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The non-metabolizable glucose analog D-glucal inhibits aflatoxin biosynthesis and promotes kojic acid production in *Aspergillus flavus*

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Abstract

Background: Aflatoxins (AFs) are potent carcinogenic compounds produced by several *Aspergillus* species, which pose serious threats to human health. As sugar is a preferred carbohydrate source for AF production, we examined the possibility of using sugar analogs to inhibit AF biosynthesis.

Results: We showed that although D-glucal cannot be utilized by *A. flavus* as the sole carbohydrate source, it inhibited AF biosynthesis and promoted kojic acid production without affecting mycelial growth when applied to a glucose-containing medium. The inhibition occurred before the production of the first stable intermediate, norsolorinic acid, suggesting a complete inhibition of the AF biosynthetic pathway. Further studies showed that exogenous D-glucal in culture led to reduced accumulation of tricarboxylic acid (TCA) cycle intermediates and reduced glucose consumption, indicating that glycolysis is inhibited. Expression analyses revealed that D-glucal suppressed the expression of AF biosynthetic genes but promoted the expression of kojic acid biosynthetic genes.

Conclusions: D-glucal as a non-metabolizable glucose analog inhibits the AF biosynthesis pathway by suppressing the expression of AF biosynthetic genes. The inhibition may occur either directly through interfering with glycolysis, or indirectly through reduced oxidative stresses from kojic acid biosynthesis.

Keywords: D-glucal, D-galactal, Aflatoxin biosynthesis, *Aspergillus flavus*, Metabolomics

Background

Aflatoxins (AFs) are highly carcinogenic secondary metabolites produced by *Aspergillus* species such as *A. flavus* and *A. parasiticus* after invading plants or stored grains. Contaminations of these toxins in the food chain pose serious threats to humans and animals [1,2]. Previous studies focused on understanding the molecular machinery of AF biosynthesis [3], which have shown that most genes involved in the production of AF are located in a co-regulated gene cluster that encodes two regulatory proteins (*aflR* and *aflS*) and at least 26 down-stream metabolic enzymes [4]. An independently regulated sugar utilization gene cluster is located adjacently [5].

Some environmental factors and chemical reagents are known to be able to inhibit AF production [6,7]. Sugar is the most frequently used carbohydrate for studying AF

production [8]. It has been proposed that the key factor determining if a carbohydrate supports AF production is its metabolic availability to the hexose monophosphate shunt and glycolysis pathway [9]. We thus speculate that sugar analogs that are unable to be utilized by *A. flavus* are candidate inhibitors for AF biosynthesis. Chemical analogs are often used to inhibit metabolism, as they may bind competitively to the active or allosteric sites of enzymes and hamper their activities [10,11]. Three glucose analogs, 2-deoxyglucose, α -methylglucoside and glucosamine, have been tested in *A. parasiticus* previously, but none of them inhibited AF production when applied to a glucose-containing medium [12].

D-glucal and D-galactal are cyclic enol ether derivatives of glucose and galactose, respectively (Additional file 1). In this study we examined in *A. flavus* for their effects on AF biosynthesis. It has been reported that D-glucal inhibits glucose oxidase (EC 1.1.3.4) [13-15], while D-galactal inhibits β -D-galactopyranosidase (EC 3.2.1.23) [16]. Whether these compounds have any effects on glycolysis and/or AF

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biosynthesis is not known. Results obtained in this study showed that D-glucal, but not D-galactal, is able to inhibit AF biosynthesis and to enhance kojic acid biosynthesis without affecting mycelial growth. The inhibition occurred before the production of norsolorinic acid (NOR), the first stable intermediate in the AF biosynthetic pathway. Metabolomics studies suggested that the glycolysis pathway was inhibited in mycelia grown in the presence of D-glucal. Using quantitative reverse transcription-PCR (qRT-PCR), we showed that exogenous D-glucal suppressed expression of AF biosynthetic genes tested but enhanced expression of kojic acid biosynthetic genes.

