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Short Communication

L-Threonine and its analogue added to autoclaved solid medium suppress trichothecene production by *Fusarium graminearum*

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Abstract

Fusarium graminearum produces trichothecene mycotoxins under certain nutritional conditions. When L-Thr and its analogue L-*allo*-threonine were added to brown rice flour solid medium before inoculation, trichothecene production after 4 days of incubation was suppressed. A time-course analysis of gene expression demonstrated that L-Thr suppressed transcription of *Tri6*, a trichothecene master regulator gene, and a terpene cyclase *Tri5* gene. Regulation of trichothecene biosynthesis by altering major primary metabolic processes may open up the possibility to develop safe chemicals for the reduction of mycotoxin contamination.

Keywords: amino acids, *Fusarium graminearum*, mycotoxin production inhibitor, nitrogen source, *Tri* gene expression

Electronic supplementary materials

The online version of this article contains supplementary material.

Introduction

Trichothecenes are mycotoxins that are known as food and feed contaminants associated with Fusarium head blight (FHB) in important cereal crops (Kimura et al. 2006; Desjardins 2009). They are also known as phytotoxins that help the causal pathogens *Fusarium graminearum* species complex and *Fusarium culmorum* infect the host plants (Bai et al. 2002; Nishiuchi et al. 2006). The major trichothecenes that accumulate in cereal grains are type B trichothecenes, such as deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), nivalenol (NIV), and 4-acetylnivalenol (4-ANIV) (Kimura et al. 2007; McCormick et al. 2011; Son and Lee 2012). These mycotoxins are often found in the grains that apparently look normal, making it difficult to discriminate between uninfected and infected grains. Unregulated marketing and consumption of cereal grains cause human and animal toxicosis through the ingestion of contaminated food and feed (Sugita-Konishi 2008), as mycotoxins are chemically stable to heating and also persist after processing. A provisional maximum tolerable daily intake (PMTDI) for DON was set at 1 μg/kg body weight for human consumption by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2001, which was later extended to include its acetylated derivatives 3-ADON and 15-ADON in 2010 (Yoshizawa 2013). For the development of methods to reduce the mycotoxin contamination, it is important to understand the regulatory mechanisms of trichothecene gene (*Tri* gene) expression (Nakajima et al. 2014).

AreAp is a GATA-type zinc finger transcription factor that is necessary for the utilization of nitrogen sources other than ammonium and L-Gln in *Aspergillus nidulans* (Todd et al. 2005). AreAp binds to DNA regions containing the 5'-HGATAR-3' sequence and activates the gene expression (Ravagnani et al. 1997). In *F. graminearum*, several consensus AreAp-binding sites are found in the promoter

region of *Tri6,* a trichothecene master regulator gene that positively regulates *Tri* gene expression (Merhej et al. 2011). In agreement with the occurrence of the AreAp-binding site, trichothecene production by *AreA* (*FgAreA*) disruption mutants (Δ*FgareA*) is markedly reduced compared to the wild type, suggesting that FgAreAp positively regulates *Tri* gene expression under the experimental conditions (Min et al. 2012; Giese et al. 2013). This led us to assume that activation of the FgAreAp transcription factor by stimulating the catabolism of FgAreAp-dependent nitrogen sources might affect trichothecene production by the fungus.

In this study, we examined the effect of feeding specific amino acids on trichothecene production by *F. graminearum*. Autoclaved brown rice flour solid medium was additionally supplemented with the amino acids, incubated with the fungus, and analyzed for trichothecene content. In contrast to our initial hypothesis that the catabolism of FgAreAp-dependent amino acids activates trichothecene biosynthesis, a suppressive effect on trichothecene production was observed with L-Thr.

Materials and methods

Strain and reagents

F. graminearum Schwabe JCM 9873, which produces 15-ADON/DON on a solid substrate (Nakajima et al. 2014), was obtained from Japan Collection of Microorganisms (JCM), Tsukuba, Japan, and mainly used in this study. *Fusarium asiaticum* O' Donnell, T. Aoki, Kistler et Geiser MAFF 240548 (strain NIV 2), a 4-ANIV/NIV producer, and *F. graminearum* Schwabe MAFF 240560 (strain ZEA 1), a 3-ADON/DON producer, were obtained from the Genetic Resource Centre, NARO (National Agriculture and Food Research Organization), Tsukuba, Japan.. L-amino

acids were obtained from Wako Pure Chemicals (Osaka, Japan). L-*allo*-threonine and L-threonine benzyl ester hydrochloride were purchased from Tokyo Kasei Co. Ltd, Japan. Kieselgel F_{254} TLC plates were from Merck Millipore (Darmstadt, Germany). 4-(*p*-nitrobenzyl)pyridine (NBP) and tetraethylene pentamine (TEPA) were from Wako Pure Chemicals. HPLC-grade acetonitrile was obtained from Sigma (Sigma-Aldrich, St. Louis, MO). HPLC-grade methanol and other organic solvents were from Kanto Chemical Co. (Tokyo, Japan).

Preparation of conidia

Conidia induced from mycelial plugs of V8 juice agar [20% (v/v) V8 juice, 0.3% (w/v) calcium carbonate, and 2% (w/v) agar] and YG agar [0.5% (w/v) Bacto™ yeast extract, 2% (w/v) glucose, and 2% (w/v) agar] do not consistently yield sufficient amount of trichothecenes when directly inoculated on autoclaved brown rice flour solid medium. Thus, fresh conidia induced from germinating spores were used as inocula for the assay. Briefly, conidial suspension, induced from the mycelial plugs and stored at -80 ^{o}C (10^7 spores/ml in 30% glycerol), was used as an inoculum [5% (v/v)] for spore germination in CMC medium [1.5% (w/v) carboxymethyl cellulose sodium salt, 0.1% (w/v) NH_4NO_3, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) yeast extract and 0.05% (w/v) $MgSO_4$ heptahydrate]; spores germinated in CMC medium subsequently developed into new conidia. The fresh conidia thus obtained in each experiment (recorded with conidial suspension lot number; Supplementary Fig. S1) were immediately used for the trichothecene assay on the solid medium.

