.

Our study demonstrates that the regulation of the trichothecene pathway is linked to the metabolism of oxidative species in *F. graminearum* and that *Fgap1* plays a central role in this regulation. According to our results, antioxidant activities controlled by *Fgap1*, such as mitochondrial MnSOD1, may be involved in OSR control, and could also play a key role in

regulation of TCTB production. Further studies are needed to fully understand the role played by *Fgap1* in the network involved in the regulation of response to stress and its link with secondary metabolism.

Supporting Information

Figure S1. Multiple sequence alignment of *Fgap1* with others *ap1* homologous sequences. Sequences from *F. graminearum* (FGSG_08800.3), *A. fumigatus* (XP_750882.1), *S. pombe* (NP_593662.1) and *S. cerevisiae* (NP_013707.1) were aligned with ClustalW online software. Conserved regions are shaded. Two domains are conserved: the b-zip domain, with the basic region and the leucine zipper and the nuclear export signal, embedded within the c-CRD. Blue arrows correspond to the cysteine residues of the c-CRD. Green arrows correspond to the n-CRD found in *Yap1* and *Pap1*. Red stars correspond to the cysteine residues found only in the C-terminal part of *Fgap1*. (DOCX)

Figure S2. Construction of $\Delta Fgap1$ and *Fgap1*^{c trunc} mutants. (A) Maps of *Fgap1wt* locus, the $\Delta Fgap1$ construct containing the hygromycin resistance cassette and the *Fgap1*^{c trunc} construction. * corresponds to the integrated codon stop. Bars represent labeled probes 1 and 2 used for the southern blot hybridization. For the $\Delta Fgap1$ construct, among the 21 hygromycin resistant transformants, only 6 contained the replacement construct integrated at the *Fgap1* locus. The transformant number 15 was selected for complementation with the wild-type copy of *Fgap1*. Both transformant number 15 and one of the wild-type complemented transformants were submitted to a control by Southern blot analysis, using probe 1 or 2. Vertical black arrows stand for *SalI* restriction sites. For the *Fgap1*^{c trunc} construct, among 27 hygromycin resistant transformants, 5 contained the truncated construction. They were cultured in GYEP medium and after 11 days of growth, 4 produced few amounts of toxins. Overexpression of *Fgap1* was analyzed by Q-RT-PCR. They were analyzed by southern blot using digestion with *PstI* and the probe 2 to ensure that the construction was ectopic and to control the number of integrations. One transformant presented one integration and was selected for further experiments (data not shown). This transformant was analyzed by Q-RT-PCR analysis, confirming that *Fgap1* was overexpressed 10 times. This mutant was called *Fgap1*^{c trunc} and retained for further experiments. (B) Southern blot hybridization. 1: pBCSK $\Delta Fgap1$ (plasmid carrying the deletion construction); 2: wild-type strain; 3: $\Delta Fgap1$; 4: $\Delta Fgap1:ap1$. In the pBCSK $\Delta Fgap1$, probe 2 reveals a fragment of 3.9 kb when digested with *BamHI*. In the wild type strain, probe 1 and 2 reveal a 3.3 kb fragment when digested with *SalI*. In the $\Delta Fgap1$ strain, probe 2 reveals a 4.8 kb fragment when digested with *SalI*. In the complemented strain $\Delta Fgap1:ap1$, probe 1 and probe 2 reveal a fragment of unknown size due to ectopic integration of the wild-type copy of *Fgap1* when digested with *SalI*. (DOCX)

Table S1. List of primers used for vector constructions and transformants screening. ^aCapitalized sequences in the YAPAmont-HY R and YAPAvall-HY F primers correspond to the hygromycin resistance cassette to allow overlapping between the 5'UTR, the hygromycin resistance cassette and the 3'UTR. ^bSequences underlined in the YapAmontIFC F and YapAvallIFC R primers correspond to the multiple cloning site of the pBCSK plasmid digested with *Bam*HI. ^cSequences underlined in the 5fpGPDdeb F and 3rTtrpcfin R primers correspond to the multiple cloning site of the pRS426 plasmid digested with *Bam*HI and *Hind*III. ^dCapitalized sequences in the Yap-pGPD F and Yap-TtrpC R primers correspond to pGPD and TtrpC sequences respectively to allow overlapping between pGPD, *Fgap1* and TtrpC.