Results

Use of D-glucal and D-galactal as the sole carbohydrate source did not support mycelial growth

The usual GMS medium used for culturing *A. flavus* contains 50 mg/mL glucose [17]. To examine if D-glucal and D-galactal could be used as the sole carbohydrate for mycelial growth, we replaced the glucose in the medium with 20 or 40 mg/mL D-glucal or D-galactal. Media containing either 20 or 40 mg/mL D-glucose were used as the control. After incubation of *A. flavus* A 3.2890 spores in these media for 3 d, we observed no mycelial growth in media with D-glucal or D-galactal, while abundant mycelial growth was observed in those two controls (Figure 1). No further growth was observed in media with D-glucal or D-galactal even when the incubation period was extended to 10 d, suggesting neither these two sugar analogs support mycelial growth when used as the sole carbohydrate.

D-glucal inhibited AF biosynthesis and sporulation without affecting mycelial growth in GMS media

To test whether D-glucal or D-galactal inhibit AF biosynthesis, spores of *A. flavus* A 3.2890 were inoculated in GMS liquid media (containing 50 mg/mL glucose) supplied with 2.5, 5, 10, 20, or 40 mg/mL of D-glucal or D-galactal and cultured at 28°C for 5 d. GMS media with the same amounts of additional D-glucose were used as controls. AFs were extracted from each sample, and the AFB1 contents were quantified using high pressure liquid chromatography (HPLC). As shown in Figure 2A, the AFB1 content was reduced significantly in samples with 2.5 to 40 mg/mL D-glucal. An almost complete inhibition was observed when 40 mg/mL D-glucal was used. In contrast, GMS media supplied with 2.5, 5 or 10 mg/mL D-glucose promoted AFB1 production (Figure 2A). In samples supplied with D-galactal only a slight inhibition on AFB1 production was detected at the concentration of 40 mg/mL (Figure 2A). Using thin layer chromatography (TLC) analyses, we showed further that production of other AFs such as AFB1 and AFG1 were also inhibited by D-glucal (Figure 2B).

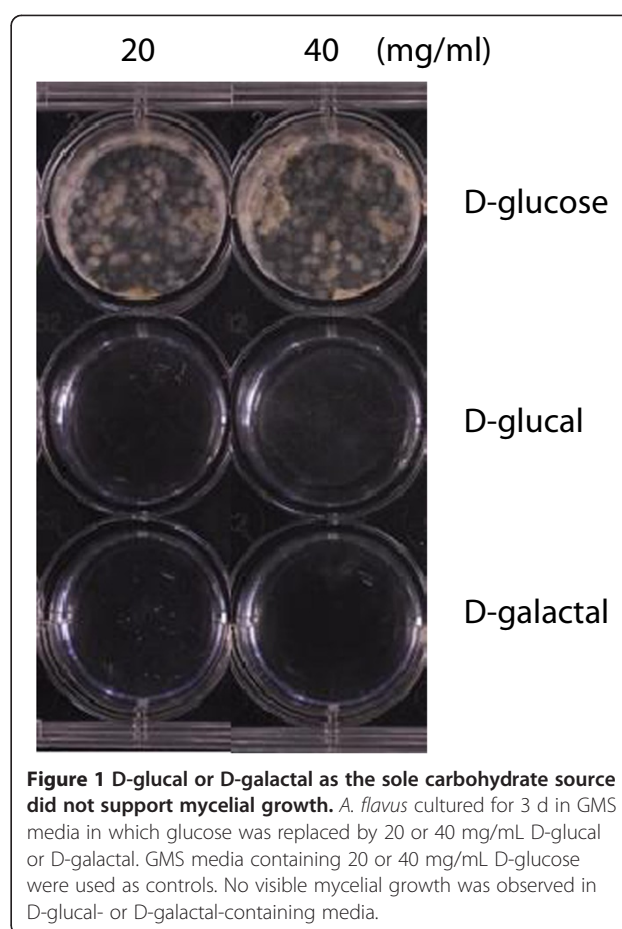


Figure 1 D-glucal or D-galactal as the sole carbohydrate source did not support mycelial growth. *A. flavus* cultured for 3 d in GMS media in which glucose was replaced by 20 or 40 mg/mL D-glucal or D-galactal. GMS media containing 20 or 40 mg/mL D-glucose were used as controls. No visible mycelial growth was observed in D-glucal- or D-galactal-containing media.

