



Degradation of salicylic acid by *Fusarium graminearum*

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ABSTRACT

Fusarium head blight (FHB) is a major cereal crop disease, caused most frequently by the fungus *Fusarium graminearum*. We have previously demonstrated that *F. graminearum* can utilize SA as sole source of carbon to grow. In this current study, we further characterized selected four fungal SA-responsive genes that are predicted to encode salicylic acid (SA)-degrading enzymes and we used a gene replacement approach to characterize them further. These included two genes predicted to encode a salicylate 1-monooxygenase, *FGSG_03657* and *FGSG_09063*, a catechol 1, 2-dioxygenase gene, *FGSG_03667*, and a 2, 3-dihydroxybenzoic acid decarboxylase gene, *FGSG_09061*. For each gene, three independent gene replacement strains were assayed for their ability to degrade salicylic acid in liquid culture. Salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* were shown to be essential for SA degradation, while a loss of 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* caused only a partial reduction of SA degradation and a loss of salicylate 1-monooxygenase *FGSG_09063* had no effect when compared to wild type culture. Salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* were identified as the first two key enzyme steps of SA degradation via catechol in the β -ketoadipate pathway. Expression profiles for all four genes were also determined in liquid culture and *in planta*. Salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* were co-expressed and their expression was substrate dependent in liquid culture; however their expression was uncoupled in *planta*. Disruption of the gene for catechol 1, 2-dioxygenase *FGSG_03667* was shown to have no effect on fungal virulence on wheat. Our results with 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* raise the possibility of an alternate non-oxidative decarboxylation pathway for the conversion of SA to catechol via 2, 3-dihydroxybenzoic acid and for a connection between the oxidative and the non-oxidative decarboxylation pathways for SA conversion.

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1. Introduction

Fusarium graminearum Schwabe (*Hypocreales: Nectriaceae*) is the main agent of fusarium head blight (FHB) in North America, causing devastating small grain crop losses and grain contamination, which are tied to enormous economic and social impacts (Windels, 2000). FHB raises concerns for food and feed safety worldwide; this has led to intense international efforts to manage the problem using multiple strategies, including genetic

crop improvement, especially in wheat (Xue et al., 2009; Zhang et al., 2011).

Salicylic acid (SA) has been studied extensively over the last decades, prominently for its significant role in plant immunity as a signaling molecule in the elicitation of systemic acquired resistance (SAR) against a broad range of phytopathogens (Gaffney et al., 1993; Rivas San Vicente and Plasencia, 2011). To date, many aspects of SA-triggered immunity have been widely described (Asai and Shirasu, 2015; Bari and Jones, 2009). Increase in activity of SA signaling pathways has been observed in wheat during the early phase of infection by *F. graminearum* (Ding et al., 2011; Erayman et al., 2015). Some reports have demonstrated that SA plays a crucial role in that pathosystem, conferring a protection against FHB (Makandar et al., 2012; Sorahinobar et al., 2015); however other experiments have shown an absence of effect of SA on FHB symptom levels (Li and Yen, 2008).

Abbreviations: SA, salicylic acid; USER, Uracil-Specific-Excision Reagent; WT, parental wild type strain; dpi, days post inoculation; SNA, synthetic nutrient deficient agar.

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Many phytopathogens have developed subtle strategies to reduce or counteract SA-dependent plant defense mechanisms, including repression of SA biosynthesis or of key regulatory components of the SA signaling pathway, degradation of SA, or alteration of the antagonistic connection between SA and jasmonic acid (Asai and Shirasu, 2015). For example, it has been shown that *Sclerotinia sclerotiorum* can degrade salicylate and that *F. graminearum* can use SA as main carbon source (Penn and Daniel, 2013; Qi et al., 2012).

In fungi, oxidative degradation pathways of phenolic compounds, including SA, proceed generally in three phases (Harwood and Parales, 1996). First, one or more ring modifications are executed, including a mono- or dioxygenation step, resulting in the formation of one of three key intermediates: catechol, protocatechuate or gentisate. In a second phase, a dioxygenase catalyses a ring fission on catechol (ortho-cleavage), protocatechuate (meta-cleavage) or gentisate (para-cleavage). The third phase consists in 2 or 3 subsequent reactions resulting in the production of β -keto-dipate, an intermediate which can enter the tricarboxylic acid cycle. Degradation via catechol or protocatechuate leads to the production of β -keto-dipate, and is referred to as the β -keto-dipate pathway (Harwood and Parales, 1996). In SA catabolism, a salicylate 1-monooxygenase (also referred to as salicylate hydroxylase) converts SA to catechol. The catechol ring is then opened by a catechol 1, 2-dioxygenase, part of a class of intradiol dioxygenases catalyzing the ortho-cleavage, producing *cis*, *cis*-muconate. A single catechol 1, 2-dioxygenase homolog, at locus *FGSG_03667*, and three potential salicylate 1-monooxygenases have previously been annotated in the genome of *F. graminearum* (Brown et al., 2012; Wong et al., 2011); the transcription of two salicylate 1-monooxygenase genes, *FGSG_03657* and *FGSG_09063* was upregulated by treatment of fungal cultures with SA (Qi et al., 2012).

Recent developments and progress in *Fusarium* pathogenomics including full genome sequence (Kasan et al., 2012; Wong et al., 2011), combined with an efficient transformation method (Frandsen et al., 2012), allowed us to investigate the genes involved in the SA catabolism pathway in *F. graminearum*. Building on the findings of Qi et al. (2012), the function of four SA responsive genes predicted to encode enzymes with a potential role in SA catabolism has been further characterized using knockout mutants and expression profiling in culture and *in planta*.

2. Materials and methods

2.1. *Fusarium graminearum* spores and mycelial growth

Macroconidia used as inoculum for all transgenic as well as the parental wild type (WT) *F. graminearum* strain DAOM 233423 (Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada, Ottawa, Canada) were produced from liquid culture of a carboxymethylcellulose sporulation medium (1 g/L NH_4NO_3 , 1 g/L KH_2PO_4 , 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g/L Yeast extract, 15 g/L carboxymethylcellulose) using the method described in Cappellini RA, Peterson (1965). After for 3–4 d at 28 °C, 270 rpm, spores were filtered onto a layer of sterile Mirocloth (Calbiochem-EMD chemicals Inc. San Diego, CA, USA) and washed twice with sterile water by centrifugation at 4500 rpm for 10 min. Spores were counted using a hemocytometer and concentration of the spore suspension adjusted as required by centrifugation and pellet resuspension in sterile water.

Mycelium was grown on modified synthetic nutrient deficient agar (SNA) plates (1 g KH_2PO_4 , 1 g KNO_3 , 0.5 g MgSO_4 , 0.5 g KCl, 1 g glucose, 1 g sucrose, and 20 g agar per liter). To test ability to use SA as a carbon source, sugars were replaced with SA at 1.5, 6 or 12 mM; all media were adjusted to pH 8, using 5 M KOH, when including SA.