References

- Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M (2007) Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. *Biosci Biotechnol Biochem* 71: 2105-2123. doi:10.1271/bbb.70183. PubMed: 17827683.
- Champeil A, Dore T, Fourbet JF (2004) *Fusarium* head blight: epidemiological origin of the effects of cultural practices on head blight attacks and the production of mycotoxins by *Fusarium* in wheat grains. *Plant Sci* 166: 1389-1415. doi:10.1016/j.plantsci.2004.02.004.
- Goswami RS, Kistler HC (2004) Heading for disaster: *Fusarium* graminearum on cereal crops. *Mol Plant Pathol* 5: 515-525. doi:10.1111/j.1364-3703.2004.00252.x. PubMed: 20565626.
- Rocha O, Ansari K, Doohan FM (2005) Effects of trichothecene mycotoxins on eukaryotic cells: A review. *Food Addit Contam* 22: 369-378. doi:10.1080/02652030500058403. PubMed: 16019807.
- Alexander NJ, Proctor RH, McCormick SP (2009) Genes, gene clusters, and biosynthesis of trichothecenes and fumonisins in *Fusarium*. *Toxin Reviews* 28: 198-215. doi:10.1080/15569540903092142.
- Desjardins AE, Hohn TM, McCormick SP (1993) Trichothecene Biosynthesis in *Fusarium* Species - Chemistry, Genetics, and Significance. *Microbiol Rev* 57: 595-604. PubMed: 8246841.
- McCormick SP, Stanley AM, Stover NA, Alexander NJ (2011) Trichothecenes: From Simple to Complex Mycotoxins. *Toxins (Basel)* 3: 802-814. doi:10.3390/toxins3070802. PubMed: 22069741.
- Seong KY, Pasquali M, Zhou X, Song J, Hilburn K et al. (2009) Global gene regulation by *Fusarium* transcription factors Tri6 and Tri10 reveals adaptations for toxin biosynthesis. *Mol Microbiol* 72: 354-367. doi:10.1111/j.1365-2958.2009.06649.x. PubMed: 19320833.
- Kazan K, Gardiner DM, Manners JM (2012) On the trail of a cereal killer: recent advances in *Fusarium* graminearum pathogenomics and host resistance. *Mol Plant Pathol* 13: 399-413. doi:10.1111/j.1364-3703.2011.00762.x. PubMed: 22098555.
- Merhej J, Richard-Forget F, Barreau C (2011) Regulation of trichothecene biosynthesis in *Fusarium*: recent advances and new insights. *Appl Microbiol Biotechnol* 91: 519-528. doi:10.1007/s00253-011-3397-x. PubMed: 21691790.
- Heller J, Tudzynski P (2011) Reactive Oxygen Species in Phytopathogenic Fungi: Signaling, Development, and Disease. In: NK VanAlfenG BrueningJE Leach. *Annual Review of Phytopathology*, Vol. 49. Palo Alto: Annual Reviews. pp. 369-390.
- Montibus M, Pinson-Gadais L, Richard-Forget F, Barreau C, Ponts N (2013) Coupling of transcriptional response to oxidative stress and secondary metabolism regulation in filamentous fungi. *Crit Rev Microbiol*. PubMed: 24041414
- Shetty N, Jørgensen H, Jensen J, Collinge D, Shetty H (2008) Roles of reactive oxygen species in interactions between plants and pathogens. *European Journal of Plant Pathology* 121: 267-280. doi:10.1007/s10658-008-9302-5.
- Guo M, Chen Y, Du Y, Dong Y, Guo W et al. (2011) The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. *PLoS Pathog* 7: e1001302. PubMed: 21383978.
- Yang F, Svensson B, Finnie C (2011) Response of germinating barley seeds to *Fusarium* graminearum: The first molecular insight into *Fusarium* seedling blight. *Plant Physiol Biochem* 49: 1362-1368. doi:10.1016/j.plaphy.2011.07.004. PubMed: 21798752.