Treatment of solid culture with amino acids and related compounds

For the inhibition assay, brown rice (5 g) was ground to a powder using a coffee mill. The brown rice flour was then mixed with water (2.5 ml) in a 100 ml beaker, covered with aluminum foil, and allowed to stand for 3 h at room temperature. The solid medium was autoclaved at 121 ^{o}C for 15 min. After cooling to room temperature, solutions (500 µl) of candidate amino acids (in water) were immediately added to 5 g of autoclaved brown rice flour and mixed well using a sterilized spatula under aseptic conditions. As mock-treated controls, sterilized water (500 µl) was added in the same manner. After 30 min of absorption, the modified solid medium was inoculated with 1 x 10^5 /ml conidial suspension (500 µl), mixed well, and incubated at 25 ^{o}C in the dark. During the incubation periods, the solid culture was mixed well with a spatula with every 24 h.

Extraction and analysis of trichothecenes

After an appropriate incubation period, an aliquot of the solid culture was taken and suspended in four times the amount of 84% (v/v) acetonitrile. The supernatant solution was placed under a gentle stream of nitrogen to remove acetonitrile, water-insoluble residues were removed by centrifugation, and the trichothecenes were extracted twice with an equal amount of ethyl acetate. The solvent was evaporated and the dried material containing the trichothecenes was dissolved in a small volume of ethanol for subsequent TLC and HPLC analyses. For semiquantitative TLC analysis, the samples spotted on a Kieselgel F_{254} plate were developed with ethyl acetate:toluene (3:1) and visualized by NBP/TEPA as the color-developing reagents (Takahashi-Ando et al. 2008b). For quantification by HPLC-UV analysis, trichothecenes were separated by reverse-phase column chromatography with PEGASIL ODS SP100 (4.6x250 mm, 5 µm, 100Å; Senshu Scientific Co., Ltd., Tokyo), as previously described (Etzerodt et al. 2015).

Analysis of ergosterol content

Half of the 4-day-old fungal culture with brown rice flour residue (ca. 3.5 g) was suspended in methanol (12.5 ml) in a 50 ml plastic tube and vigorously mixed using a vortex mixer. The 50 ml tube was then attached to a floating rack "Jellyfish" (Watson Biolabs, Kobe, Japan), floated in a model EYELA MUS-10D sonication bath (Tokyo Rikakikai Co., Ltd., Tokyo), and treated for 3 min at maximum sonication amplitude and 100% duty cycle. The methanol solution was filtered through filter paper (filter paper No. 1; Advantech Toyo Co., Tokyo) and a portion of the recovered sample (500 µl) was transferred to a 1.5-ml plastic tube. The sample was then mixed with potassium chloride (70 mg), *n*-hexane (250 µl) was added to the tube, and the mixture was incubated at 65 ^{o}C for 30 min. After cooling to room temperature, water (125 µl) and *n*-hexane (250 µl) were added, mixed well, and the solvent layer was recovered by centrifugation. To the remaining water-methanol phase, *n*-hexane (250 µl) was added and the same extraction process was repeated. The combined solvent layers were dried under a gentle stream of nitrogen, the dried sample was dissolved in HPLC-grade ethanol (500 µl), and an aliquot (10 µl) was used for ergosterol analysis using a PEGASIL ODS column (diameter, 4.6 mm; length, 250 mm; Senshu Scientific Co., Tokyo) with acetonitrile+methanol+water (49+49+2 by volume) at a flow rate of 1 ml/min. The eluates were monitored at 282 nm, and the peak area of samples was normalized to an ergosterol standard (Wako) of known concentration eluted at 21.1 min.

Analysis of *Tri* gene expression by quantitative real-time reverse-transcription (RT)-PCR

The expression of *Tri6* (Proctor et al. 1995) and *Tri5* (Hohn and Beremand 1989) were analyzed by real-time RT-PCR using a LightCycler 1.5 Instrument (Roche Diagnostics Japan, Tokyo) with THUNDERBIRD® Probe qPCR Mix (Toyobo Co., Ltd., Otsu, Japan). Ten 100-ml beakers containing the inoculated solid medium (see the third section of **Material and methods**), five for the L-Thr treatment and five for a mock treatment, were sampled after incubation for 36, 48, 60, 72, and 96 h. At each sampling time, half of the fungal culture sample (ca. 3.5 g) was collected and used for trichothecene analysis, and the remaining half (except at t = 96 h) was used for RNA analysis. cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix (Toyobo Co., Ltd.) using total RNA prepared as previously described (Etzerodt et al. 2015). A single standard DNA (Ohno et al. 2012) containing cDNA fragments of *Tri10*, *Tri6*, *Tri5*, *Tri4*, and a ubiquitin conjugating enzyme gene (FGSG_10805; *Ubc*) (Supplementary Fig. S2A) was used as a reference to determine the number of cDNA molecules in the reverse-transcribed samples. *Ubc* was previously identified as a suitable reference gene for the normalization of *Tri* gene expression (Kim and Yun 2011; Faltusová et al. 2015). Specific primers and Universal ProbeLibrary probes (Roche Applied Science, Basel, Switzerland) used for the amplification analysis are described in Supplementary Fig. S2B. The reactions were carried out in LightCycler® capillaries (20 µl) with 0.5 µl of the reverse transcription reaction mixture, 0.2 µM specific primers, and a Universal ProbeLibrary probe for each gene, as previously reported (Etzerodt et al. 2015). The concentrations of sample cDNA were determined by applying to the standard curves of the crossing point (Cp) values *versus* concentrations of 10-fold serial dilutions of a single standard DNA. Expression levels of *Tri6* and *Tri5* were normalized to the expression level of *Ubc*, which was used as an endogenous reference in the same RNA sample by dividing target *Tri* gene cDNA copy number by the *Ubc* cDNA copy number.

Results

L-Thr as a trichothecene biosynthesis inhibitory amino acid when added to autoclaved rice flour medium

Previous study revealed that an *FgAreA* disruption mutant of *F. graminearum* is not able to grow on the medium containing L-Asp, L-Cys, L-Gly, L-Glu, L-Ile, L-Leu, L-Lys, L-Thr, L-Trp, L-Tyr, and L-Val as a sole nitrogen source (Giese et al. 2013). The result implies that an activated FgAreAp transcription factor is necessary for utilization of these amino acids. To investigate whether these FgAreA-dependent nitrogen sources facilitate binding of FgAreAp to the *Tri6* promoter and activate trichothecene biosynthesis, each amino acid was added to autoclaved brown rice flour solid medium at a concentration of 10 μmol/g medium.