2.2. Generation of *Fusarium graminearum* gene-disrupted mutants

2.2.1. Target gene replacement plasmid construction

Plasmids to generate *F. graminearum* mutants were constructed using the Uracil-Specific-Excision Reagent (USER) friendly cloning methodology developed by Frandsen et al. (2012), with some modifications. The non-coding upstream and downstream regions of each gene were obtained by PCR amplification from 20 ng of genomic DNA of *F. graminearum* DAOM 233423, using the proof-reading Pfu Turbo Cx Hotstart DNA polymerase (Agilent Technologies, Canada) using Uracil-containing primers (0.2 mM each; Table S1). The primers were designed using the Primer Select program (DNASTAR software, Canada) and *F. graminearum* genome sequence annotation by Wong et al. (2011), then synthesized by Sigma–Aldrich (Canada). The PCR conditions were: 94 °C for 3 min, followed by 37 cycles of 94 °C for 30 s, 54 °C to 58 °C for 30 s (depending of the melting temperature of the primers), 72 °C for 1.5 min, and a final extension at 72 °C for 10 min. Each amplified DNA fragment was cleaned up using the QIAquick PCR Purification kit (Qiagen Inc Canada). One hundred ng of the pRF-HU2 binary vector (Frandsen et al., 2008), digested with Pac1, nicked with NtBbvC1 (New England Biolabs, Canada) and purified using the QIAquick PCR Purification Kit (Qiagen), were mixed with the two PCR fragments for upstream and downstream regions of a gene (260 ng each), 1 μl of USER reagent (New England Biolabs) and 3 μl of 10X Pfu Turbo buffer in a final volume of 30 μl . Incubation was carried out at 37 °C for 20 min and at 25 °C for an additional 20 min. Five μl of USER reaction were used to transform the gene replacement cassette into 50 μl of *E. coli* Top10 competent cells (Invitrogen Life Technologies, Canada) by the heat shock method. Transformed cells were plated onto LB agar supplemented with 30 $\mu\text{g}/\text{mL}$ of kanamycin (Sigma Aldrich). Plasmid DNA was isolated from kanamycin positive *E. coli* colonies and screened by PCR amplification using combinations of upstream or downstream gene specific primers and primers for the hygromycin B gene present in the replacement cassette, to confirm presence and orientation of all fragments in the plasmids. Identity of selected clones and absence of PCR-induced mutations was confirmed by sequencing using the following reaction: 700 ng of plasmid DNA, 0.5 μl of 3.2 μM primer, 2 μl of 5X sequencing buffer, 0.5 of 2.5X Big Dye Terminator (Thermo Fisher Scientific, Canada) in 10 μl .

2.2.2. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium graminearum*

The gene replacement plasmids were electroporated into competent *Agrobacterium tumefaciens* LBA4404 as described in Frandsen et al. (2012), using a GENE PULSII from Bio-Rad (1.5 μF , 2.5 kV and 200 Ω). Cultures of transformed *A. tumefaciens* strains were used to transform *F. graminearum* DAOM 233423 spores following the detailed method described in Frandsen et al. (2012). Mycelium from isolated transformed colonies was scraped off and processed for DNA preparation using E.Z.N.A Fungal DNA Mini kit (OMEGA Biotek USA). Resulting genomic DNA was used for PCR amplification screening to distinguish transformed fungal isolates where the target gene was replaced by the marker gene *hph* (referred to as ΔFGSG_03657 -salicylate 1-monooxygenase; ΔFGSG_03667 -catechol 1, 2-dioxygenase; ΔFGSG_09061 -2, 3-dihydroxybenzoic acid decarboxylase; ΔFGSG_09063 -salicylate 1-monooxygenase) from those transformants with non-homologous chromosomal insertion of part or the full gene replacement cassette somewhere else in the genome (ectopic insertion mutants). Single conidiated cultures were prepared from each positively screened *F. graminearum* transformant by spreading spores on 2 % water agar plates and selecting single germinating macroconidia using an inverted microscope (Evos, Thermo Fisher

Scientific) for transfer onto fresh PDA plates supplemented with hygromycin B (150 µg/mL). For each gene, three gene replacement mutant strains and three ectopic insertion mutant strains (as controls) were selected for further characterization, except for gene 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* for which only one ectopic insertion strain was identified.

2.3. In culture salicylic acid degradation assay

The culture assay was adapted from [Subramaniam et al. \(2015\)](#). Briefly, 40 µl of macroconidial suspension, at 12.5×10^6 spores/mL in sterile water, was inoculated in 4 mL of nutrient-rich medium and incubated in the dark in 6-wells culture trays (Falcon Multiwell, cat. #35 304) at 28 °C, in an Innova-44 shaker (New Brunswick Scientific) set at 170 rpm. After 24 h of incubation, the mycelia from each well were transferred to a sterile 15 mL Falcon tube and centrifuged at 4000 rpm in an Eppendorf centrifuge 5804R for 10 min. The washed pellet was resuspended in 4 mL of mycotoxin-inducing medium without sucrose nor glycerol, supplemented with 1 mM SA at pH 8, transferred to a new 6-well culture tray and incubated as above for 0, 2, 4 or 6 h. Cultures were harvested, centrifuged and supernatants filtered onto 0.45 µm Nylon syringe filters (Mandel Scientific) then were kept at –20 °C until HPLC analysis. Mycelia were either collected on Whatman filter paper no 1 and dried at 37 °C to measure dry weight, or immediately frozen in liquid nitrogen and kept at –80 °C until used for RNA extraction. The catechol assay was performed in the same way and at the same concentration, using a freshly prepared pyrocatechol (Sigma Aldrich) stock solution to avoid risk of catechol condensation. Strains were assayed in groups of three including one gene replacement strain, one ectopic insertion strain and the WT strain, using duplicate wells (as technical replicates) for each strain per plate. The three independent mutants (either for gene replacement or ectopic insertion) for a given gene were treated as biological replicates for that gene.

2.4. High performance liquid chromatography (HPLC) analysis

All HPLC analyses were carried out on an ATKA P-10 HPLC system (GE Healthcare, Canada) equipped with an autosampler A-900. Each sample mixture for chromatographic separation consisted in 950 µl of a filtrated fungal liquid culture supernatant combined with 50 µL of acetonitrile; the mixtures were bottled into 12 × 32 mm Amber vials (ThermoFisher Scientific Inc). Both solvents, water (containing trifluoroacetic acid at 0.1 %) and acetonitrile were degassed for 10 min before use. Separation conditions were adapted from [Mradu et al. \(2012\)](#). One hundred µL of each sample mixture and reference compounds (HPLC grade salicylic acid & pyrocatechol, Sigma Aldrich) were separated through a 5 micron C18 Hypersil Reverse Phase Column (ThermoFisher Scientific Inc) using a one-step gradient from 5 to 95 % acetonitrile in water over 25 min at a flow rate of 1 mL/min. The column was washed with 100 % acetonitrile for 5 min followed by 05:95 acetonitrile: water for 15 min in between samples. The eluted SA or catechol was detected by UV at 300 nm and quantified in mAU*min using peak integration with the Unicorn software (Version 5.01, GE Healthcare, Canada). SA and catechol were the unique compounds in the fungal supernatants that was observed under the growth and HPLC separation conditions used. SA retention time was 16.29 min while catechol was 8.3 min.

