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The antioxidant methyl gallate inhibits fungal growth and deoxynivalenol production in *Fusarium graminearum*

Xin Liu^{1,2}, Xin Fang^{1,2}, Shuang Wang^{1,2}, Deliang Wu¹, Tao Gao¹, Yin-Won Lee^{1,3}, Sherif Ramzy Mohamed⁴, Fang Ji^{1,2*}, Jianhong Xu^{1,2} and Jianrong Shi^{1,2*} 

Abstract

Production of the *Fusarium* toxin deoxynivalenol (DON) is associated with oxidative stress and has been indicated to be part of an adaptive response to oxidative stress in the important wheat fungus *Fusarium graminearum*. In this study, we found that the antioxidant methyl gallate (MG) displays inhibitory effects against mycelial growth, conidial formation and germination, and DON biosynthesis in *F. graminearum* in a dose-dependent manner. Treatment with 0.05% (w/v) MG resulted in an abnormal swollen conidial morphology. The expression of the *TRI* genes involved in DON biosynthesis was significantly reduced, and the induction of Tri1-GFP green fluorescence signals in the spherical and crescent-shaped toxisomes was abolished in the MG-treated mycelium. RNA-Seq analysis of MG-treated *F. graminearum* showed that 0.5% (w/v) MG inhibited DON production by possibly altering membrane functions and oxidoreductase activities. Coupled with the observations that MG treatment decreases catalase, POD and SOD activity in *F. graminearum*. The results of this study indicated that MG displays antifungal activity against DON production by modulating its oxidative response. Taken together, the current study revealed the potential of MG in inhibiting mycotoxins in *F. graminearum*.

Keywords: *Fusarium graminearum*, Antioxidant methyl gallate, Deoxynivalenol production, Mechanism of action

Introduction

Members within the *Fusarium graminearum* species complex (FGSC) can infect wheat, barley and other small-grain cereal plants and cause Fusarium head blight (FHB) worldwide. Epidemics of FHB led to very large yield losses and a reduction in cereal quality. Additionally, *Fusarium* toxins (e.g., type B trichothecenes) commonly produced by *Fusarium* spp. during growth in host plants poses a serious threat to the health of

humans and livestock. Deoxynivalenol (DON) is one of the most prevalent type B trichothecenes and has been proven to be toxic to eukaryotic cells by inhibiting protein synthesis (Arunachalam & Doohan 2013). DON is frequently and ubiquitously found worldwide (Ji et al. 2014, 2019). Owing to its toxicity, arising food safety concerns and its ubiquitous nature, many countries and regions, including China, have set maximum levels for DON in wheat and other cereal-based products, thus, an effective control strategy for FHB and DON contamination is urgently needed worldwide.

Due to the lack of wheat cultivars highly resistant to FHB and DON (Jansen et al. 2005), the application of systemic fungicides during the wheat flowering stage has been one of the primary tools for controlling disease and DON contamination in China (Chen et al. 2019). Currently, fungicides including the sterol demethylation

* Correspondence: jifang625@126.com; jianrong63@126.com

¹Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base, Ministry of Science and Technology/Key Laboratory for Control Technology and Standard for Agro-product Safety and Quality, Ministry of Agriculture and Rural Affairs/Key Laboratory for Agro-product Safety Risk Evaluation (Nanjing), Ministry of Agriculture and Rural Affairs/Collaborative Innovation Center for Modern Grain Circulation and Safety/Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, Jiangsu, China
Full list of author information is available at the end of the article



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inhibitors tebuconazole and prothioconazole (Yin et al. 2009), the novel myosin inhibitor phenamacril (Zhang et al., 2010, 2015) and the carboxamide fungicide pydiflumetofen (Chen et al. 2020; Sun et al. 2020) widely used in China are effective in controlling FHB and DON accumulation in wheat. However, due to extensive use, field *Fusarium asiaticum* strains with low tebuconazole resistance have been isolated, and some provinces of China have been reported to have moderate to high or high resistance risk for the fungicides phenamacril and pydiflumetofen (Chen et al. 2020; Chen & Zhou 2009; Sun et al. 2020). Moreover, previous studies have indicated that *Fusarium* isolates conferring resistance to carbendazim, a fungicide widely used since the 1970s in China to control FHB, tend to produce more DON in infected grains (Zhang et al. 2009; Zhou et al. 2020). Additionally, the application of certain fungicides below their recommended dosage could trigger DON production (Audenaert et al. 2010; Duan et al. 2020).