- Zhou WC, Kolb FL, Riechers DE (2005) Identification of proteins induced or upregulated by *Fusarium* head blight infection in the spikes of hexaploid wheat (*Triticum aestivum*). *Genome* 48: 770-780. doi:10.1139/g05-041. PubMed: 16391683.
- Ponts N, Pinson-Gadais L, Barreau C, Richard-Forget F, Ouellet T (2007) Exogenous H₂O₂ and catalase treatments interfere with Tri genes expression in liquid cultures of *Fusarium graminearum*. *FEBS Lett* 581: 443-447. doi:10.1016/j.febslet.2007.01.003. PubMed: 17250833.
- Ponts N, Pinson-Gadais L, Verdal-Bonnin MN, Barreau C, 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 Microbiol Lett* 258: 102-107. doi:10.1111/j.1574-6968.2006.00200.x. PubMed: 16630263.
- Ponts N, Pinson-Gadais L, Richard-Forget F (2003) H₂O₂ effects on trichothecenes B (DON, ADON) production by *Fusarium graminearum* in liquid culture. *Aspects of Applied Biology* 68.
- Moye-Rowley WS (2003) Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. *Eukaryotic Cell* 2: 381-389. doi:10.1128/EC.2.3.381-389.2003. PubMed: 12796283.
- Vivancos AP, Jara M, Zuin A, Sansó M, Hidalgo E (2006) Oxidative stress in *Schizosaccharomyces pombe*: different H₂O₂ levels, different response pathways. *Molecular Genetics and Genomics* 276: 495-502. doi:10.1007/s00438-006-0175-z. PubMed: 17043891.
- Rodrigues-Pousada C, Menezes RA, Pimentel C (2010) The Yap family and its role in stress response. *Yeast* 27: 245-258. doi:10.1002/yea.1752. PubMed: 20148391.
- Kuge S, Jones N, Nomoto A (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J* 16: 1710-1720. doi:10.1093/emboj/16.7.1710. PubMed: 9130715.
- Yan C, Lee LH, Davis LI (1998) Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. *EMBO J* 17: 7416-7429. doi:10.1093/emboj/17.24.7416. PubMed: 9857197.
- Jamieson DJ (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast* 14: 1511-1527. doi:10.1002/(SICI)1097-0061(199812)14:16. PubMed: 9885153.
- Qiao J, Liu W, Li R (2010) Truncated Afpap1 Attenuates Antifungal Susceptibility of *Aspergillus fumigatus* to Voriconazole and Confers Adaptation of the Fungus to Oxidative Stress. *Mycopathologia*. Springer. pp. 155-160.
- Reverber M, Gazzetti K, Punelli F, Scarpari M, Zjalic S et al. (2012) Aoyap1 regulates OTA synthesis by controlling cell redox balance in *Aspergillus ochraceus*. *Appl Microbiol Biotechnol* 95: 1293-1304. doi:10.1007/s00253-012-3985-4. PubMed: 22410746.
- Reverber M, Zjalic S, Ricelli A, Punelli F, Camera E et al. (2008) Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the AfpapA gene. *Eukaryotic Cell* 7: 988-1000. doi:10.1128/EC.00228-07. PubMed: 18441122.
- Ilgen P, Haderl B, Maier FJ, Schäfer W (2009) Developing Kernel and Rachis Node Induce the Trichothecene Pathway of *Fusarium graminearum* During Wheat Head Infection. *Molecular Plant-Microbe Interactions* 22: 899-908. doi:10.1094/MPMI-22-8-899.
- Cappellini R, Peterson J (1965) CMC medium for stimulation of sporulation in *Fusarium graminearum*. *Mycologia* 57: 962-966. doi:10.2307/3756895.