We next examined if D-glucal or D-galactal inhibited mycelial growth, and found that neither D-glucal nor D-galactal affected mycelial growth at the concentration of 40 mg/mL (Figure 2C). In contrast, additional D-glucose enhanced mycelial growth significantly, especially from the 3rd day onwards (Figure 2C). We next performed experiments on solid GMS media with 40 mg/mL D-glucal or D-galactal to assess if these sugar analogs have any effect on sporulation, and observed that exogenous D-glucal inhibited sporulation significantly, while additional D-glucose enhanced sporulation (Figure 2D). No effect was observed for D-galactal.

D-glucal promoted kojic acid biosynthesis, but inhibited fatty acid biosynthesis and glucose consumption

We performed metabolomics analyses of mycelia of *A. flavus* A 3.2890 grown in media with or without 40 mg/mL D-glucal. The gas chromatography time-of-flight mass spectrometry (GC-TOF MS) based metabolomics technology developed in our lab has been shown to be a powerful tool to elucidate metabolic changes in *A. flavus* [18]. For statistical analyses, we used nine replicates for each treatment. Partial least-squares (PLS) analyses of metabolite peak areas showed clustering of two distinct

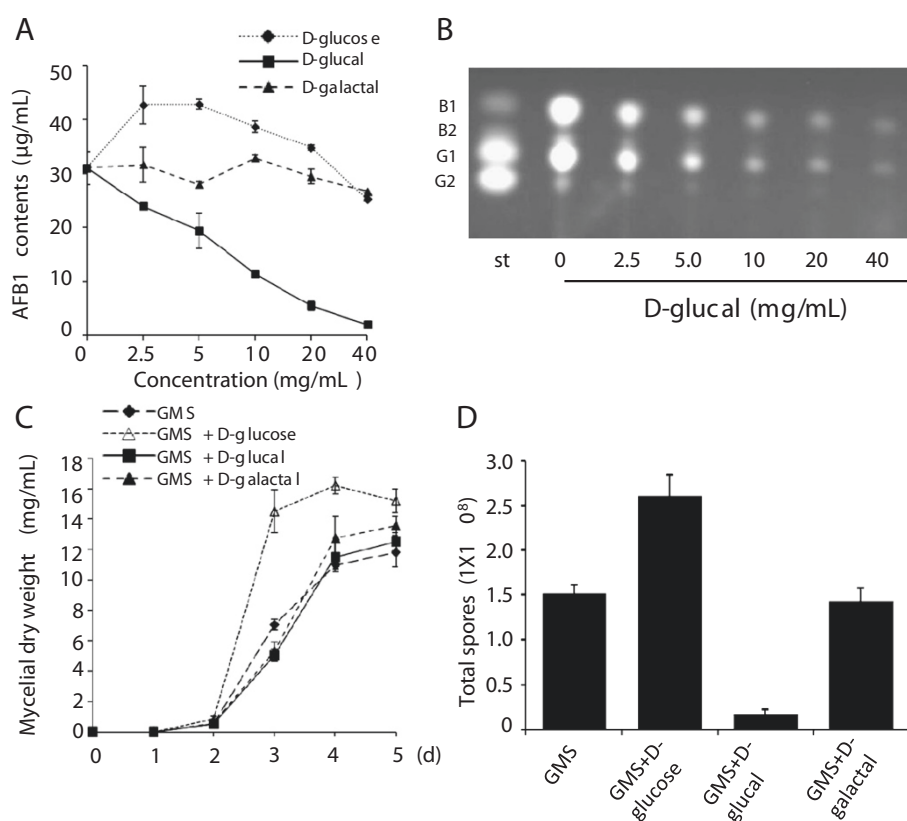


Figure 2 Effects of D-glucose and D-galactal on AF production and sporulation. **(A)** Amounts (μg per mL media) of AFB1 produced by *A. flavus* with different concentrations of D-glucose, D-glucal, or D-galactal (0, 2.5, 5, 10, 20 or 40 mg/mL). Data are presented as means \pm S.D. ($n = 3$), from 3 independent experiments. **(B)** TLC analyses of AF production by *A. flavus* cultured in GMS media with different concentrations of D-glucose (0, 2.5, 5, 10, 20 or 40 mg/mL). **(C)** Growth curves of mycelia cultured in media with 40 mg/mL D-glucose, D-glucal, or D-galactal for 5 d. **(D)** Numbers of spores produced per mL culture with D-glucose, D-glucal, or D-galactal. Data are presented as means \pm S.D. ($n = 3$).