Therefore, it is extremely urgent to develop new, efficient and sustainable tools or new classes of chemicals with different modes of action for the control of FHB and DON contamination in wheat. Plant secondary metabolites with multiple biological activities are of great interest in the search for safe and environmentally friendly antifungals. Among them, metabolites with antioxidant properties have been shown to have important roles in the plant defense response against invading microorganisms (Atanasova-Penichon et al. 2016; Balmer et al. 2013), thus displaying antifungal activities, and some can interfere with toxin synthesis (Gauthier et al., 2016; Zhao et al. 2018).

Methyl gallate (MG) is a ubiquitous phenolic compound widely distributed in medicinal and food plants (Farhoosh & Nyström 2018). MG possesses valuable biological effects, including antioxidant, anti-inflammatory, antiapoptotic, and antitumor activities (Kamatham et al. 2015; Kang et al. 2009). MG is known for its capacities to protect cells against DNA damage caused by oxidative stress (Rahman et al. 2016) and prevent lipid oxidation. In addition, MG is a potent antibacterial compound against multidrug-resistant *Shigella* spp., nalidixic acid-resistant bacteria, and the plant-pathogenic bacterium *R. solanacearum* (Acharyya et al. 2015; Choi et al. 2009; Fan et al. 2014). MG also shows potential as an antifungal chemical and is effective against the rice fungal pathogen *Magnaporthe oryzae* (Ahn et al. 2005).

The objectives of this current study were i) to characterize the inhibitory activity of MG on fungal growth and DON production in *F. graminearum*; ii) to investigate the molecular mechanism by which MG inhibits fungal growth and toxin synthesis in *F. graminearum*, which will investigate the potential of the potent antioxidant MG as an antifungal agent and

provided guidance for the further development and utilization of MG as a safe and natural pathogen inhibitor.

Materials and methods

Strains, media and culture conditions

Fusarium graminearum strain PH-1 (NRRL 31084, originally isolated from Michigan) was used as the parental wild-type (Cuomo et al. 2007). Mycelial growth was compared in PDA (Potato Dextrose Agar) plates with or without MG at the concentrations indicated in the figures and incubated at 25 °C. Fungal biomass was compared by collecting mycelia from 3-day-old PDB (Potato Dextrose Broth) culture treated with or without MG at the concentrations indicated in the figures incubated at 25 °C in a shaker (180 rpm). Conidial formation was evaluated by inoculating five 5-mm mycelial plugs from the edge of a 3-day-old colony into a 150 ml triangular flask containing 50 ml of CMC (carboxymethyl cellulose) medium with or without MG treatment at the concentrations indicated in the figures and incubated at 25 °C, 180 rpm for 4 days in a shaker with light (Iida et al., 2008; Zhang et al. 2015). The number of conidia in CMC medium was counted using a hemacytometer and conidial morphology was observed under microscopy stained with calcofluor white (CFW). Conidial germination rates were compared by re-suspending conidia in 2% sucrose solutions amended with or without 0.05% or 0.1% (w/v) MG at 25 °C for 3 h or 5 h. The experiment was repeated three times, each sample in triplicate.

RNA extraction and gene expression analysis (RNA-Seq)

For RNA-Seq analysis, conidia (10^5) of *F. graminearum* strain PH-1 were inoculated into 50 ml FMM (*Fusarium* minimum medium) (Leslie & Summerell 2007) in triplicates and cultured at 25 °C, 180 rpm for 2 days in a shaker. Mycelia was collected by filtering through sterile miracloth, washed and then equally divided. One half mycelia was grown under the same conditions with the other half shifted to a 0.05% (w/v) MG treatment for 24 h. After that, mycelia was collected, washed, frozen with liquid nitrogen and lyophilized. Total RNA of *F. graminearum* strains was extracted using TaKaRa RNAiso Plus (TaKaRa, Dalian, China) and cDNA was reverse transcribed using TaKaRa PrimeScript™ RT Master Mix (TaKaRa, Dalian, China).

RNA samples were prepared to perform digital transcriptome analysis by the RNA-seq approach (BGL, Shenzhen, China) using BGISEQ-500 Platform. Clean reads were mapped to the *F. graminearum* PH-1 genome (https://www.ncbi.nlm.nih.gov/assembly/GCF_000240135.3) by using Bowtie2 (Langmead & Salzberg 2012) and RSEM (Li & Dewey 2011). Tags with a frequency greater than 3 tag counts per million (TPM)

were used in data analysis (Jongeneel et al. 2003; Liu et al. 2010). Frequencies of each sequenced tag in samples treated or untreated with 0.05% (w/v) MG were compared and the statistical significance (Q-value) was calculated. Differentially expressed genes were selected using DEGseq and genes with fold change ≥ 2 and Q-value ≤ 0.001 were considered as significantly differentially expressed genes (DEGs) (Wang et al. 2009). GO and KEGG analysis were performed by using phyper (R package).