2.5. *Fusarium graminearum* infection experiments on wheat *Triticum aestivum* cv *Roblin*

F. graminearum WT, three Δ*FGSG_0367*-catechol 1, 2-dioxygenase and three ectopic insertion strains for the same gene

were inoculated onto heads of the FHB-susceptible wheat cv *Roblin* as described previously ([Harris et al., 2016](#)). A preliminary experiment was also done with Δ*FGSG_03657*-salicylate 1-monooxygenase strains and ectopic insertion strains. Mock inoculation with water was also done as control for gene expression analysis. For disease rating, only one spikelet was inoculated per head and 24–26 heads were inoculated per fungal strain; progression of infection was noted at 4, 8 and 12 d post inoculation (dpi), including discoloration up and down the rachis from the inoculation point, and browning and bleaching of spikelets. For RNA extraction, all fully developed spikelets on each head were inoculated and 4 to 5 heads were used per replicate, 3 replicates per fungal strain; treated heads were harvested at 4 dpi, flash-frozen in liquid nitrogen and stored at –80 °C until used.

2.6. RNA isolation, cDNA synthesis and quantitative RT-PCR analysis

Total RNA samples from either fungal mycelium or infected plant tissues were extracted with Tri-Reagent (Molecular Research Center Inc.) following the instructions provided by the manufacturer. The aqueous phase separation was technically implemented with phase-lock gel tubes (5 PRIME Inc., Gaithersburg, MD, USA) before the isopropanol precipitation. Crude RNA samples were cleaned up onto columns of the RNeasy Mini kit (Qiagen, Mississauga, Canada) and treated with DNase I from RNase-free DNase set (Qiagen). Total RNA were quantified with QJAXpert instrument (Qiagen, Mississauga, Canada). Reverse Transcriptase reactions were carried out with 3 µg of RNA per tube and an oligo-dT primed cDNA synthesis was performed using the RETROscript kit (Ambion – Thermo Fisher Scientific). RT-qPCR amplifications were carried out with cDNA diluted 1:25, using the Brilliant II SYBR®Green QPCR master mix (Agilent Technologies, USA) according to the cycling procedure in [Wang et al. \(2010\)](#). Three gene replacement mutants and three ectopic insertion mutants (except for 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* ectopic insertion) were analyzed for each gene, the three mutants in each group being treated as biological replicates. Two RT-qPCR technical replicates were performed for each RNA sample. Primers used for RT-qPCR analysis are listed in [Table S2](#). Expression values were calculated based on ($E^{-\Delta Cq}$), as described in [Wang et al. \(2010\)](#). For liquid culture experiments and the plant experiment, the relative expression of fungal genes was normalized with the geometric mean of expression values of two *F. graminearum* housekeeping genes, β-tubulin (*FGSG_09530*) and GAPDH (*FGSG_06257*). For estimation of the *F. graminearum* level in *planta*, the relative expression of quantification of the fungal genes β-tubulin and GAPDH was normalized using the wheat housekeeping genes heterogeneous nuclear ribonucleoprotein Q (hnRNP-Q; identified as *Traes_2AL_45601830C* in the Wheat Genome IWGSC release 2.25, [website reference 1](#)) and amine oxidase (Aox; *Traes_2AL_CD28AB70E*). All the Cq values of the normalizer genes were similar, with minimal sample to sample variation in the datasets, except for β-tubulin at 0 h which showed some variation. RT-qPCR data represent the relative level of mRNA transcript for a given gene after rescaling with either the 0 h time point (liquid culture experiments) or water-inoculated wheat head tissues (*in planta* experiments).

2.7. Bioinformatics and statistical analysis

Statistical analyses were performed using the one-tailed Student T-test comparison for two independent means at [website reference 2](#), comparing the group composed of WT and ectopic insertion strains to the gene replacement mutant group for a given gene.

Keyword searches for relevant metabolic functions in the official annotation for the *F. graminearum* genome (website reference 3) were supplemented with information from UniProtKB (website reference 4) and KEGG (website reference 5). Positions of proteins in *F. graminearum* genome were obtained from website reference 4.

3. Results

3.1. Selection of *Fusarium graminearum* candidate genes for SA catabolism

With the aim of better understanding the catabolism of SA in *F. graminearum*, we have focused on enzymes that could contribute to the first two steps of SA catabolism towards β -keto adipate via catechol (Cain et al., 1968; Harwood and Parales, 1996). Keyword searches for the targeted metabolic functions revealed that fifteen genes were annotated either as salicylate 1-monooxygenase or salicylate 1-hydroxylase (EC 1.14.13.1), the enzyme responsible for the oxidative decarboxylation of SA into catechol. Only two of those genes, salicylate 1-monooxygenases *FGSG_03657* and *FGSG_09063*, were shown to be transcriptionally induced by treatment of *F. graminearum* liquid cultures with SA (Qi et al., 2012). Of the other genes, two had expression signals above hybridization background level however were not induced by SA treatment; eleven were below the hybridization background level. An additional gene encoding a potential 2, 3-dihydroxybenzoic acid decarboxylase (EC 4.1.1.46), *FGSG_09061*, predicted to convert dihydroxybenzoic acid substrates to catechol via a non-oxidative decarboxylation (Anderson and Dagley, 1980; Kamath and Vaidyanathan, 1990), was also induced by SA. Three genes were annotated as a potential catechol/hydroxyquinol 1, 2-dioxygenase (EC 1.13.11.1); although all three had detectable expression signals, only catechol 1, 2-dioxygenase *FGSG_03667* was strongly expressed and induced by SA (Qi et al., 2012); this enzyme would catalyze the fission of the catechol phenyl ring to produce *cis*, *cis*-muconate. Table 1 summarizes the functional annotation and genome localization of the 4 genes that were selected for further characterization, and Fig. 1 illustrates their potential role in SA catabolism.

To identify which of those *F. graminearum* genes were factually involved in SA metabolism, single gene replacement mutant (identified by Δ) strains were produced for each of the candidate genes. There was no difference in mycelial growth for all gene replacement mutant and ectopic insertion strains when grown on SNA plates. Although all strains also grew at a similar rate on SNA plates supplemented with SA (as a substitute for sugars), the mycelial density was noticeably reduced for all three Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains; no significant effect on mycelial density was observed for the other gene replacement mutant strains when compared to ectopic insertion strains (Fig. S1, data not shown). This suggested that catechol 1, 2-dioxygenase *FGSG_03667* contributed to SA catabolism.

3.2. Loss of ability to utilize SA or catechol by gene replacement mutants for three *F. graminearum* genes

For each gene, the ability to utilize supplemented SA in a liquid culture assay was compared quantitatively between three

replacement mutants and one ectopic insertion mutant strains from the same transformation batch. The WT strain was also included in the comparisons. The remaining SA in the culture supernatant was measured at 0, 2, 4 and 6 h of incubation. Although little or no change in the amount of SA was observed between 0 and 2 h for the WT and ectopic insertion strains, most or all of SA had been utilized after 4 h of incubation (Fig. 2). No significant change in the level of remaining SA was observed between 0 and 6 h in the gene replacement mutant strains for salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667*, demonstrating that those two genes were essential for SA catabolism in the experimental conditions used (Fig. 2).