After 4 days of incubation, trichothecenes produced by JCM 9873 were quantified as described in **Materials and methods**. Contrarily to our initial hypothesis, some amino acids suppressed trichothecene production (Supplementary Fig. S3). Among the amino acids, L-Thr most significantly suppressed trichothecene production by *F. graminearum*. There were apparently no growth and morphological differences among the fungal culture supplemented with the amino acids (data not shown). The result suggested that catabolism of L-Thr afforded considerably negative impacts on induction of trichothecene biosynthesis. We thus examined the inhibitory activity of L-Thr in more detail and carefully, as demonstrated below.

Effects of L-Thr concentration on trichothecene production by *F. graminearum* on the solid medium

L-Thr solution (diluted to an appropriate concentration from a 200 mM stock solution) was added to autoclaved brown rice flour at a final concentration of 2, 10, and 20 μmol/g and strain JCM 9873 was cultured for 4 days. Growth of the fungus was unaffected at these L-Thr concentrations, as measured by the amount of ergosterol extracted from the solid culture (Table 1). However, trichothecene accumulation was significantly reduced when L-Thr was used at a concentration of 2 μmol/g brown rice flour solid medium for the assay. The amount of trichothecene decreased as the concentration of L-Thr increased; the total amount of trichothecene after treatment with 10 and 20 μmol/g brown rice flour solid medium of L-Thr was approximately 1/4 and 1/6 that of the mock-treated control, respectively (Table 1).

Although the suppressive effects of L-Thr were dose-dependent, as shown by the average concentration of trichothecenes, no statistical significance was detected between the range of 2 to 20 μmol/g brown rice flour solid medium in our experiments (n = 4). The lack of significance is caused due to very high standard deviations in the amount of trichothecenes produced by each conidial lot (Supplementary Fig. S1). When toxin/ergosterol ratios to that of a mock-treated control were used for the analysis, statistical significance between 2 and 20 μmol/g brown rice flour solid medium was detected (Table 1).

The suppressive effect of L-Thr was also significant in fungal strain MAFF 240548 (strain NIV 2) that produces a moderate amount of 4-ANIV/NIV (Supplementary Fig. S4). Trichothecene accumulation also decreased after L-Thr treatment in the 3-ADON/DON chemotype MAFF 240560 (strain ZEA 1) that produces a large amount of the mycotoxin (Supplementary Fig. S4), although the difference was not significant.

Effects of L-Thr analogues on trichothecene accumulation on autoclaved brown rice flour solid medium

In addition to L-Thr, treatment of the medium with L-*allo*-threonine (10 µmol/g brown rice flour solid medium) also had a suppressive effect on trichothecene production by the fungus (Table S1). Conversely, L-threonine benzyl ester stimulated trichothecene production without morphological difference of the fungal culture (data not shown), suggesting that the metabolism of a benzyl ester serves as the stimulus for mycotoxin biosynthesis. We thus focused on the effect of L-threonine benzyl ester on fungal growth and measured the amount of ergosterol and trichothecenes after 4 days of incubation in independent experiments. As shown in Table 2, there were no significant differences in fungal mass but the mycotoxin accumulation significantly increased to 1.9-fold to that of the mock-treated control. Since a benzyl alcohol arises by hydrolysis of L-threonine benzyl ester and serves as a substrate of *Fusarium* galactose oxidase, its metabolism could yield H_2O_2 as a byproduct (Whittaker and Whittaker 2001; Yin et al. 2015). Indeed, we previously demonstrated that addition of H_2O_2 at concentrations that do not inhibit fungal growth activate trichothecene biosynthesis (Ochiai et al. 2007). Thus, L-threonine benzyl ester could have stimulated accumulation of trichothecenes in a similar manner (Ponts et al. 2006).

Expression analysis of *Tri6* and *Tri5*

Suppression of *Tri6* expression inhibits the expression of *Tri* pathway genes, such as *Tri5* encoding a trichodiene synthase (Proctor et al. 1995). To determine the effect of L-Thr (10 µmol/g brown rice flour solid medium) on *Tri* gene expression, we estimated the transcript levels of *Tri6* and *Tri5* using real-time RT-PCR with locked nucleic acid probes. The experiment was repeated three times using different conidial suspension lots as an inoculum, and all yielded similar results in four biologically independent experiments (data not shown). A representative result with conidial suspension lot

#KM1010 (n = 3) is shown in Fig. 1. Strong suppression of both *Tri6* and *Tri5* expression was observed after 36-h incubation, which supports the suppressive effect of L-Thr for mycotoxin accumulation observed for incubation at 48, 60, 72, and 96 h (Fig. 1). After 48 h of incubation, the differences of the *Tri* gene expression levels between L-Thr-treated and mock-treated cultures became smaller. Despite the small differences of the *Tri* gene expression levels, the trichothecene yield was not recovered during the incubation period of the experiment by 96 h.

Discussion

By adding L-Thr and L-*allo*-threonine solutions to sterilized grain substrate, trichothecene production by *F. graminearum* decreased considerably. Since catabolism of L-Thr on the solid culture did not affect fungal growth, the reduced mycotoxin production after 4 days of incubation (Table 1) is most likely to be attributed to a small change in the physiological conditions of the fungus grown in the presence and absence of the amino acid. In other similar instances, increasing percentage of galactose relative to total carbon source of the medium negatively affected trichothecene biosynthesis induction by an inducing sugar sucrose (Nakajima et al. 2016), and acivicin treatment at non-growth-inhibitory concentrations in minimal media suppressed trichothecene production (Maeda et al. 2014; Maeda et al. 2016). Thus, subtle differences in metabolic conditions of the fungus appear to be responsible for the failure to activate signaling pathways necessary for trichothecene biosynthesis.