DON analysis

Conidia (10^5) of *F. graminearum* strain PH-1 were inoculated into 30 ml trichothecene biosynthesis inducing (TBI) liquid amended with or without MG at different concentrations indicated in the figures and cultured at 28 °C for 7 days in the dark. There were three replicates for each treatment. The filtrate and fungal mass were then collected separately, frozen with liquid nitrogen and lyophilized for 24 h. The filtrate was re-dissolved in methanol and DON concentration was determined using a high-performance liquid chromatography-mass spectrometry-mass spectrometry (HPLC-MS/MS) system (Shimadzu 30A LC system coupled to a Triple Quad 6500 plus, Sciex, USA). Parameters of MS analysis were according to a previously described method (Qiu et al. 2016). The amounts of DON were normalized to the dry mycelium weight of each sample. The experiment was repeated three times.

Relative gene expression levels determined by qRT-PCR

Expression of *TRI* genes involved in DON biosynthesis was determined by using quantitative Real-Time PCR (qRT-PCR) assays with primer pairs listed in Table S1. qRT-PCR amplifications were performed in a LightCycler® 96 (Roche Molecular Systems) using the TaKaRa SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Dalian, China). Relative expression levels of each gene were calculated using a $2^{-\Delta\Delta CT}$ method and normalized with *F. graminearum* β -tubulin gene (Livak & Schmittgen 2001).

Analysis of lipid oxidation levels and antioxidant enzymes activities

To gain insights of the impacts of MG treatment on antioxidant responsive system in *F. graminearum*, activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) were compared. Mycelia were collected as previously described from samples treated or untreated with MG at a final concentration of 0.1% or 0.5% (w/v) and were used for enzyme activity determination as described previously (Zhang et al. 2010; Zhao et al. 2018). The

enzymatic activities were normalized to the weight of dry mycelia.

Statistical analysis

All data were presented as the means \pm standard deviation (SD). Statistical significance differences were determined by unpaired Student's *t*-test with software GraphPad Prism (La Jolla, CA, USA). A *p*-value less than 0.05 was labelled as statistically significant. Hierarchical clustering analysis was performed to compare the correlation relationships of the six RNA-Seq samples.

Results

Inhibitory effects of MG on *F. graminearum* mycelial growth

MG treatment at different concentrations (0.05, 0.1, 0.5 and 1%, w/v) led to a significant reduction in *F. graminearum* mycelial growth in PDA plates compared to the control in a dose-dependent manner ($p < 0.05$) (Fig. 1B-C). The addition of 1% (w/v) MG completely inhibited *F. graminearum* mycelial growth. The fungal biomasses of the MG-treated samples at different concentrations harvested from PDB were also significantly reduced compared to those of the control ($p < 0.001$) (Fig. 1D).

MG treatment affected the conidial morphogenesis of *F. graminearum*

Treatment with MG at 0.05% or 0.1% (w/v) in the liquid conidia-producing medium CMC caused morphological changes in the conidia of *F. graminearum* strain PH-1. MG-treated conidia showed swollen structures and disrupted tips with shorter sizes and fewer septa, as confirmed by calcofluor white (CFW) staining (Fig. 2A). Conidiation in liquid CMC was reduced significantly after treatment with MG at 0.05, 0.1, 0.5 and 1% (w/v) ($p < 0.001$) (Fig. 2B). Conidial germination rates were compared by resuspending equal amounts of conidia in 2% sucrose solutions with or without MG at 0.05 and 0.5% (w/v). The results showed that MG treatment significantly reduced conidial germination of *F. graminearum* at 3 h and 5 h at both concentrations tested (Fig. 2C).

MG inhibited DON biosynthesis in *F. graminearum*

Equal amounts of *F. graminearum* strain PH-1 conidia were inoculated in liquid TBI with 0, 0.05, 0.01, 0.5% or 1% (w/v) MG for 7 days. The filtrate and mycelium were collected separately. DON production was normalized to dry mycelial weight (mg DON/g dry mycelium). As shown in Fig. 3A, MG treatment significantly inhibited DON production in *F. graminearum* in a dose-dependent manner. Furthermore, the expression levels of six *TRI* genes involved in the trichothecene biosynthesis gene cluster were determined by qRT-PCR.