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Acknowledgements

The authors thank Vance C. Huskins for editing and proofreading this research article.

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Conceived and designed the experiments: MM CB FF NP JB. Performed the experiments: MM CD MNBV JB. Analyzed the data: MM CB FF NP. Wrote the manuscript: MM CB FF NP JB.

31. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410. doi:10.1016/S0022-2836(05)80360-2. PubMed: 2231712.
32. Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics Chapter 2: Unit 2 3*
33. Marek ET, Schardl CL, Smith DA (1989) Molecular transformation of *Fusarium solani* with an antibiotic resistance marker having no fungal DNA homology. *Curr Genet* 15: 421-428. doi:10.1007/BF00376799. PubMed: 2550150.
34. Punt PJ, Dingemans MA, Kuyvenhoven A, Soede RDM, Pouwels PH et al. (1990) Functional elements in the promoter region of the *Aspergillus nidulans* *gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene* 93: 101-109. doi:10.1016/0378-1119(90)90142-E. PubMed: 2121607.
35. Colopy PD, Colot HV, Park G, Ringelberg C, Crew CM et al. (2010) High-throughput construction of gene deletion cassettes for generation of *Neurospora crassa* knockout strains. *Methods Mol Biol* 638: 33-40. PubMed: 20238259.
36. Carroll AM, Sweigard AJ, Valent B (1994) Improved vectors for selecting resistance to hygromycin. *Fungal Genet Newsl* 41: 22.
37. Javerzat JP, Bhattacherjee V, Barreau C (1993) Isolation of telomeric DNA from the filamentous fungus *Podospora anserina* and construction of a self-replicating linear plasmid showing high transformation frequency. *Nucleic Acids Res* 21: 497-504. doi:10.1093/nar/21.3.497. PubMed: 8441663.
38. Florent M, Noël T, Ruprich-Robert G, Da Silva B, Fitton-Ouhabi V et al. (2009) Nonsense and Missense Mutations in *FCY2* and *FCY1* Genes Are Responsible for Flucytosine Resistance and Flucytosine-Fluconazole Cross-Resistance in Clinical Isolates of *Candida lusitanae*. *Antimicrob Agents Chemother* 53: 2982-2990. doi:10.1128/AAC.00880-08. PubMed: 19414575.
39. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30: 1-10. doi:10.1093/nar/30.1.1. PubMed: 11752241.
40. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: 2002-2007. PubMed: 11328886.
41. Jenczmionka NJ, Schäfer W (2005) The *Gpmk1* MAP kinase of *Fusarium graminearum* regulates the induction of specific secreted enzymes. *Curr Genet* 47: 29-36. doi:10.1007/s00294-004-0547-z. PubMed: 15549317.
42. Rodrigues-Pousada CA, Nevitt T, Menezes R, Azevedo D, Pereira J et al. (2004) Yeast activator proteins and stress response: an overview. *FEBS Lett* 567: 80-85. doi:10.1016/j.febslet.2004.03.119. PubMed: 15165897.
43. Alarco AM, Raymond M (1999) The *bZip* transcription factor *Cap1p* is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J Bacteriol* 181: 700-708. PubMed: 9922230.
44. Giles SS, Stajich JE, Nichols C, Gerrald QD, Alspaugh JA et al. (2006) The *Cryptococcus neoformans* Catalase Gene Family and Its Role in Antioxidant Defense. *Eukaryotic Cell* 5: 1447-1459. doi:10.1128/EC.00098-06. PubMed: 16963629.