groups for mycelia grown in media with or without D-glucal, suggesting that exogenous D-glucal imposed significant metabolic changes in mycelia (Figure 3). In particular, in the presence of D-glucal, the content of glucose, ribitol, glycerol and galactose were increased significantly, while the content of TCA intermediates (succinic acid, malic acid and fumaric acid) and fatty acids (FAs) including palmitic acid, stearic acid, oleic acid and linoleic acid were decreased (Table 1). We also noticed that, in the presence of D-glucal, the content of two secondary metabolites, kojic acid and furanacetic acid, were increased by 2 and 159 fold, respectively. These results together suggest that D-glucal interferes with both primary and secondary metabolism.

We next cultured *A. flavus* A 3.2890 in GMS media with or without 40 mg/mL D-glucal, and measured kojic acid contents in media using a colorimetric method [19]. During the 5-d culture period the kojic acid contents in media with D-glucal were always higher (about 4 to 5 folds) than the control (Figure 4A). We also measured glucose content in the media and observed that, in the presence of D-glucal, the glucose content on the 4th and

the 5th d were about 30% higher than those in the control media lacking D-glucal, suggesting that exogenous D-glucal inhibited the consumption of glucose (Figure 4B).

D-glucal inhibited NOR production

We used the *A. flavus* strain Papa 827 to decipher at which step D-glucal inhibits AF biosynthesis. The lack of functional NOR reductase in this strain results in the accumulation of the first stable compound, NOR, in the AF biosynthetic pathway [20]. NOR is pinkish in color. After 4-d cultures, the control plate was pink in color, while no color was observed in the plate with 40 mg/mL D-glucal. Spectrophotometric analyses showed that NOR productions were significantly inhibited by D-glucal at concentrations of 10 mg/mL or higher (Figure 4C). These results suggest that D-glucal inhibits the AF biosynthesis pathway prior to the production of NOR.

D-glucal inhibited expression of AF biosynthetic genes, but promoted expression of kojic acid biosynthetic genes

To examine the effect of D-glucal on AF biosynthesis at the transcriptional level, we analyzed expression of several

Table 1 Metabolites with significant differences between mycelia of *A. flavus* grown in media with or without D-glucal (40 mg/mL)

Compounds ^a	Relative peak area ^b		Fold increase ^c	P value ^d
	Control	D-glucal		
Organic acids				
Furanacetic acid	0.0184 ± 0.0039	2.9291 ± 0.2771	159.10	<0.01
Kojic acid	0.0942 ± 0.0333	0.2076 ± 0.0293	2.20	<0.01
Sugar metabolism				
Ribitol	0.0066 ± 0.0038	0.0168 ± 0.0051	2.56	<0.01
Glycerol	0.0219 ± 0.0055	0.0514 ± 0.0350	2.34	<0.01
D-glucose	0.0133 ± 0.0060	0.1233 ± 0.0400	9.27	<0.01
D-galactose	0.0317 ± 0.0096	0.1750 ± 0.0743	5.53	<0.01
TCA intermediates				
Succinic acid	0.0053 ± 0.0016	0.0020 ± 0.0005	0.37	<0.01
Malic acid	0.0023 ± 0.0013	ND	ND	ND
Fumaric acid	0.0003 ± 0.0001	0.0002 ± 0.0000	0.53	<0.01
Fatty acids				
Palmitic acid	0.1428 ± 0.0116	0.0856 ± 0.0144	0.60	<0.01
Stearic acid	0.0702 ± 0.0150	0.0468 ± 0.0072	0.66	<0.01
Oleic acid	0.1957 ± 0.0159	0.0377 ± 0.0093	0.19	<0.01
Linoleic acid	0.2647 ± 0.0219	0.1281 ± 0.0212	0.48	<0.01
Others				
Glycine	0.0010 ± 0.0004	0.0004 ± 0.0002	0.39	<0.01
Pyrimidine	0.0018 ± 0.0005	0.0009 ± 0.0001	0.53	<0.01