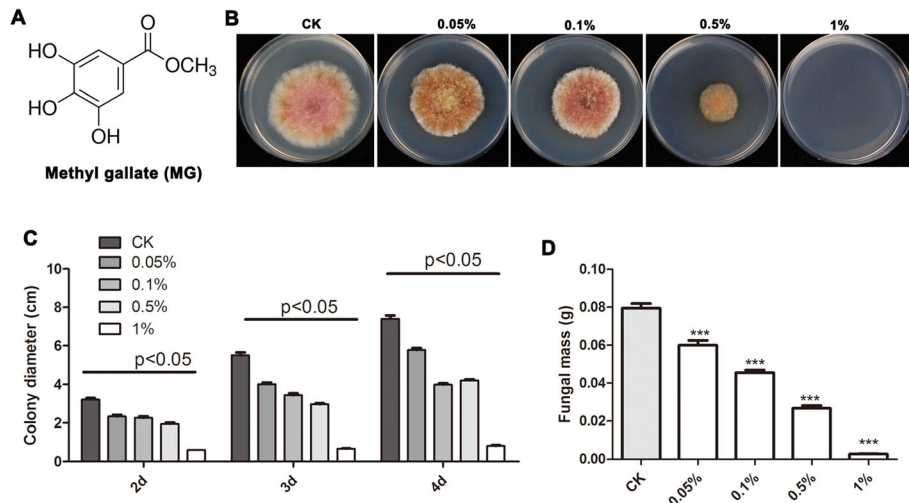


Fig. 1 MG inhibits the mycelial growth of *F. graminearum*. **A** Chemical structure of methyl gallate. **B** Colony morphology of *F. graminearum* strain PH-1 grown on PDA plates amended with different concentrations (w/v) of MG indicated in the figure and cultured at 25 °C, and colony diameters were measured at 2 d, 3 d, and 4 d after inoculation **C**. **D** Five mycelial plugs were inoculated into 100 mL PDB amended with different concentrations (w/v) of MG indicated in the figure and cultured in a shaker at 180 rpm, 25 °C for 3 days, fungal biomass was compared. Each treatment has three replicates, ***, $p < 0.001$

Compared to the nontreatment control, the relative expression levels of *TRI3*, *TRI5*, *TRI8*, *TRI10*, *TRI11* and *TRI12* were significantly downregulated ($p < 0.001$) in the MG-treated mycelia.

Recent studies have shown that DON is synthesized in organized endoplasmic reticulum structures called

toxosomes in *F. graminearum*. When cultured in DON biosynthesis-inducing conditions (e.g., liquid TBI or during infection in wheat heads), the expression of *TRI* genes was upregulated, and the translated Tri was found to colocalize with DON-toxisome. Tri1, a cytochrome P-450 oxygenase involved in the late steps of trichothecene

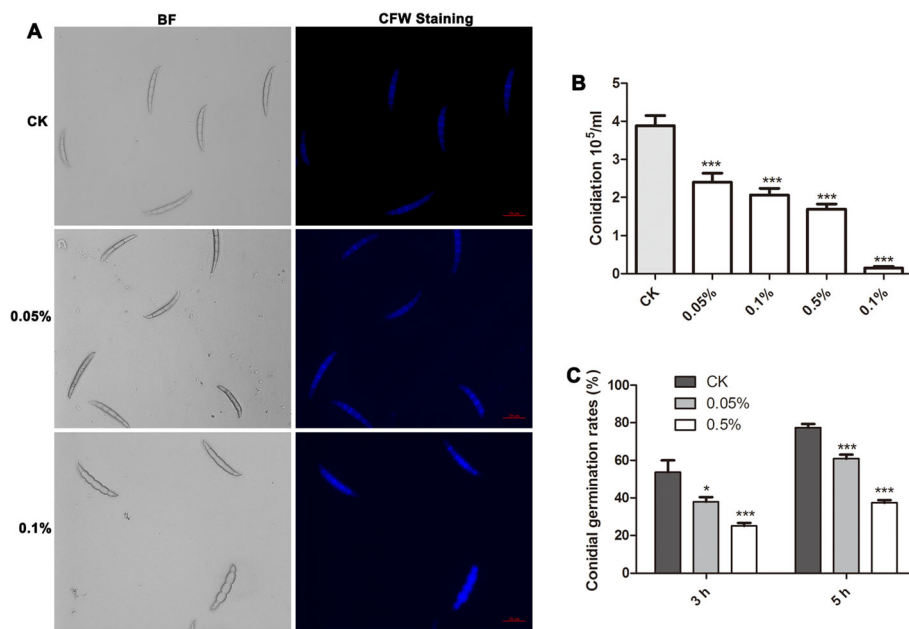


Fig. 2 MG treatment altered conidial morphology, formation and germination. **A** Conidia were then harvested and examined under fluorescence microscope stained by CFW. **B** Conidial amounts and **C** conidial germination were compared between control and MG treatment at the concentration indicated in the figures. Each treatment has three replicates, * means $p < 0.05$ and *** means $p < 0.001$. Bar = 20 μm