Three catabolic pathways of L-Thr are known to occur in living organisms (Edgar 2005; Nelson and Cox 2008), the first steps of which are catalyzed by one of the following rate-limiting enzymes: dehydrogenase [L-threonine 3-dehydrogenase (TDH); EC 1.1.1.103], aldolase [L-threonine aldolase (TA); EC 4.1.2.5 and low-specificity TA;

EC 4.1.2.48], and deaminase [threonine ammonia-lyase (threonine dehydratase); EC 4.3.1.19 (originally EC 4.2.1.16)]. In the catabolic pathways with TDH and TA, acetyl-CoA and Gly are provided as common products after the second steps, which are catalyzed by glycine C-acetyltransferase (EC 2.3.1.29) and acetaldehyde dehydrogenase (acetylating) (EC 1.2.1.10), respectively. Gly is then ultimately catabolized to pyruvate and ammonia through a series of reactions, including C1 unit charging of tetrahydrofolate (THF) *via* the Gly cleavage system. In the degradation route with the threonine ammonia-lyase, L-Thr is first converted to 2-oxobutanoate and then metabolized to succinyl-CoA *via* propionyl-CoA and methylmalonyl-CoA. Among the catabolic genes in these L-Thr degradation pathways, probable TA and the ammonia-lyase genes were identified in the MIPS *Fusarium graminearum* Genome Database. Our preliminary microarray analysis [using the RNA from the L-Thr-treated mycelia after 36-h incubation (Fig. 1)] revealed upregulated transcription of a probable low-specificity TA gene (FGSG_12344), but not of probable threonine ammonia-lyase genes (FGSG_00296, FGSG_03147, FGSG_07162, FGSG_10119). Thus, we speculate that enhanced L-Thr catabolism stimulated production of both acetyl-CoA and Gly in *F. graminearum*. Such a metabolic state is somewhat similar to that of mouse embryonic stem (ES) cells, in which activated L-Thr metabolism is essential for maintenance of the pluripotency (Wang et al. 2009). Indeed, recycling of *S*-adenosylhomocysteine (SAH) to *S*-adenosylmethionine (SAM) is significantly upregulated in mouse ES cells through the increased supplies of Gly and acetyl-CoA, which provide a C1 unit for the THF charging and optimal energy for SAM synthesis, respectively (Shyh-Chang et al. 2013). Abundance of SAM affects histone H3 methylations on lysines, which could account for a possible mechanistic link between the L-Thr metabolic pathway and epigenetic state of the cells. In this context, it is interesting to investigate the ratio of SAM/SAH and methylation profile of proteins in the mycelia treated with L-Thr.

Although several fungicides have been used to control FHB, their preventive effects are unsatisfactory. In an attempt to alleviate the problems associated with mycotoxin contamination, synthetic and natural products have been searched for to identify chemicals that reduce the level of trichothecene production by the fungus (Sakuda et al. 2016). Continuing efforts have resulted in the identification of ancymidol (Desjardins et al. 1987), furanocoumarins (Desjardins et al. 1988), flavones (Desjardins et al. 1988), catechins (Takahashi-Ando et al. 2008a), phenolic acids (Boutigny et al. 2009; Boutigny et al. 2010; Pani et al. 2014), benzoxazinoid hydroxamic acid (Etzerodt et al. 2015), lignins (Kulik et al. 2014), acivicin (Maeda et al. 2014), and precocenes (Yaguchi et al. 2009). However, the effects of these inhibitors are restricted to weak trichothecene-inducing conditions, high inhibitor concentrations, specific fungal strains, and/or limited incubation period. To date, none of them has been developed as practical protective additives against mycotoxin contamination.

The suppressive effect of L-Thr exogenously added to brown rice flour medium on trichothecene production was not maintained after a longer incubation period (data not shown). The lack of long-lasting effect of L-Thr is not surprising as predominant catabolism of this nutrient is limited at an earlier stage of the batch culture; decrease of L-Thr in the medium leads to increased use of other nitrogen sources (*i.e.*, similar to the mock-treated condition) and eventual recovery of the secondary metabolism. However, this may not be a particular disadvantage compared with other mycotoxin production inhibitors listed above, considering that none of them show the suppressive activities with prolonged incubation periods under strong trichothecene-production inducing conditions (Takahashi-Ando et al. 2008a; Pagnussatt et al. 2014; Etzerodt et al. 2015; Maeda et al. 2016). In most studies, effectiveness of the inhibitors was demonstrated under weak-to-moderate mycotoxin inducing-conditions by using liquid cultures in Erlenmyer flasks (Pinson-Gadais et al. 2008; Boutigny et al. 2009; Ponts et al. 2011; Aristimuño Ficoseco et al. 2014; Pani et al. 2014). The concept

of regulating secondary metabolism by alteration of the major primary metabolic processes may provide an alternative possible means for the development of environmentally safe strategies to reducing mycotoxin contamination.

References

Aristimuño Ficoseco ME, Vattuone MA, Audenaert K, Catalan CA, Sampietro DA (2014) Antifungal and antimycotoxigenic metabolites in *Anacardiaceae* species from northwest Argentina: isolation, identification and potential for control of *Fusarium* species. J Appl Microbiol 116:1262-1273 doi: 10.1111/jam.12436

Bai GH, Desjardins AE, Plattner RD (2002) Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. Mycopathologia 153:91-98 doi: Doi 10.1023/A:1014419323550

Boutigny AL, Atanasova-Penichon V, Benet M, Barreau C, Richard-Forget F (2010) Natural phenolic acids from wheat bran inhibit *Fusarium culmorum* trichothecene biosynthesis *in vitro* by repressing *Tri* gene expression. Eur J Plant Pathol 127:275-286 doi: Doi 10.1007/S10658-010-9592-2

Boutigny AL, Barreau C, Atanasova-Penichon V, Verdal-Bonnin MN, Pinson-Gadais L, Richard-Forget F (2009) Ferulic acid, an efficient inhibitor of type B trichothecene biosynthesis and *Tri* gene expression in *Fusarium* liquid cultures. Mycol Res 113:746-753 doi: 10.1016/j.mycres.2009.02.010

Desjardins AE (2009) From yellow rain to green wheat: 25 years of trichothecene biosynthesis research. J Agric Food Chem 57:4478-4484 doi: 10.1021/jf9003847

Desjardins AE, Plattner RD, Beremand MN (1987) Ancymidol blocks trichothecene biosynthesis and leads to accumulation of trichodiene in *Fusarium sporotrichioides* and *Gibberella pulicaris*. Appl Environ Microbiol 53:1860-1865