In contrast, SA disappeared as rapidly in the Δ *FGSG_09063*-salicylate 1-monooxygenase strains as it did in the WT and ectopic insertion strains; this suggested that this second salicylate 1-monooxygenase, although induced by SA treatment, was not required for SA catabolism (Fig. 2). A partial utilization of SA at 6 h, representing approximately 50 % of the starting amount of SA, was observed in the Δ *FGSG_09061-2*, 3-dihydroxybenzoic acid decarboxylase strains (Fig. 2), indicating that the gene, encoding a possible 2, 3-dihydroxybenzoic acid decarboxylase, could contribute to SA catabolism. Surprisingly, our results showed an unexpected activity pattern between the salicylate 1-monooxygenase *FGSG_03657* and the 2, 3-dihydroxybenzoic acid decarboxylase. In Δ *FGSG_09061-2*, 3-dihydroxybenzoic acid decarboxylase strains, significant reduction in SA level was observed only at 6 h, although the gene for salicylate 1-monooxygenase *FGSG_03657* was intact in those strains and its activity should have been observed at 4 h. Conversely, Δ *FGSG_03657*-salicylate 1-monooxygenase strains contain an intact 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061*-gene, yet no reduction in SA level was observed in those strains at 6 h. Further experiments will be required to unravel the relationship between those two genes.

To confirm that the predicted catechol 1, 2-dioxygenase *FGSG_03667* could utilize catechol, the liquid culture assay was supplemented with catechol instead of SA. As observed for SA, the catechol substrate in the culture supernatants had mostly disappeared by 4 h post-exposure in WT and ectopic insertion strains but not in Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains, consistent with the predicted functional activity of the gene (Fig. 3).

3.3. Substrate-induced expression profile of the candidate genes for SA catabolism

To further characterize the four genes, their RNA expression profile was measured in the same liquid culture system, following treatment with either SA or catechol in WT, ectopic insertion and Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. The salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* genes were both induced rapidly by SA in WT and ectopic insertion strains, with the maximum expression observed in the 2 h samples (Fig. 4). The reductions in expression levels at 4 and 6 h correlated with the rapid disappearance of SA in the culture supernatants of those strains (Fig. 2). The response of salicylate 1-monooxygenase *FGSG_03657* to SA was however delayed and reduced by about 100 fold in the Δ *FGSG_03667*-catechol 1, 2-

Table 1
Candidates genes for SA catabolism in *Fusarium graminearum*.

Locus name	Annotation	Chromosome and Position (bp)
<i>FGSG_03657</i>	Salicylate 1-monooxygenase	2: 6333690..6335066
<i>FGSG_03667</i>	catechol 1, 2-dioxygenase	2: 6306791..6307922
<i>FGSG_09061</i>	2, 3-dihydroxybenzoic acid decarboxylase	4: 7617220..7618277
<i>FGSG_09063</i>	salicylate 1-monooxygenase	4: 7614350..7615755

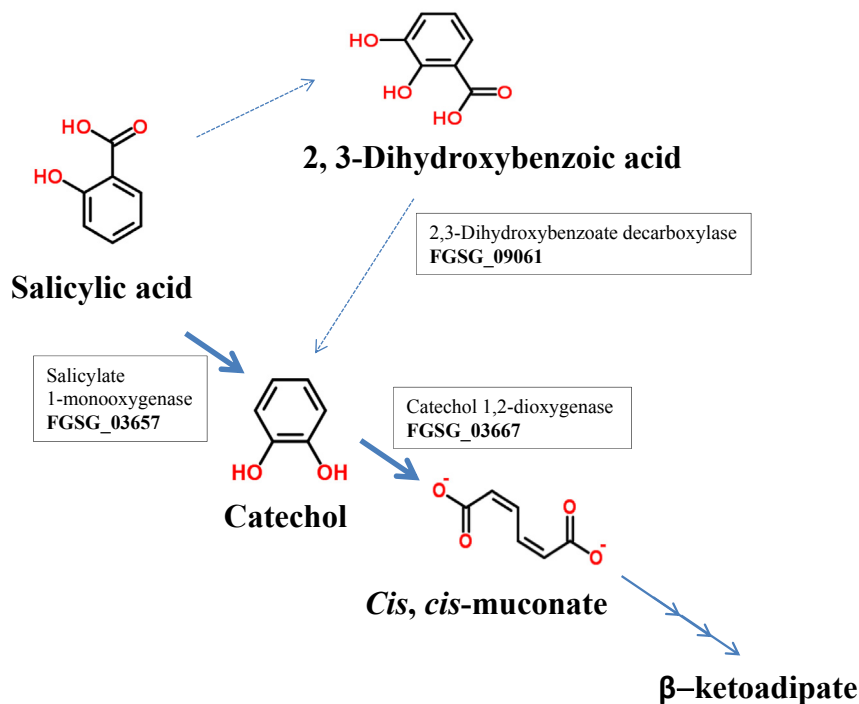


Fig. 1. Proposed pathway for SA catabolism in *F. graminearum*. The genes responsible for the oxidative decarboxylation of SA to catechol and the ortho cleavage of catechol to *cis, cis*-muconate were identified in this study and those steps are indicated with bold arrows. A proposed alternate non-oxidative decarboxylation of SA to catechol via 2, 3 dihydroxybenzoic acid is indicated with dotted arrows.