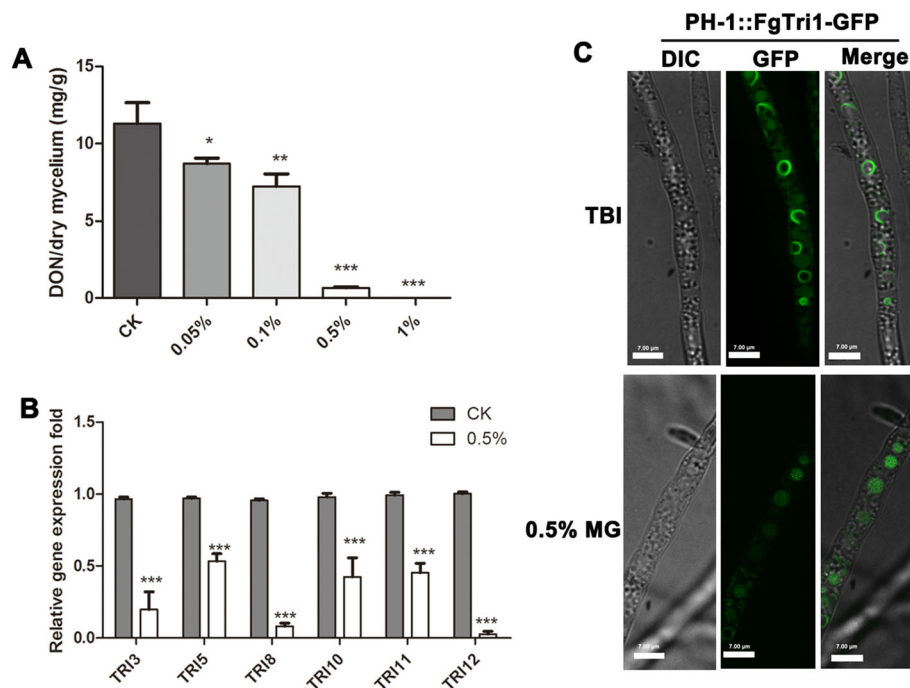


Fig. 3 MG treatment affects DON biosynthesis. **A** DON production was normalized with the dry mycelium collected from the culture. **B** Relative expression of *TRI* genes involved in DON biosynthesis was determined by qRT-PCR. **C** DON-toxisome was examined in the hyphae harvested from 3 day-old liquid TBI culture with or without 0.5% (w/v) MG treatment under fluorescence confocal microscope. Each treatment has three replicates, * means $p < 0.05$, ** means $p < 0.01$ and *** means $p < 0.001$. Bar = 10 μ m

DON biosynthesis, was confirmed to localize in DON-toxisome (Menke et al. 2013; Zhang et al. 2015). When cultured in liquid TBI, colocalized fluorescence could be detected in the *Tri1* GFP (green fluorescent protein)-tagged mutant (Δ Tri1:: FgTri1-GFP) with DON-toxisome; however, 0.5% (w/v) MG treatment strongly inhibited DON-toxisome formation, as only faint fluorescent signals were observed in the MG-treated mycelia (Fig. 3C).