Desjardins AE, Plattner RD, Spencer GF (1988) Inhibition of trichothecene toxin biosynthesis by naturally occurring shikimate aromatics. Phytochemistry 27:767-771

Edgar AJ (2005) Mice have a transcribed L-threonine aldolase/GLY1 gene, but the human GLY1 gene is a non-processed pseudogene. BMC Genomics 6:32 doi: 10.1186/1471-2164-6-32

Etzerodt T, Maeda K, Nakajima Y, Laursen B, Fomsgaard IS, Kimura M (2015) 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) inhibits

trichothecene production by *Fusarium graminearum* through suppression of *Tri6* expression. Int J Food Microbiol 214:123-128 doi: 10.1016/j.ijfoodmicro.2015.07.014

Faltusová Z, Chrpová J, Salačová L, Džuman Z, Pavel J, Zachariášová M, Hajšlová J, Ovesná J (2015) Effect of *Fusarium culmorum Tri* gene transcription on deoxynivalenol and D3G levels in two different barley cultivars. J Phytopathol 163:593-603 doi: 10.1111/jph.12359

Giese H, Sondergaard TE, Sorensen JL (2013) The AreA transcription factor in *Fusarium graminearum* regulates the use of some nonpreferred nitrogen sources and secondary metabolite production. Fungal Biol 117:814-821 doi: 10.1016/j.funbio.2013.10.006

Hohn TM, Beremand PD (1989) Isolation and nucleotide sequence of a sesquiterpene cyclase gene from the trichothecene-producing fungus *Fusarium sporotrichioides*. Gene 79:131-138

Kim HK, Yun SH (2011) Evaluation of potential reference genes for quantitative RT-PCR analysis in *Fusarium graminearum* under different culture conditions. Plant Pathol J 27:301-309 doi: 10.5423/Ppj.2011.27.4.301

Kimura M, Takahashi-Ando N, Nishiuchi T, Ohsato S, Tokai T, Ochial N, Fujimura M, Kudo T, Hamamoto H, Yamaguchi I (2006) Molecular biology and biotechnology for reduction of *Fusarium* mycotoxin contamination. Pestic Biochem Physiol 86:117-123 doi: 10.1016/j.pestbp.2006.02.008

Kimura M, Tokai T, Takahashi-Ando N, Ohsato S, Fujimura M (2007) Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: pathways, genes, and evolution. Biosci Biotechnol Biochem 71:2105-2123 doi: 10.1271/bbb.70183

Kulik T, Busko M, Pszczolkowska A, Perkowski J, Okorski A (2014) Plant lignans inhibit growth and trichothecene biosynthesis in *Fusarium graminearum*. Lett Appl Microbiol 59:99-107 doi: 10.1111/lam.12250

Maeda K, Nakajima Y, Motoyama T, Kitou Y, Kosaki T, Saito T, Nishiuchi T, Kanamaru K, Osada H, Kobayashi T, Kimura M (2014) Effects of acivicin on growth, mycotoxin production and virulence of phytopathogenic fungi. Lett Appl Microbiol 59:377-383 doi: 10.1111/lam.12289

Maeda K, Nakajima Y, Tanahashi K, Kosaki T, Kitou Y, Kanamaru K, Kobayashi T, Nishiuchi T, Kimura M (2016) Characterization of the acivicin effects on

trichothecene production by *Fusarium graminearum* species complex. J Gen Appl Microbiol 62:272-276 doi: 10.2323/jgam.2016.04.002

McCormick SP, Stanley AM, Stover NA, Alexander NJ (2011) Trichothecenes: from simple to complex mycotoxins. Toxins 3:802-814 doi: 10.3390/toxins3070802

Merhej J, Richard-Forget F, Barreau C (2011) Regulation of trichothecene biosynthesis in *Fusarium*: recent advances and new insights. Appl Microbiol Biotechnol 91:519-528 doi: 10.1007/s00253-011-3397-x

Min K, Shin Y, Son H, Lee J, Kim JC, Choi GJ, Lee YW (2012) Functional analyses of the nitrogen regulatory gene *areA* in *Gibberella zeae*. FEMS Microbiol Lett 334:66-73 doi: 10.1111/j.1574-6968.2012.02620.x

Nakajima Y, Maeda K, Jin Q, Takahashi-Ando N, Kanamaru K, Kobayashi T, Kimura M (2016) Oligosaccharides containing an α-(1→2) (glucosyl/xylosyl)-fructosyl linkage as inducer molecules of trichothecene biosynthesis for *Fusarium graminearum*. Int J Food Microbiol doi: 10.1016/j.ijfoodmicro.2016.09.011

Nakajima Y, Tokai T, Maeda K, Tanaka A, Takahashi-Ando N, Kanamaru K, Kobayashi T, Kimura M (2014) A set of heterologous promoters useful for investigating gene functions in *Fusarium graminearum*. JSM Mycotoxins 64:147-152 doi: 10.2520/myco.64.147

Nelson DL, Cox MN (2008) Lehninger Principles of Biochemistry, 5 edn. Freeman, New York, NY, USA

Nishiuchi T, Masuda D, Nakashita H, Ichimura K, Shinozaki K, Yoshida S, Kimura M, Yamaguchi I, Yamaguchi K (2006) *Fusarium* phytotoxin trichothecenes have an elicitor-like activity in *Arabidopsis thaliana*, but the activity differed significantly among their molecular species. Mol Plant Microbe Interact 19:512-520 doi: 10.1094/MPMI-19-0512

Ochiai N, Tokai T, Takahashi-Ando N, Fujimura M, Kimura M (2007) Genetically engineered *Fusarium* as a tool to evaluate the effects of environmental factors on initiation of trichothecene biosynthesis. FEMS Microbiol Lett 275:53-61 doi: 10.1111/j.1574-6968.2007.00869.x

Ohno M, Tsuda K, Sakaguchi M, Sugahara Y, Oyama F (2012) Chitinase mRNA levels by quantitative PCR using the single standard DNA: acidic mammalian chitinase is a major transcript in the mouse stomach. PLoS One 7:e50381 doi:

10.1371/journal.pone.0050381

Pagnussatt FA, Del Ponte EM, Garda-Buffon J, Badiale-Furlong E (2014) Inhibition of *Fusarium graminearum* growth and mycotoxin production by phenolic extract from *Spirulina* sp. Pestic Biochem Physiol 108:21-26 doi: 10.1016/j.pestbp.2013.11.002

Pani G, Scherm B, Azara E, Balmas V, Jahanshiri Z, Carta P, Fabbri D, Dettori MA, Fadda A, Dessi A, Dallocchio R, Migheli Q, Delogu G (2014) Natural and natural-like phenolic inhibitors of type B trichothecene *in vitro* production by the wheat (*Triticum* sp.) pathogen *Fusarium culmorum*. J Agric Food Chem 62:4969-4978 doi: 10.1021/jf500647h

Pinson-Gadais L, Richard-Forget F, Frasse P, Barreau C, Cahagnier B, Richard-Molard D, Bakan B (2008) Magnesium represses trichothecene biosynthesis and modulates *Tri5*, *Tri6*, and *Tri12* genes expression in *Fusarium graminearum*. Mycopathologia 165:51-59 doi: 10.1007/s11046-007-9076-x

Ponts N, Pinson-Gadais L, Boutigny AL, Barreau C, Richard-Forget F (2011) Cinnamic-derived acids significantly affect *Fusarium graminearum* growth and in vitro synthesis of type B trichothecenes. Phytopathology 101:929-934 doi: Doi 10.1094/Phyto-09-10-0230

Ponts N, Pinson-Gadais L, Verdal-Bonnin MN, Barreau C, Richard-Forget F (2006) Accumulation of deoxynivalenol and its 15-acetylated form is significantly modulated by oxidative stress in liquid cultures of *Fusarium graminearum*. FEMS Microbiol Lett 258:102-107 doi: 10.1111/j.1574-6968.2006.00200.x

Proctor RH, Hohn TM, McCormick SP, Desjardins AE (1995) *Tri6* encodes an unusual zinc finger protein involved in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. Appl Environ Microbiol 61:1923-1930

Ravagnani A, Gorfinkiel L, Langdon T, Diallinas G, Adjadj E, Demais S, Gorton D, Arst HN, Jr., Scazzocchio C (1997) Subtle hydrophobic interactions between the seventh residue of the zinc finger loop and the first base of an HGATAR sequence determine promoter-specific recognition by the *Aspergillus nidulans* GATA factor AreA. EMBO J 16:3974-3986 doi: 10.1093/emboj/16.13.3974

Sakuda S, Yoshinari T, Furukawa T, Jermnak U, Takagi K, Iimura K, Yamamoto T, Suzuki M, Nagasawa H (2016) Search for aflatoxin and trichothecene production inhibitors and analysis of their modes of action. Biosci Biotechnol

Biochem 80:43-54 doi: 10.1080/09168451.2015.1086261

Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, Asara JM, Daley GQ, Cantley LC (2013) Influence of threonine metabolism on S-adenosylmethionine and histone methylation. Science 339:222-226 doi: 10.1126/science.1226603

Son H, Lee Y-W (2012) *Fusarium graminearum* mycotoxins and their biosynthetic genes. Mycotoxins 62:29-40 doi: 10.2520/myco.62.29

Sugita-Konishi Y (2008) Toxicity and control of trichothecene mycotoxins. Mycotoxins 58:23-28 doi: 10.2520/myco.58.23

Takahashi-Ando N, Ochiai N, Tokai T, Ohsato S, Nishiuchi T, Yoshida M, Fujimura M, Kimura M (2008a) A screening system for inhibitors of trichothecene biosynthesis: hydroxylation of trichodiene as a target. Biotechnol Lett 30:1055-1059 doi: 10.1007/s10529-008-9649-x

Takahashi-Ando N, Tokai T, Yoshida M, Fujimura M, Kimura M (2008b) An easy method to identify 8-keto-15-hydroxytrichothecenes by thin-layer chromatography. Mycotoxins 58:115-117 doi: 10.2520/myco.58.115

Todd RB, Fraser JA, Wong KH, Davis MA, Hynes MJ (2005) Nuclear accumulation of the GATA factor AreA in response to complete nitrogen starvation by regulation of nuclear export. Eukaryot Cell 4:1646-1653 doi: 10.1128/EC.4.10.1646-1653.2005

Wang J, Alexander P, Wu L, Hammer R, Cleaver O, McKnight SL (2009) Dependence of mouse embryonic stem cells on threonine catabolism. Science 325:435-439 doi: 10.1126/science.1173288

Whittaker MM, Whittaker JW (2001) Catalytic reaction profile for alcohol oxidation by galactose oxidase. Biochemistry 40:7140-7148

Yaguchi A, Yoshinari T, Tsuyuki R, Takahashi H, Nakajima T, Sugita-Konishi Y, Nagasawa H, Sakuda S (2009) Isolation and identification of precocenes and piperitone from essential oils as specific inhibitors of trichothecene production by *Fusarium graminearum*. J Agric Food Chem 57:846-851 doi: 10.1021/jf802813h

Yin DT, Urresti S, Lafond M, Johnston EM, Derikvand F, Ciano L, Berrin JG, Henrissat B, Walton PH, Davies GJ, Brumer H (2015) Structure-function characterization reveals new catalytic diversity in the galactose oxidase and

glyoxal oxidase family. Nat Commun 6:10197 doi: 10.1038/ncomms10197
Yoshizawa T (2013) Thirty-five years of research on deoxynivalenol, a trichothecene mycotoxin: with special reference to its discovery and co-occurrence with nivalenol in Japan. Food Safety 1:doi: 10.14252/foodsafetyfscj.2013002 doi: 10.14252/foodsafetyfscj.2013002

Table 1. Dose-dependent effects of L-Thr on fungal growth and trichothecene production on autoclaved brown rice flour solid medium[a]

Dose (μmol/g medium)	Ergosterol (μg/g medium)	15-ADON + DON (μg/g medium)	Toxin/ergosterol ratio to mock-treated control
0	172.9 ± 16.2 a	34.3 ± 10.2 a	1.00 a
2	187.0 ± 9.5 a	16.4 ± 10.7 b	0.42 ± 0.17 b
10	188.7 ± 18.2 a	8.6 ± 2.1 b	0.25 ± 0.09 bc
20	184.2 ± 19.2 a	5.4 ± 3.4 b	0.14 ± 0.08 c