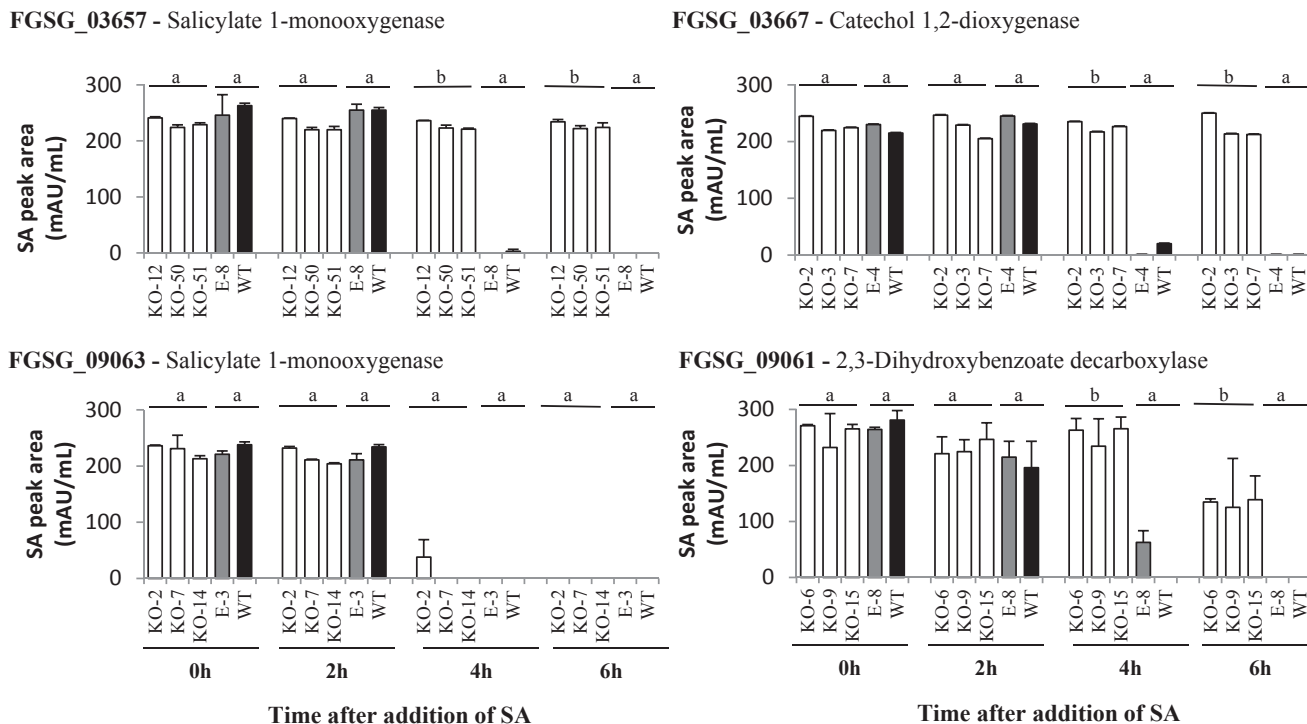


Fig. 2. Residual SA in fungal culture supernatants for WT, ectopic insertion and gene replacement mutant strains for 4 candidate genes for SA catabolism. Genes included salicylate 1-monooxygenases *FGSG_03657* and *FGSG_09063*, catechol 1, 2-dioxygenase *FGSG_03667* and 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061*. Bars show means \pm standard deviation of peak area after HPLC separation (n = 2 technical replicates for each of the gene replacement and ectopic insertion strain). KO, gene replacement strain; E, ectopic insertion strain; WT, parental wild type strain. Different lower case letters above the panels indicate statistical differences between the group including WT + E and the gene replacement group of strains at p = 0.0001 for salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667*, and p = 0.038 for 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061*.

FGSG_03667 - Catechol 1,2-dioxygenase

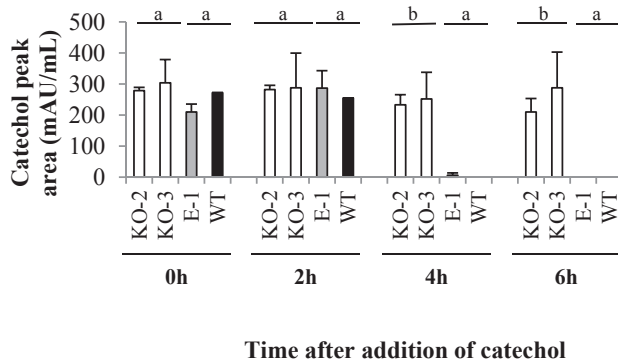


Fig. 3. Residual catechol in fungal culture supernatants for WT, ectopic insertion and gene replacement strains for catechol 1, 2-dioxygenase *FGSG_03667*. Bars show mean \pm standard deviation of peak area after HPLC separation ($n = 2$ technical replicates for each of the gene replacement and ectopic insertion strain). KO, gene replacement strain; E, ectopic insertion strain; WT, parental wild type strain. Different lower case letters above the panels indicate statistical differences at $p = 0.0001$ between the group including WT + E and the gene replacement group of strains.

dioxygenase strains when compare to the WT and ectopic insertion strains, suggesting that an intact catechol 1, 2-dioxygenase *FGSG_03667* was required for proper induction of transcription of salicylate 1-monooxygenase *FGSG_03657*. This is consistent with the observed lack of conversion of SA to catechol in the Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains, even though the salicylate 1-monooxygenase *FGSG_03657* gene was intact (Fig. 2). In catechol-treated ectopic insertion and WT strains, catechol 1, 2-dioxygenase *FGSG_03667* and salicylate 1-monooxygenase *FGSG_03657* had a more transient up-regulation profile with maximum at 2 h followed by a faster reduction in expression levels than what was observed in SA-treated cultures (Fig. 4).

Expression profiles for the salicylate 1-monooxygenase *FGSG_09063* and 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* genes were also measured in the SA and catechol-treated cultures (Fig. 5). In ectopic insertion and WT strains treated with SA, the two genes were induced rapidly and their expression level did not changed significantly over the rest of the sampling period. This suggested that SA could induce expression of those two genes; however it was not required to sustain their induction as SA had been utilized in the 4 and 6 h samples (Fig. 2). Induction of both genes was delayed in the Δ *FGSG_03667*-catechol 1, 2-dioxygenase strain, reaching its maximum at 4 h. In catechol-treated cultures, expression of both genes was delayed in all strains, reaching a maximum level at 6 h only. The expression profiles of those two genes were similar to each other while being markedly different from those of salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667*.

3.4. *In planta* gene expression and pathogenicity of the Δ *FGSG_03667*-catechol 1, 2-dioxygenase replacement mutant strains in inoculated wheat heads

Expression of the four candidate genes for SA catabolism was also determined *in planta*, at 4 dpi in wheat heads inoculated with WT, ectopic insertion and Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. At that sampling time, the fungal infection was well established yet it had not spread to the whole head tissues. The gene for catechol 1, 2-dioxygenase *FGSG_03667* was strongly induced when growing in the wheat tissues infected with ectopic insertion or WT strains (Fig. 6) while no transcripts were detected in the gene replacement mutant strains, as expected. The genes for salicylate 1-monooxygenases *FGSG_03657* and *FGSG_09063*, and 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* were all induced *in planta* at a relatively consistent level in all infected tissues, including those inoculated with Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. However, induction of the salicylate 1-monooxygenase *FGSG_09063* gene was much more modest than

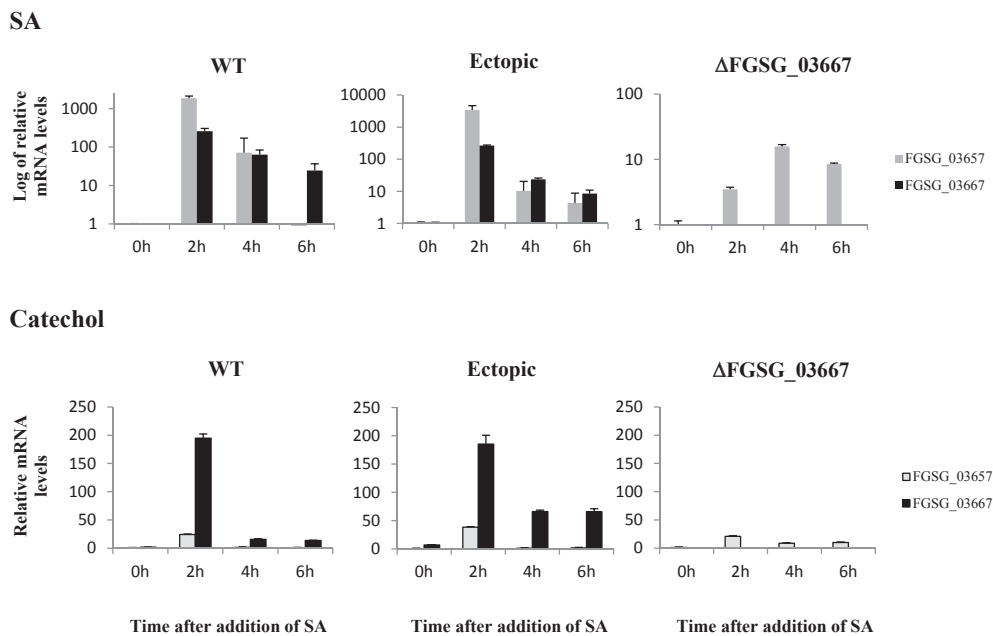


Fig. 4. Relative levels of mRNA transcripts for salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* over time in liquid cultures of *F. graminearum* WT, ectopic insertion and Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. Cultures were supplemented with 1 mM SA or 1 mM catechol. Bars represent the mean \pm standard deviation of the relative levels from RT-qPCR assays. For SA, $n = 3$ strains for gene replacement, two for ectopic insertion strains and one for WT; for catechol, only one ectopic strain was tested. For each strain, 2 technical replicates were done in RT-qPCR. Panels are presented at scales optimizing the viewing of the results for individual experiments; note the use of a log scale for the upper row of panels.