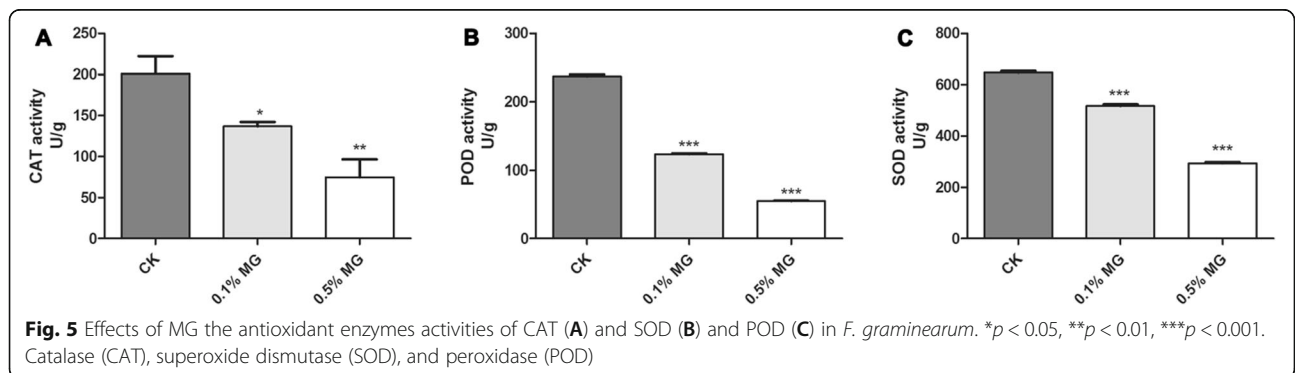
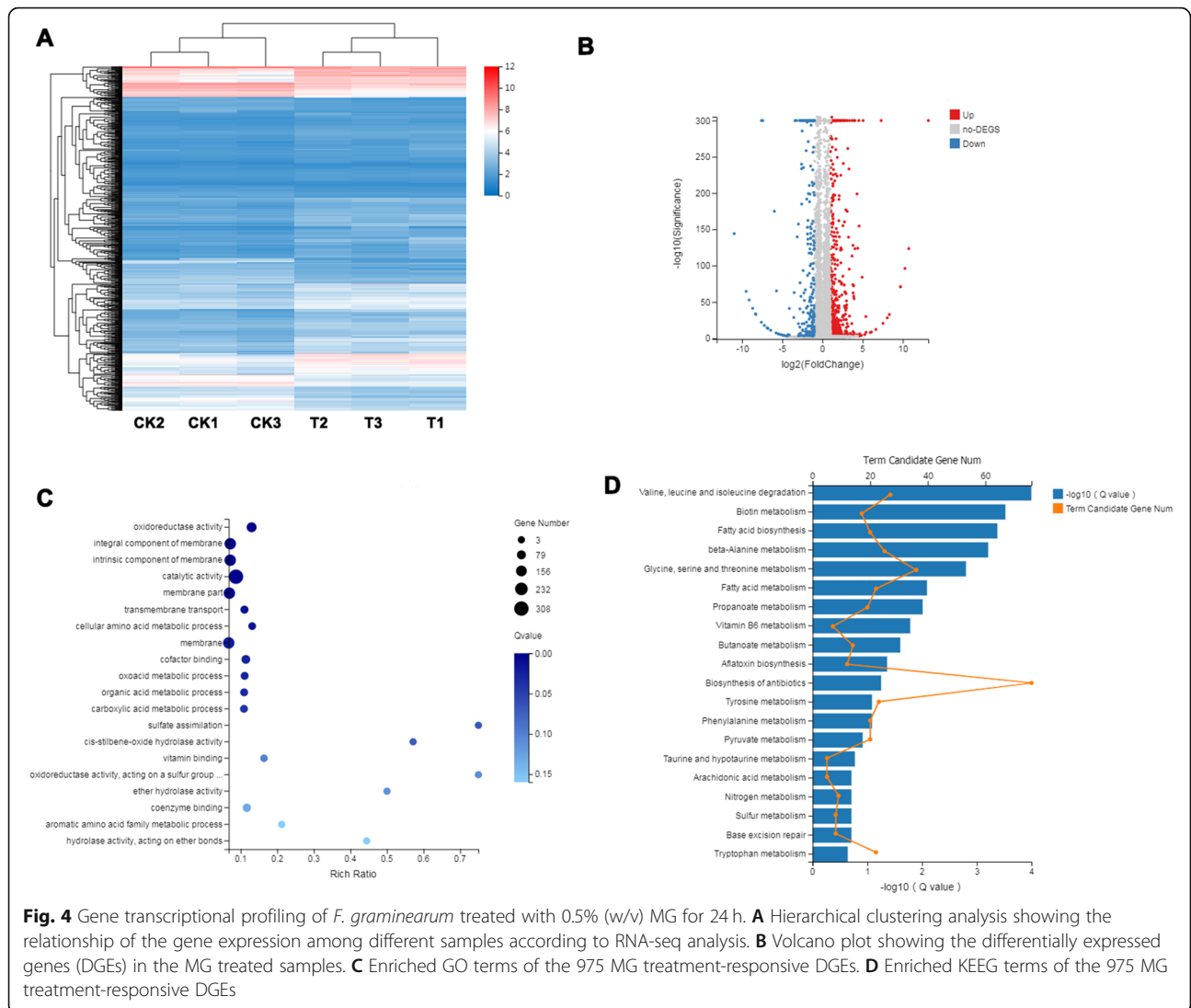
Gene transcriptional profiling of *F. graminearum* treated with MG