^aIndividual metabolites were identified by GC-TOF MS, as described in the Methods. ^bRelative peak area as normalized to the peak area of heptadecanoic acid. ^cFolds represents the relative peak areas in D-glucal-treated samples/peak areas in the control. ^dStatistical differences between control and D-glucal treated samples were calculated by two-tailed Student's t-test. N.D.: not detected.

growth and metabolic activities based on the availability of glucose [28]. Genomic sequencing of *A. flavus* revealed 55 putative secondary metabolism gene clusters that are differentially regulated through global transcriptional regulators such as LaeA and VeA [2]. Individual secondary metabolic pathways may further be regulated independently by transcriptional regulators located in individual gene clusters for example, *aflR* and *aflS* in AF biosynthesis and *kojR* in kojic acid biosynthesis [2,29,30].

Non-metabolizable chemical analogs have been used in the past to inhibit metabolic pathways and to study metabolism [25]. In this study, we examined D-galactal and D-glucal, non-metabolizable chemical analogs of D-glucose and galactose, respectively, for their effects on AF biosynthesis in *A. flavus*. We observed that 40 mg/mL D-galactal as a galactose analog did not have much effect on AF production. This is not surprising as though galactose supports mycelial growth, it cannot be utilized efficiently for AF biosynthesis [8,31], suggesting galactose utilization might be independent from the AF biosynthesis pathway. In contrast, 40 mg/mL D-glucal effectively inhibited AF biosynthesis. In the presence of D-glucal, glucose consumption and FA biosynthesis were reduced;

the concentrations of TCA cycle intermediates were also reduced. In contrast, the production of kojic acid, a secondary metabolite produced directly from glucose, and furanacetic acid, a secondary metabolite of unknown function, were increased. At the metabolic level, we observed that D-glucal inhibited AF biosynthesis before production of the first stable intermediate, NOR. Based on these observations, we propose that, as depicted in route ① of Figure 6, D-glucal may interfere directly with enzymes such as hexokinase in glycolysis to prevent sufficient acetyl-CoA to be produced for TCA cycling, and for AF and FA biosynthesis in *A. flavus*. Consequently this has led to the increased glucose level observed in media and possibly in mycelia as well, which may enhance kojic acid biosynthesis. This hypothesis is in agreement with some previous observations that showed that active AF production usually correlates with increased accumulation of TCA cycle intermediates and active FA biosynthesis [26,32,33].

Alternatively, since kojic acid is synthesized from glucose without going through glycolysis [34], exogenous D-glucal may interfere with the sugar sensing machinery to enhance kojic acid production directly. The accumulation