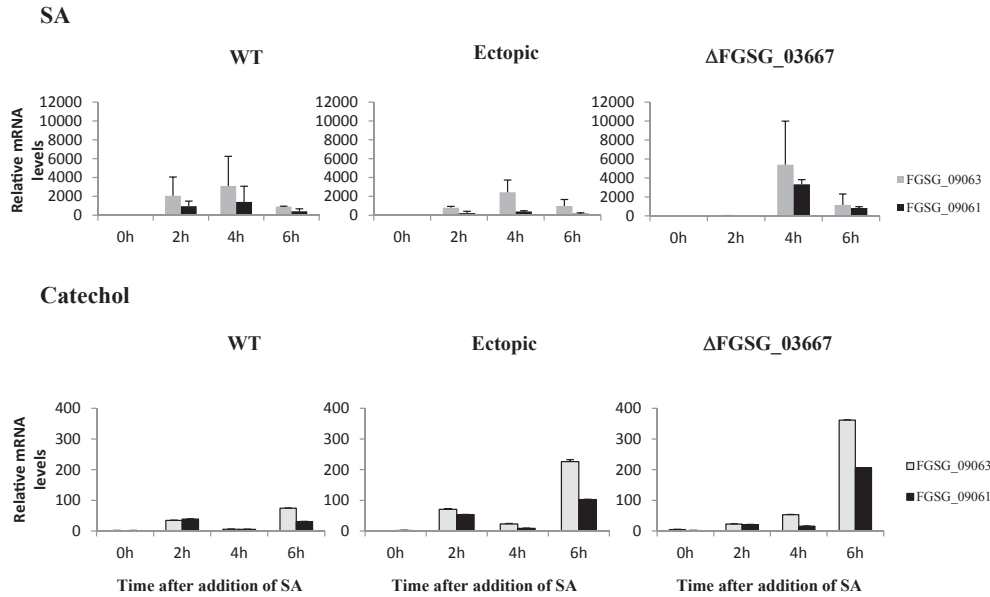


Fig. 5. Relative levels of mRNA transcripts for salicylate 1-monooxygenase *FGSG_09063* and 2, 3-dihydroxybenzoate decarboxylase *FGSG_09061* over time in liquid cultures of *F. graminearum* WT, ectopic insertion and Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. Cultures were supplemented with 1 mM SA or 1 mM catechol. Bars represent the mean \pm standard deviation of the relative levels from RT-qPCR assays. For SA, n = three strains for gene replacement, two for ectopic insertion strains and one for WT; for catechol, only one ectopic strain was tested. For each strain, 2 technical replicates were done in RT-qPCR. Panels are presented at scales optimizing the viewing of the results for individual genes.

what was observed for the other 3 genes. The absence of an intact catechol 1, 2-dioxygenase *FGSG_03667* gene did not affect the extent of expression of salicylate 1-monooxygenase *FGSG_03657* in the infected wheat tissues, contrary to what was observed in liquid culture. This difference in expression profiling between the liquid culture and wheat tissues experiments suggest that salicylate 1-monooxygenase *FGSG_03657* is modulated by other compounds, in addition to SA, in the plant tissues.

The level of *F. graminearum* infection in the 4 dpi wheat samples was estimated by measuring the relative levels of GAPDH and β -tubulin transcripts (Fig. 7, data not shown). The same expression profile was observed with both genes. Variations within biological replicates and between strains of the same group (gene replacement or ectopic insertion + WT) were observed; however no statistically significant differences were observed between the groups. Progression of disease symptoms, including browning and bleaching of spikelets, and discoloration of rachis above and below the inoculation point was also noted at 4, 8 and 12 dpi for inoculated wheat heads (Fig. 8). As expected, there was a large difference

in symptoms between 4, 8 and 12 dpi; all heads were completely infected by 12 dpi. No significant difference (at $\alpha = 0.05$) in infection symptoms was observed between WT, ectopic insertion and Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. In a preliminary experiment with Δ *FGSG_03657*-salicylate 1-monooxygenase strains, similar disease rating results were obtained, although a transient browning at the base of the inoculated florets was observed at 2 dpi only with Δ *FGSG_03657* strains (data not shown). Quantitative transcript measurements and visual symptoms all suggest that catechol 1, 2-dioxygenase *FGSG_03667* and possibly salicylate 1-monooxygenase *FGSG_03657* are not required for pathogenicity on wheat in the conditions used.

4. Discussion

Most of the research studies on SA have focused on its role as a plant resistance inducer (Rivas-San Vicente and Plasencia, 2011). However recent publications have shown that fungal pathogens have evolved strategies and stealthy ways to overcome SA-

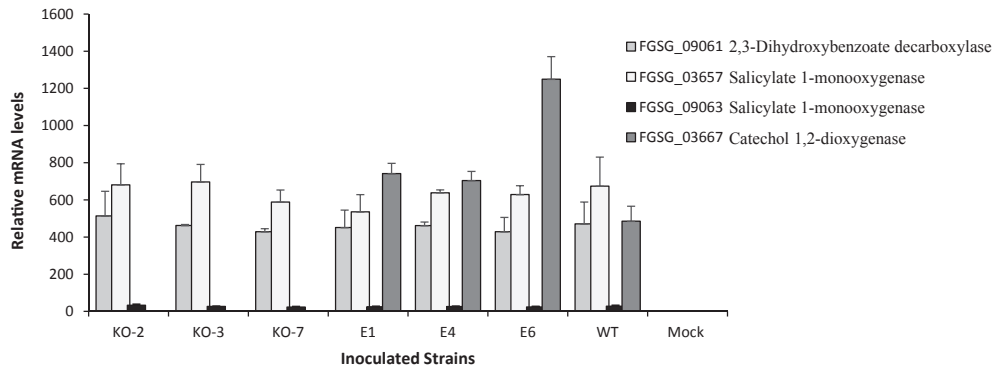


Fig. 6. Relative levels of mRNA transcripts *in planta* for the 4 *F. graminearum* candidate genes. Heads at mid-anthesis of FHB-susceptible spring wheat Roblin were inoculated with *F. graminearum* WT, ectopic insertion or Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains and sampled at 4 dpi. Bars represent the mean \pm standard deviation of the relative levels from RT-qPCR assays. For each strain, 2 technical replicates were done in RT-qPCR.