To further gain insights into how MG inhibits fungal growth and DON biosynthesis in *F. graminearum*, RNA-seq was performed to identify genes differentially expressed after MG treatment at 0.5% (w/v) for 24 h. Three replicates were used for both the control and MG treatments. Clustering analysis based on the correlation relationships of the six samples suggested that the genes expressed in the control and MG treatment groups were distinct (Fig. 4A). Genes with fold change ≥ 2 and an adjusted Q value ≤ 0.001 were considered differentially expressed genes (DEGs). Compared to the control, 664 and 312 genes were upregulated and downregulated, respectively, in the MG-treated samples (Fig. 4B, Table S2). The enriched Gene Ontology (GO) terms of the DEGs in the MG-

treated samples are listed in Table S3. Genes involved in catalytic activity, integral/intrinsic components of the membrane, membrane part, and membrane and oxidoreductase activity were enriched in MG treatment-responsive DEGs (Fig. 4C). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the DEGs caused by MG treatment in *F. graminearum* were concentrated in amino acid metabolism (glycine serine and threonine, branched-chain amino acids, tryptophan), fatty acid biosynthesis and metabolism. Genes involved in the MAPK signaling pathway, antibiotic biosynthesis and peroxisomes were also enriched in MG treatment-responsive DEGs (Fig. 4D, Table S4).

Effects of MG on antioxidant enzyme activities in *F. graminearum*

The activities of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), were compared in *F. graminearum* hyphae treated with or without MG at 0.1% or 0.5% (w/v). As shown in Fig. 5, the results demonstrated that the activities of these three antioxidant enzymes were significantly reduced in the MG-treated *F. graminearum* samples in a dose-dependent manner (Fig. 5A-C).



Discussion

Efficient and substantial control of FHB and toxin contamination caused by members of the FGSC remains a challenge in the wheat industry. Due to the lack of resistant wheat cultivars, the limitations of effective fungicides and the emerging resistance risk, the development and application of an integrated management strategy is urgently needed. The potential of antioxidants in plant disease resistance has been reported previously (Pani et al. 2014; Picot et al. 2013). Inhibitory effects of antioxidants on fungal growth and toxin production were observed in aflatoxin-producing *Aspergillus flavus* (Zhao et al. 2018) and fumonisin-producing *Fusarium verticillioides* and *F. proliferatum* (Picot et al. 2013). In the present study, we showed that the antioxidant MG effectively suppressed fungal growth and DON synthesis in the important wheat fungus *F. graminearum* in a dose-dependent manner. The antimicrobial activity of MG was previously reported in other plant and human pathogenic bacteria and fungi (Acharyya et al. 2015; Ahn et al. 2005; Choi et al. 2009). Until now, the mechanism of the action of the antioxidant MG has been poorly understood. Further elucidation of the mechanism of MG to inhibit fungal growth and DON synthesis is necessary for the efficient application of this compound in disease and toxin control in agriculture.

Natural antioxidants are crucially involved in the plant defense response to oxidative stress caused by fungal invasion (Pani et al. 2014; Picot et al. 2013). DON, a type-B trichothecene, is a crucial virulence factor, and its synthesis is induced when confronting reactive oxygen species (ROS) that are produced by host plants as a defense response during infection in *F. graminearum*. DON production is associated with oxidative stress and has been indicated to be part of an adaptive response to oxidative stress in this fungus. Upon MG treatment, reduced DON production normalization to the fungal biomass in liquid DON-inducing medium was observed. At the transcriptional level, we found that the expression of the DON biosynthetic *TRI* genes was downregulated after MG treatment. Recently, *Tri1*, the key enzyme involved in the late DON biosynthesis pathway, was mainly localized to the DON-toxisome derived from the endoplasmic reticulum under toxin-inducing conditions or during infection (Menke et al. 2013; Tang et al. 2018, 2020). Fluorescence confocal microscopy indicated that MG also has inhibitory effects against the formation of cellular DON-toxisome in *F. graminearum*.