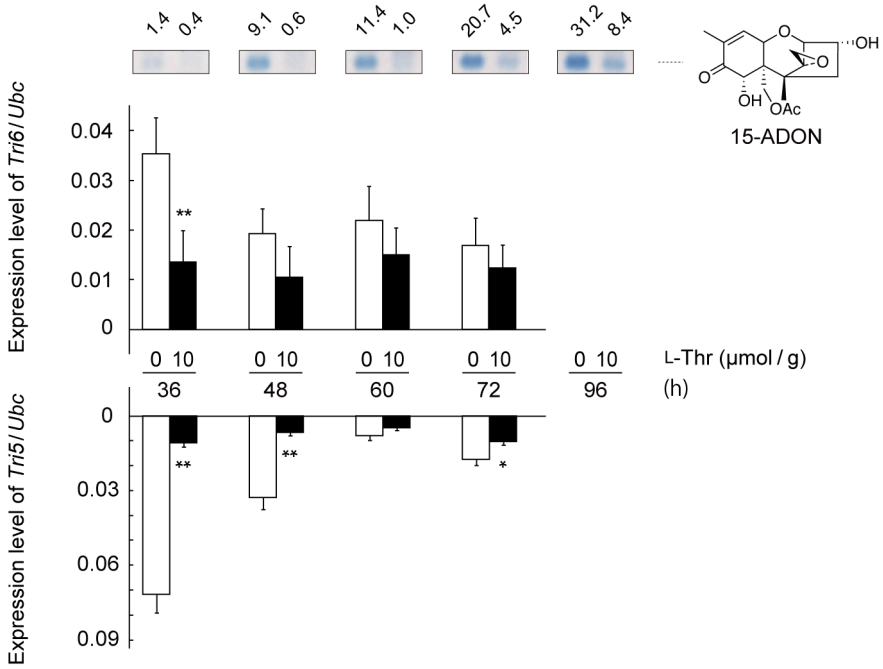
[a] Mean ± standard deviation of the mean (SD) of four replicates (conidial suspension lot numbers #KM1001, #KM1022, #KM1102, and #KM1104). The difference was analyzed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer test. Values followed by the same letter in each column are not significantly different; $P < 0.05$.

Table 2. Effects of L-Thr and L-threonine benzyl ester (10 μmol/g brown rice flour solid medium) on trichothecene production on autoclaved brown rice flour solid medium[a]

Amino acid	Ergosterol (μg/g medium)	15-ADON + DON (μg/g medium)	Toxin/ergosterol ratio to mock-treated control
Mock-treated control	207.3 ± 22.9 a	9.1 ± 2.9 a	1.00 a
L-Thr	213.5 ± 20.8 a	3.8 ± 1.8 a	0.40 ± 0.13 b
L-threonine benzyl ester	213.0 ± 1.4 a	17.2 ± 3.5 b	1.87 ± 0.05 c

[a] Mean ± SD in triplicate (conidial suspension lot numbers #YN0828, #YN0830, and #YN0831). The difference was analyzed by single-factorial ANOVA followed by a Tukey-Kramer test. Values followed by the same letter in each column are not significantly different; $P < 0.05$.

Fig. 1 Real-time RT-PCR analysis of *Tri6* and *Tri5* expression in *F. graminearum* JCM 9873 with or without L-Thr (10 μmol/g) added to the solid medium.



Tri gene expression levels were expressed relative to *Ubc* expression at each time point ($n = 3$). Filled and empty bars indicate the relative expression levels of *Tri* genes in mycelia grown on autoclaved brown rice flour solid medium amended with or without (*i.e.*, mock-treated control) 10 μmol/g medium of L-Thr, respectively. Asterisks on filled bars denote a significant difference (*$P < 0.05$, **$P < 0.01$; Student's *t* test) relative to the corresponding control samples (empty bars). Trichothecenes that accumulated in the medium at each time point were extracted with 84% (v/v) acetonitrile, separated by TLC, and visualized as blue spots, as described in **Materials and methods**. The position of 15-ADON on a TLC plate is shown above the filled and empty bars at each time point, with slanted letters representing combined amounts of 15-ADON and DON (μg/g medium) determined by HPLC. Only the spot of 15-ADON is shown for simplicity of presentation, because the amount of DON was marginal during the incubation period. Each lane in TLC contains metabolites extracted from 1/20 (ca. 0.35 g) of the solid culture.

Table S1 Inhibitory effects of L-Thr and its analogues (10 μmol/g brown rice flour solid medium) on trichothecene production on autoclaved brown rice flour solid medium[a]

Amino acid	15-ADON + DON (μg/g medium)
Mock-treated control	16.3 ± 7.0
L-Thr	2.9 ± 2.1 *
L-*allo*-threonine	2.3 ± 1.1 *
L-threonine benzyl ester	31.7 ± 4.8 *
L-Ser	7.3 ± 1.6

[a] Mean ± SD in triplicate (conidial suspension lot numbers #KM0911, #KM0914, and #KM1016). The difference was analyzed by single-factorial ANOVA followed by a Dunnett's test. Asterisk indicates a significant difference between mock-treated control and treated brown rice flour solid medium; *$P < 0.01$.

Fig. S1 Preparation of conidia used for the assay.