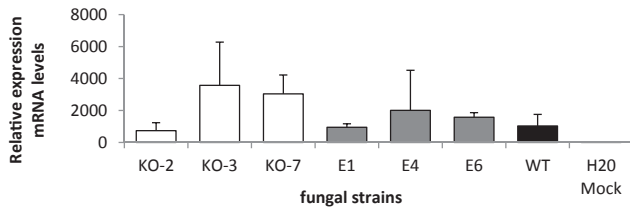


Fig. 7. Relative levels of mRNA transcript for FgGAPDH *in planta*. Heads at mid-anthesis of FHB-susceptible spring wheat Roblin were inoculated with *F. graminearum* WT, ectopic insertion and Δ FGSG_03667-catechol 1, 2-dioxygenase strains and sampled at 4 dpi. RT-qPCR values were normalised with 2 reference plant genes. Bars represent the mean \pm standard deviation of the relative levels from RT-qPCR assays. For each strain, 2 technical replicates were done in RT-qPCR.

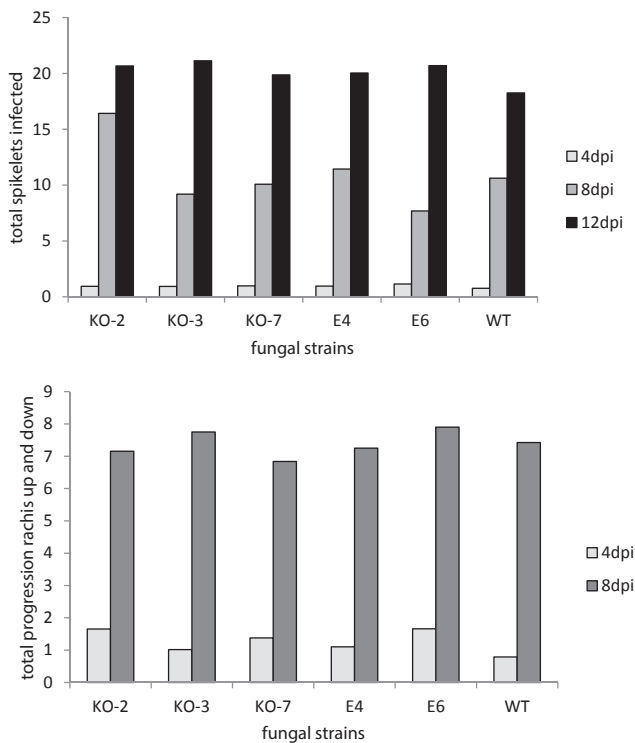


Fig. 8. FHB disease symptom progression on wheat heads inoculated with *F. graminearum* WT, ectopic insertion and Δ FGSG_03667-catechol 1, 2-dioxygenase strains at 4, 8 and 12 dpi. The total number of spikelets infected and the total number of rachis nodes infected above and below the inoculation point are shown. Bars represent mean ($n = 24$ to 26).

triggered immunity metabolism in order to better invade their plant host (Asai et al., 2015; Dodds and Rathjen, 2010; Rabe et al., 2016). A limited number of studies have examined the fungal response to SA and the impact of SA degradation on the interplay between plant and fungus (Ambrose et al., 2015; Penn and Daniel, 2013; Qi et al., 2012; Rabe et al., 2013, 2016). SA levels are known to be strictly and tightly controlled in plant, consistent with its key roles in plant growth, development and defense (Rivas-San Vicente and Plasencia, 2011). On the other hand, fungi are experts in aromatic compound degradation, a much conserved ability throughout the fungal kingdom and can utilize SA as sole source of carbon and energy (Harwood and Parales, 1996). Here we have investigated in more details the capacity of *F. graminearum* to degrade SA and asked if that capacity contributed to its pathogenicity on wheat.

In a previous study, we have shown that *F. graminearum* can use SA as sole carbon source (Qi et al., 2012). Four *F. graminearum* genes,

induced by SA and with predicted function suggesting a role in SA catabolism, have been further characterized to confirm their function and gain understanding of their expression profile in culture and *in planta*. Experiments with gene replacement mutant strains showed that the salicylate 1-monoxygenase gene FGSG_03657 and the catechol 1, 2-dioxygenase gene FGSG_03667 were required for degradation of SA in liquid culture, while a deletion of the 2, 3-dihydroxybenzoic acid decarboxylase gene FGSG_09061 showed only a partial loss of ability to degrade SA; no reduction in SA degradation was observed when the salicylate 1-monoxygenase gene FGSG_09063 was disrupted. Catechol 1, 2-dioxygenase FGSG_03667 was also essential for use of catechol. Our experiments clearly demonstrated that SA catabolism proceeded through the classical ortho-catechol pathway in *F. graminearum* (Fig. 1). This strengthens earlier findings using an unspecified species of *Fusarium* (Dodge and Wackett, 2005).

Salicylate hydroxylase (or salicylate 1-monoxygenase) genes have been characterized in *Epichloë festucae* and *Ustilago maydis* (Ambrose et al., 2015; Rabe et al., 2013). In *U. maydis*, only one of three predicted salicylate hydroxylase genes encoded an enzyme that could use sodium salicylate as a substrate and was required for growth on plates containing SA as sole source of carbon (Rabe et al., 2013). In contrast, *S. sclerotiorum* could not use SA for growth, even though it could degrade salicylate in liquid culture (Penn and Daniel, 2013). Those findings, together with our results, exemplify the need to validate bioinformatics annotation of genes with biochemical and/or genetics experiments. Catechol 1, 2-dioxygenase activity has been reported in fungi (Boominathan and Mahadevan, 1989; Dodge and Wackett, 2005; Varga and Neujahr, 1970; Vilimkova et al., 2009); however very few fungal genes for it have been characterized. A gene for catechol 1, 2-dioxygenase was identified in the yeast *Candida albicans* using the sequence of peptide fragments from the purified enzyme (Tsai and Li, 2007). Three genes for catechol 1, 2-dioxygenase were identified in the blue-stain fungus *Endoconidiophora polonica* and their biochemical activity characterized individually using heterologous expression in *E. coli* (Wadke et al., 2016). Those authors found that the ability of a collection of *E. polonica* strains to use astringin and catechin, two substrates used by the characterized catechol 1, 2-dioxygenases, had a significant positive correlated with the virulence of those strains, suggesting that the associated pathway was important for pathogenicity. This is the first report identifying genes for salicylate 1-monoxygenase and catechol 1, 2-dioxygenase in the important plant pathogen *F. graminearum*.