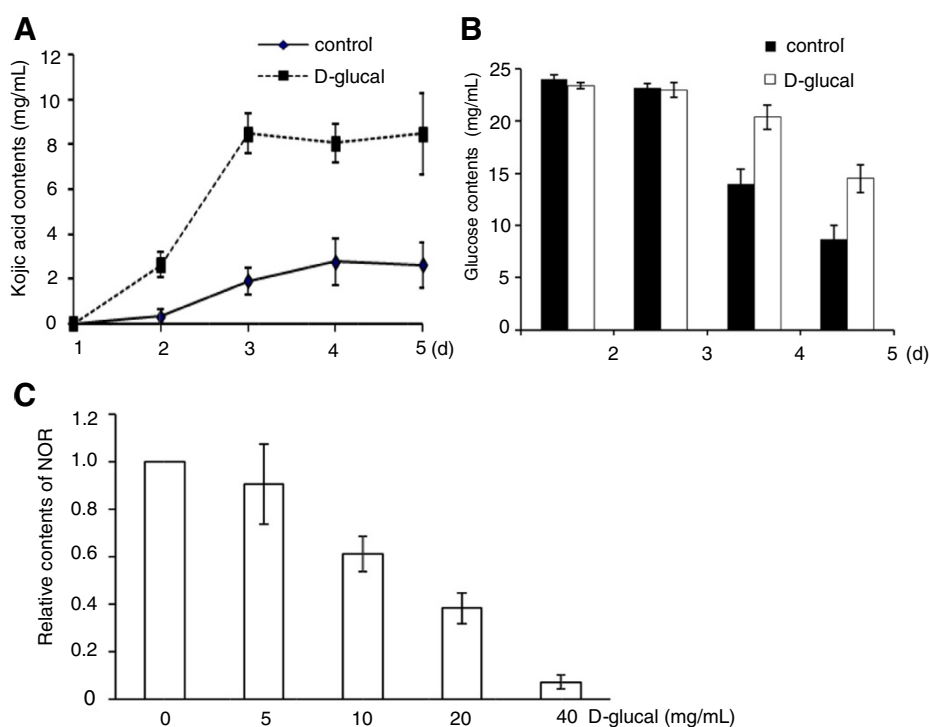


Figure 4 Effects of D-glucal on kojic acid production, glucose consumption and NOR accumulation. **(A)** Production of kojic acid by *A. flavus* grown in GMS media with or without 40 mg/mL D-glucal. Values are presented as means \pm S.D. ($n = 3$), from three independent experiments. **(B)** Glucose contents in media at different time points when cultured in the presence of 40 mg/mL D-glucal. **(C)** D-glucal inhibited NOR accumulation. The amount of NOR in GMS medium lacking D-glucal was set to 1, those in other samples were calculated accordingly. Values are presented as means \pm S.D. ($n = 3$).

of kojic acid may have then relieved the oxidative stress in the fungus, which consequently inhibits AF biosynthesis at the transcriptional level, as depicted in route ② of Figure 6. It is known that kojic acid is a potent antioxidant that is able to scavenge reactive oxygen species [35], and oxidative stress is a prerequisite for AF production [36]. As reported previously, antioxidants such as eugenol, saffron and caffeic acid are able to inhibit AF biosynthesis [37-39]. A negative correlation between kojic acid and AF production has been shown before. D-xylose, ethanol, Diocatin A and high temperature are factors known to promote kojic acid production, but inhibit AF biosynthesis [40,41].

We also showed that, although neither D-glucal nor D-galactal supported mycelial growth when used as the sole carbohydrate source, D-glucal inhibited sporulation without affecting mycelial growth. Secondary metabolism is usually associated with sporulation in fungi [42], a G-protein signaling pathway is involved in coupling these two processes [43,44]. The coupling does not seem to be very tight, as molasses promotes sporulation but suppresses AF production in *Aspergillus flavus* [45]. It will be interesting to study if D-glucal acts independently in AF production and sporulation, or if a common signaling pathway is involved in both processes.