45. Toone WM, Kuge S, Samuels M, Morgan BA, Toda T et al. (1998) Regulation of the fission yeast transcription factor *Pap1* by oxidative stress: requirement for the nuclear export factor *Crm1* (Exportin) and the stress-activated MAP kinase *Sty1/Spc1*. *Genes Dev* 12: 1453-1463. doi:10.1101/gad.12.10.1453. PubMed: 9585505.
46. Temme N, Tudzynski P (2009) Does botrytis cinerea ignore H(2)O(2)-induced oxidative stress during infection? Characterization of Botrytis activator protein 1. *Mol Plant Microbe Interact* 22: 987-998. doi:10.1094/MPMI-22-8-0987. PubMed: 19589074.
47. Yang SL, Lin C-H, Chung K-R (2009) Coordinate control of oxidative stress tolerance, vegetative growth, and fungal pathogenicity via the AP1 pathway in the rough lemon pathotype of *Alternaria alternata*. *Physiological and Molecular Plant Pathology* 74: 100-110. doi:10.1016/j.pmpp.2009.09.007.
48. Lev S, Hadar R, Amedeo P, Baker SE, Yoder OC et al. (2005) Activation of an AP1-like transcription factor of the maize pathogen *Cochliobolus heterostrophus* in response to oxidative stress and plant signals. *Eukaryotic Cell* 4: 443-454. doi:10.1128/EC.4.2.443-454.2005. PubMed: 15701806.
49. Nguyen TV, Kröger C, Bönninghausen J, Schäfer W, Bormann J (2013) The ATF/CREB transcription factor *Atf1* is essential for full virulence, deoxynivalenol production and stress tolerance in the cereal pathogen *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*.
50. Nagao K, Taguchi Y, Arioka M, Kadokura H, Takatsuki A et al. (1995) *bfr1+*, a novel gene of *Schizosaccharomyces pombe* which confers brefeldin A resistance, is structurally related to the ATP-binding cassette superfamily. *J Bacteriol* 177: 1536-1543. PubMed: 7883711.
51. Ponts N, Couedelo L, Pinson-Gadais L, Verdal-Bonnin MN, Barreau C et al. (2009) *Fusarium* response to oxidative stress by H2O2 in trichothecene chemotype-dependent. *FEMS Microbiol Lett* 293: 255-262. doi:10.1111/j.1574-6968.2009.01521.x. PubMed: 19239497.
52. Lee J, Godon C, Lagniel G, Spector D, Garin J et al. (1999) *Yap1* and *Skn7* control two specialized oxidative stress response regulons in yeast. *J Biol Chem* 274: 16040-16046. doi:10.1074/jbc.274.23.16040. PubMed: 10347154.
53. Osiewacz HD (2011) Mitochondrial quality control in aging and lifespan control of the fungal aging model *Podospora anserina*. *Biochem Soc Trans* 39: 1488-1492. doi:10.1042/BST0391488. PubMed: 21936839.
54. Kiel JA, van der Klei IJ, van den Berg MA, Bovenberg RA, Veenhuis M (2005) Overproduction of a single protein, *Pc-Pex11p*, results in 2-fold enhanced penicillin production by *Penicillium chrysogenum*. *Fungal Genet Biol* 42: 154-164. doi:10.1016/j.fgb.2004.10.010. PubMed: 15670713.
55. Maggio-Hall LA, Wilson RA, Keller NP (2005) Fundamental contribution of beta-oxidation to polyketide mycotoxin production in planta. *Mol Plant Microbe Interact* 18: 783-793. doi:10.1094/MPMI-18-0783. PubMed: 16134890.
56. Proctor RH, Hohn TM, McCormick SP (1995) Reduced virulence of *Gibberella zeae* caused by disruption of a trichothecene toxin biosynthetic gene. *Mol Plant Microbe Interact* 8: 593-601. doi:10.1094/MPMI-8-0593. PubMed: 8589414.
57. Gardiner DM, Kazan K, Manners JM (2009) Nutrient profiling reveals potent inducers of trichothecene biosynthesis in *Fusarium graminearum*. *Fungal Genet Biol* 46: 604-613. doi:10.1016/j.fgb.2009.04.004. PubMed: 19406250.