The enzyme 2, 3-dihydroxybenzoic acid decarboxylase has been shown to convert 2, 3-dihydroxybenzoic acid to catechol through a non-oxidative decarboxylation (Anderson and Dagley, 1980; Kamath and Vaidyanathan, 1990). In *Aspergillus niger*, that enzyme was efficiently induced by salicylate; however it did not use salicylate as a substrate (Kamath and Vaidyanathan, 1990). Our experiments in culture showed that a loss of function of the 2, 3-dihydroxybenzoic acid decarboxylase FGSG_09061 resulted in a delayed, partial degradation of SA. It is possible that some SA was transiently converted to 2, 3-dihydroxybenzoic acid in our culture assay, even though that compound was not observed in the culture supernatants; this would explain our results with Δ FGSG_09061-2, 3-dihydroxybenzoic acid decarboxylase strains. Hydroxylation of SA to 2, 3-dihydroxybenzoic acid has been reported before in fungi (Wright, 1993); however no information documenting the characterization of such a fungal hydroxylase was found. It has also been shown that SA can react with highly reactive OH* radicals to produce 2, 3-dihydroxybenzoic acid in non-enzymatic systems (Floyd et al., 1984). In plants, 2, 3-dihydroxybenzoic acid has been shown to be produced in significant amount during the plant defense response, and exogenous SA can be converted to 2, 3-dihydroxybenzoic acid by

a SA 3-hydroxylase in *Arabidopsis thaliana* (Bartsh et al., 2010; Mustafa et al., 2009; Zhang et al., 2013). Additional experiments will be required to exclude the possibility that 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* can use SA as a substrate.

Surprisingly, the activity of the salicylate 1-monooxygenase *FGSG_03657*, fully functional in the Δ *FGSG_09061-2*, 3-dihydroxybenzoic acid decarboxylase strains, could not be observed in those culture assays. Similarly, no delayed degradation of SA was observed in the cultured Δ *FGSG_03657*-salicylate 1-monooxygenase mutant assays, although 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* was not disrupted in them. These results suggest an interconnection between the two potential pathways of SA degradation to catechol, oxidative and non-oxidative decarboxylation, which has not been reported before. Further experiments will be required to confirm the occurrence of the proposed conversion of SA to 2, 3-dihydroxybenzoic acid (Fig. 1) in *F. graminearum* and to determine the mechanism associated with the observed connection between the two degradation pathways.

Transcript profiling showed that salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* were both induced rapidly and transiently after SA treatment in liquid culture. A similar rapid and strong induction of three potential salicylate hydroxylases was observed in the smut fungus *Ustilago maydis* after SA treatment (Rabe et al., 2013). Catechol 1, 2-dioxygenase *FGSG_03667* was also induced rapidly by catechol, its presumed substrate, while induction was much more modest for the other three genes, compared to levels of expression after induction by SA. Expression profiles for salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* were very similar to each other and were dependent of the level of SA or catechol in the liquid media, suggesting a fine level of regulation of the first two steps in SA catabolism in *F. graminearum*. A key regulator of SA sensing, the gene *Rss1*, has recently been identified in *U. maydis* (Rabe et al., 2016); however no homolog of that gene has been found in the *F. graminearum* genome using a BLAST search (data not shown). Interestingly, salicylate 1-monooxygenase *FGSG_03657* was not induced by SA in Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains grown in culture while it was induced *in planta* in WT, ectopic insertion and gene replacement mutant strains of catechol 1, 2-dioxygenase *FGSG_03667*. Our gene expression profiling experiments suggest that salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* are co-regulated in some growth conditions, however not *in planta*. Other compounds, in addition of SA, may modulate their expression *in planta*.

In our expression profiling experiments, 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* and salicylate 1-monooxygenase *FGSG_09063* had a similar slower and longer induction profile than salicylate 1-monooxygenase *FGSG_03657* and catechol 1, 2-dioxygenase *FGSG_03667* after SA or catechol treatment. It was shown previously that 2, 3-dihydroxybenzoic acid decarboxylase *FGSG_09061* and salicylate 1-monooxygenase *FGSG_09063* were part of an expression cluster regulated by the MADS-box transcription factor *FgMcm1* (Yang et al., 2015). Co-regulation was also observed for two genes annotated as salicylate monooxygenases in the yeast *Candida parapsilosis* (Holesova et al., 2011). Those authors also determined that the two monooxygenases had substrate specificity for 3-hydroxybenzoate and 4-hydroxybenzoate, not salicylate (also called 2-hydroxybenzoate). Similarly, the salicylate 1-monooxygenase encoded by *FGSG_09063* may be specific for a different benzoate compound than SA, even though the gene is induced by it, as we observed no reduction of SA degradation in cultures with Δ *FGSG_09063*-salicylate 1-monooxygenase strains. Additional experiments will be required to confirm or identify the substrates for 2, 3-dihydroxybenzoic acid decarboxylase

FGSG_09061 and salicylate 1-monooxygenase *FGSG_09063*, and to determine the optimum conditions to characterize them. Co-induction by SA of gene expression or activity for many of the enzymes in the β -keto adipate pathway, including enzymes that cannot use SA, has been reported before in fungi (Martins et al., 2015; Kamath and Vaidyanathan, 1990; Qi et al., 2012; Rabe et al., 2013).

The ability of *F. graminearum* to catabolize SA raised a question about its possible contribution to pathogenicity. To test this possibility, wheat plants were inoculated with gene replacement strains of catechol 1, 2-dioxygenase *FGSG_03667*. No impact on the pathogenicity of the strains was observed when infecting the very FHB-susceptible wheat Roblin. A preliminary experiment with gene replacement mutants of salicylate 1-monooxygenase *FGSG_03657* showed a similar absence of impact on pathogenicity. However, when Δ *FGSG_03657*-salicylate 1-monooxygenase strains were tested *in planta* (data not shown), some browning was transiently observed at 2 dpi at the base of inoculated florets, suggesting a transient yet unsuccessful defense response by the wheat plants. Further experiments will be required to confirm a differential response of wheat to Δ *FGSG_03657*-salicylate 1-monooxygenase and Δ *FGSG_03667*-catechol 1, 2-dioxygenase strains. Variation in catabolic activity occurs not only between fungal species but also between isolates of the same species; additional isolates of *F. graminearum* with a different genetic background could be tested for their ability to utilize SA vs their pathogenicity on wheat, to thoroughly ascertain the relationship between SA degradation and pathogenicity in that species.

The nutritional complexity of the wheat head is much larger than that of the media used for culture of *F. graminearum* in our experiments, including the presence of simple and complex sugars and numerous phenolic compounds; so we cannot exclude that the mobilization of other pathways overshadowed the possible contribution of the SA catabolism to pathogenicity of *F. graminearum* in our experimental conditions. The wheat head is an environment rich in phenolic compounds; *F. graminearum*'s ability to utilize SA and potentially additional phenolic derivatives as source of energy via the β -keto adipate pathway could provide a growth advantage in particular conditions not tested in our experiments.

Deletion of a 3-carboxy-cis-cis-muconate lactonizing enzyme gene in *Fusarium oxysporum* caused a complete loss of pathogenicity for the mutant strains on tomato; that gene catalyses the conversion of 3-carboxy-cis-cis-muconate to 3-carboxymuconolactone in a parallel branch of the β -keto adipate pathway for the catabolism of protocatechuate (Michielse et al., 2012). In contrast, a salicylate hydroxylase identified in *U. maydis* as required for growth on plates supplemented with SA was not required for virulence in a seedling assay (Rabe et al., 2013). Experiments by Ambrose et al. (2015) showed no evidence that the expression of the *E. festucae* salicylate hydroxylase modulated the level of SA in infected plant tissues; however no gene replacement experiments were done in that study. The ability to degrade SA by pathogenic fungi appears to have a different contribution to their pathogenicity on plants depending on the species studied; further investigation will be required to understand the nature of that difference.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funbio.2018.11.002>.

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