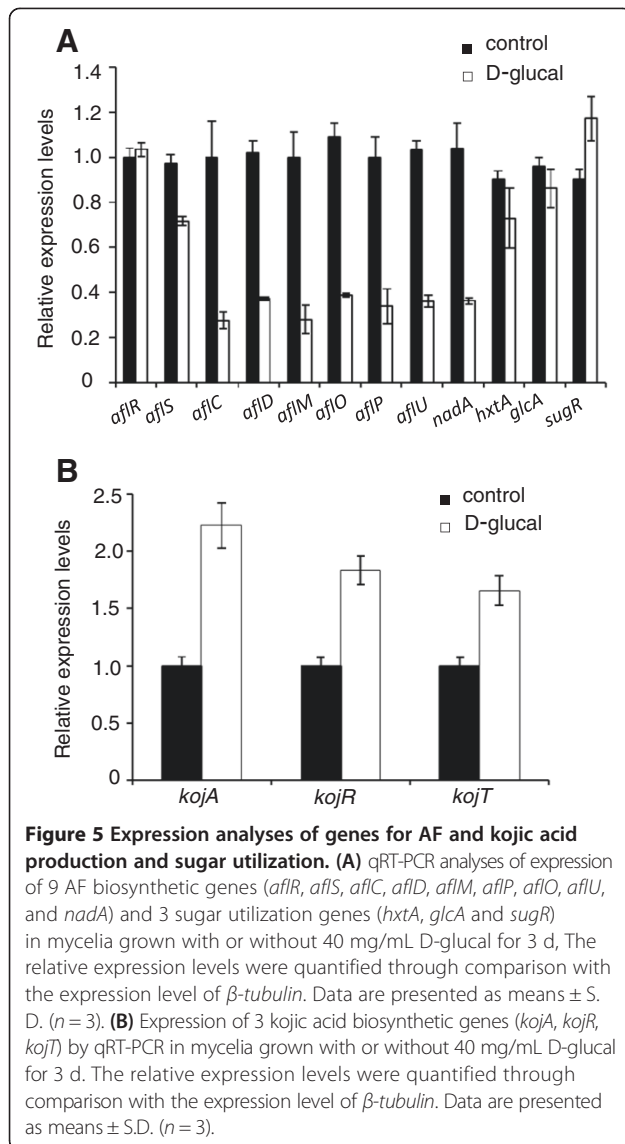
Conclusions

We showed in this study that D-glucal effectively inhibited AF biosynthesis and promoted kojic acid biosynthesis through modulating expression of genes in these two secondary metabolic pathways. The inhibition may occur either directly through interfering with glycolysis, or indirectly through reduced oxidative stresses from kojic acid biosynthesis.

Methods

Fungal strains and culture conditions

A. flavus A3.2890 was obtained from the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences. *A. flavus* Papa 827 was provided by Gary Payne [20]. All strains were maintained in glycerol stocks and grown on potato dextrose agar (PDA) medium at 37°C for 4 d before spores were collected to initiate new cultures. The PDA medium was also used for the examination of NOR accumulation. For all other experiments, Adye and Mateles' GMS medium was used (containing 5% glucose) [17]. D-glucal and D-galactal were purchased from Chemsynlab (Beijing, China). AF standards were purchased from Sigma (St. Louis, USA).



Determination of fungal dry weights

Mycelia cultured for 2, 3, 4 and 5 days were harvested by filtration through two layers of filter paper, washed by sterilized water, and freeze-dried before weighing.

AF extractions and analyses

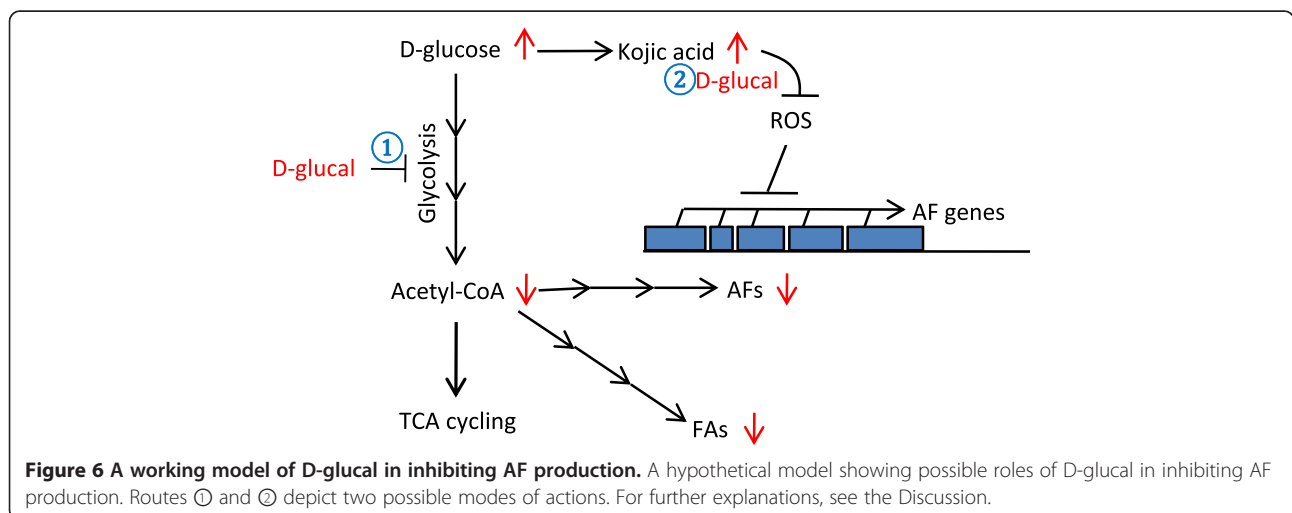
Mycelia grown in 1 mL GMS media were extracted using 1 mL chloroform/water (1:1). After vortexing for 2 min, the mixture was centrifuged at 12,000 rpm for 10 min. The organic phase was then collected and filtered through a 0.22 μ m filter, dried under nitrogen gas, and re-dissolved in 200 μ L chloroform before being analyzed by TLC as described previously [18]. The AFB1 content was measured by HPLC (Agilent 1200, Waldbronn, Germany) using a reverse phase C18 column (150 mm in length and 4.6 mm in internal diameter, 5 μ m particle size, Agilent), eluted initially with 25% methanol/20% acetonitrile water solution for 3 min, and then with 38% methanol for 2.9 min, detected by a DAD analyzer at 360 nm. Quantifications were performed by measuring peak areas and comparing with an AFB1 standard calibration curve.