Based on this finding, RNA-seq was further conducted to elucidate the mechanisms of the antifungal activity of MG. The results indicated that MG treatment at 0.5% (w/v) inhibited DON production, possibly by the antioxidant activities of MG and the cleavage of oxidant stresses and by altering enzyme function involved in

oxidoreductase activities, lipid oxidation and the membrane. Coupled with the observations that MG treatment decreases the enzymatic activities of CAT, SOD and POD in *F. graminearum*, the results of this study indicated that MG displays antifungal activity against DON production by modulating the fungal oxidative response.

Fungal conidial spores are specialized reproductive structures. Conidial formation and germination are crucial first steps in the asexual life cycle of *F. graminearum* and are important for successful plant colonization (Seong et al. 2008). After successful initiation of infection, hyphae were formed on the exterior surfaces of wheat florets and glumes and penetrated through stomata or other sites (Bushnell et al., 2003). Abnormal conidial morphology was also observed in MG-treated spores. MG significantly suppressed conidial formation and germination and mycelial growth at the concentrations indicated in Fig. 2 indicating the potential of MG to inhibit fungal colonization and spread within plants to effectively control disease.

Conclusions

In summary, the antioxidant methyl gallate (MG) was found to display inhibitory effects against mycelial growth, conidial morphogenesis, and DON biosynthesis in *F. graminearum* in a dose-dependent manner. RNA-Seq analysis identified DEGs responsive to 0.5% (w/v) MG treatment that were enriched in membrane functions and oxidoreductase activities. MG also affects the enzymatic activities of CAT, SOD and POD in *F. graminearum*. The results of this study indicated that MG displays antifungal activity against DON production, possibly by modulating the oxidative response of the fungus. Taken together, this study revealed the potential of antioxidants to inhibit mycotoxins in *F. graminearum* and to control FHB in wheat and their possible mechanisms of action.

Abbreviations

MG: Methyl gallate; FGSC: *Fusarium graminearum* species complex; FHB: Fusarium head blight; DON: Deoxynivalenol; PDA: Potato dextrose agar; PDB: Potato dextrose broth; CMC: Carboxymethyl cellulose; CFW: Calcofluor white; FMM: Fusarium minimum medium; TPM: Tag counts per million; TBI: Trichothecene biosynthesis inducing; qRT-PCR: Quantitative Real-Time PCR; CAT: Catalase; SOD: Superoxide dismutase; POD: Peroxidase; SD: Standard deviation; GFP: Green fluorescent protein; DGEs: Differentially expressed genes; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; ROS: Reactive oxygen species

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43014-021-00070-0>.

Additional file 1: Table S1. Oligonucleotide primers used in this study and their relevant characteristics. **Table S2.** Differentially expressed genes (DGEs) responsive to 0.5% (w/v) MG treatment in *F. graminearum*. **Table S3.** GO analysis of the DGEs responsive to 0.5% (w/v) MG treatment in *F.*

graminearum. **Table S4.** KEEG analysis of the DGEs responsive to 0.5% (w/v) MG treatment in *F. graminearum*.

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Authors' contributions

Xin Liu was involved in data acquisition, draft and critical revision of article, and final approval; Xin Fang, Shuang Wang, Deliang Wu and Tao Gao were involved in data analysis; Yin-Won Lee, Sherif Ramzy Mohamed and Jianhong Xu were involved in data acquisition and data consulting; Fang Ji and Jianrong Shi conceived and designed the paper. All authors read and approved the manuscript.

Authors' information

Xin Liu, Xin Fang, Shuang Wang, Fang Ji, Jianhong Xu, and Jianrong Shi (Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences; Jiangsu University); Deliang Wu and Tao Gao (Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences), Yin-Won Lee (Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences and Seoul National University), Sherif Ramzy Mohamed (National Research Centre, Egypt).

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest. Dr. Jianrong Shi and Dr. Yin-Won Lee are members of Editorial Board of Food Production, Processing and Nutrition and they were not involved in the journal's review of, or decisions related to this manuscript.

Author details

¹Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base, Ministry of Science and Technology/Key Laboratory for Control Technology and Standard for Agro-product Safety and Quality, Ministry of Agriculture and Rural Affairs/Key Laboratory for Agro-product Safety Risk Evaluation (Nanjing), Ministry of Agriculture and Rural Affairs/Collaborative Innovation Center for Modern Grain Circulation and Safety/Institute of Food Safety and Nutrition, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, Jiangsu, China. ²School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, Jiangsu, China. ³School of Agricultural Biotechnology, Seoul National University, Seoul 08826, Republic of Korea. ⁴Department of Food Toxicology and Contaminant, National Research Centre of Egypt, Giza 12411, Egypt.

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