Spore counting

Three mL of sterile water with 0.05% Tween-20 was added to the surface of PDA plates on which *A. flavus* were grown for 3 d. Spores were scraped with a cell scraper before being counted with a haemocytometer.

qRT-PCR

Mycelia grown in GMS media with or without 40 mg/mL D-glucal for 3 d were collected and ground in liquid nitrogen, and total RNA was extracted using a Trizol solution (Invitrogen, CA, USA). PolyA mRNA was purified from mycelia with the PolyAT Rack mRNA isolation system (Promega, Madison, WI). Template cDNA was synthesized by reverse transcription with ReverTra Ace- α -^o (Toyobo, Japan) at 42°C for 1 h, followed by incubation



at 85°C for 15 min to terminate the reaction. qRT-PCR was performed using SYBR Green I (Takara, Japan) and a Rotor-Gene 3000 (Corbett, Australia) with primers described in Additional file 2: Table S1. PCR programs used are 94°C for 30 sec, 40 cycles at 94°C for 30 sec, followed by annealing (55°C for *aflO*, *aflR*, *aflS*, *aflD* and β -*tubulin*; 62.5°C for *aflU* and *nadA*; 58°C for *kojA*, *kojR* and *kojT*; 61°C for *hxtA*, *glcA* and *sugR*; 60°C for *aflC*, *aflM* and *aflP*) for 30 sec, and 72°C for 30 sec. The relative expression levels were quantified by comparing the expression level of β -*tubulin*.

Kojic acid and glucose measurements

A. flavus A3.2890 was cultured in a GMS liquid medium plus 40 mg/mL D-glucal for 5 d. Media samples were harvested by centrifugation at 12,000 rpm for 10 min before kojic acid was quantified according to Bentley [19]. Glucose contents in media were measured by using a glucose determination kit (Appligen, Beijing). The absorbance was measured at 550 nm using a multimode plate reader (Tecan Infinite M200 PRO, Switzerland), and calculated against a glucose standard curve.

Metabolomics analyses

Metabolites in mycelia of *A. flavus* A3.2890 cultured in a GMS liquid medium with or without 40 mg/mL D-glucal for 5 d were purified, silyl-derivatized and analyzed with GC-TOF MS as described previously [18], with minor modifications. The column temperature was held at 100°C for 3 min, and raised to 150°C at a rate of 10°C/min, then to 250°C at 5°C/min, finally to 300°C at 10°C/min, and held for 15 min at 300°C. PLS analysis was performed using SIMCA-P V12.0 (Umetrics, Sweden).

NOR analyses

A. flavus Papa 827 was cultured for 4 d on PDA media containing 0, 5, 10, 20, or 40 mg/mL D-glucal. Quantification of NOR was performed as reported [46] with modifications. Briefly, media samples were mixed with 0.5 mL 90:10 methanol/1 N NaOH (pH 10). NOR is pinkish at this pH, which allows for spectrophotometric measurement at 595 nm with a 96-well Tecan plate reader.

Statistical analyses

All experiments were conducted with at least 3 replicates and statistical significance was evaluated using Student's *t*-tests.

Additional files

Additional file 1: Structures of D-glucose, D-glucal and D-galactal.

Additional file 2: Table S1. Primers used for qRT-PCR.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JDZ designed and performed the experiments; JDZ and LDH analyzed the data; SJY helped to develop some analysis tools; JDZ and CML wrote the manuscript. All authors read and approved the final manuscript.

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