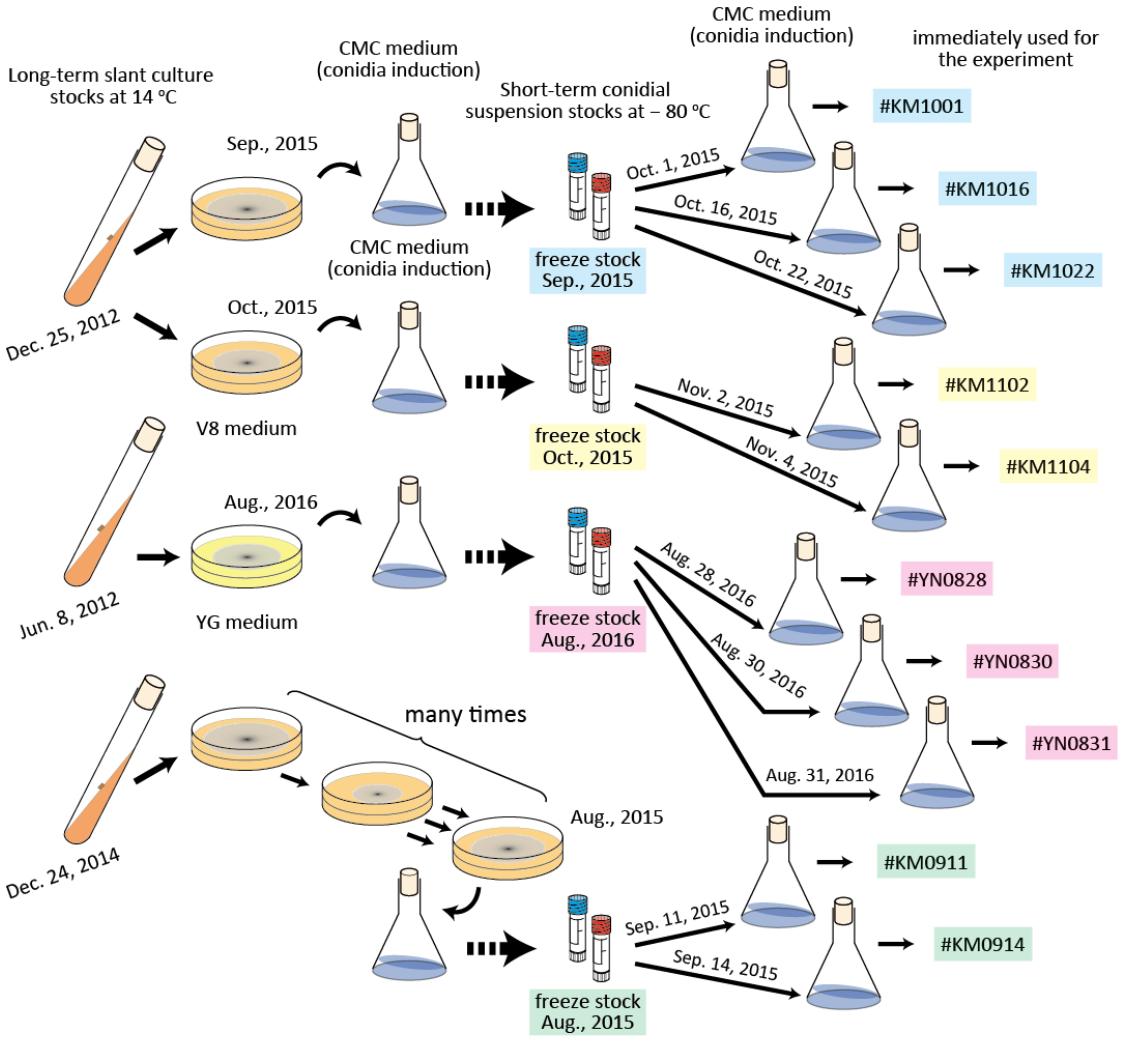


Table 1

Dose (μmol/g medium)	15-ADON + DON (Ergosterol) (μg/g medium)			
0	30.5 (150.6)	30.9 (183.8)	49.2 (171.3)	26.5 (185.9)
2	9.6 (187.7)	9.4 (187.6)	32.1 (198.0)	14.5 (174.8)
10	11.8 (165.4)	7.7 (199.7)	7.8 (206.1)	7.3 (183.6)
20	8.8 (166.3)	2.1 (190.7)	7.8 (208.4)	2.8 (171.5)
inoculation onto brown rice flour solid medium	2015/10/4	2015/10/25	2015/11/5	2015/11/7
conidia lot # used for inoculation	#KM1001	#KM1022	#KM1102	#KM1104

Table 2

Amino acid	15-ADON + DON (Ergosterol) (μg/g medium)		
Mock-treated control	6.9 (201.5)	8.0 (187.9)	12.4 (232.6)
L-Thr	1.8 (209.9)	4.7 (235.9)	4.9 (194.8)
L-threonine benzyl ester	13.6 (212.8)	17.5 (214.5)	20.5 (211.8)
inoculation onto brown rice flour solid medium	2016/8/31	2016/9/2	2016/9/3
conidia lot # used for inoculation	#YN0828	#YN0830	#YN0831

Table S1

Amino acid	15-ADON + DON (μg/g medium)		
Mock-treated control	8.7	17.8	22.4
L-Thr	1.0	2.6	5.2
L-*allo*-Thr	1.4	2.0	3.4
L-threonine benzyl ester	26.9	36.5	31.6
L-Ser	7.1	5.8	8.9
inoculation onto brown rice flour solid medium	2015/9/14	2015/9/17	2015/10/19
conidia lot # used for inoculation	#KM0911	#KM0914	#KM1016

The scheme of conidial suspension preparation is depicted. The results of ergosterol and trichothecene analyses (raw data) are provided below the scheme.

Fig. S2 Use of a single standard DNA for copy number determination of reverse-transcribed cDNA in expression analysis.

(A)

#1, GA_pUC18-Tri10_Fw: 5′- TCGAGCTCGGTACCCATGGCCTCAGAAAGTCATAAG -3′
#2, GA_Tri10-6_Rev: 5′- CATGTAATCTTCTCTTCCAAGCGTCTTC -3′
#3, GA_10-Tri6_Fw: 5′- AGAGAAGATTACATGGAGGCCGAATCTCA -3′
#4, GA_Tri6-5_Rev: 5′- AAGTGCCTGGCAGGATCGGGAGACGCA -3′
#5, GA_6-Tri5_Fw: 5′- TCCTGCCAGGCACTTGTGCGACGCTCGAT -3′
#6, GA_Tri5-4_Rev: 5′- AATACGGCCCGAACGTTTGCCAGTTGT -3′
#7, GA_5-Tri4_Fw: 5′- CGTTCGGGCCGTATTATACCTCTTGACCC -3′
#8, GA_Tri4-ubc_Rev: 5′- AAGAGCCCCTCATGGATGACACCGTTC -3′
#9, GA_4-UBC_Fw: 5′- CCATGAGGGGCTCTTAAGCGTATCAACAAG -3′
#10, GA_ubc-pUC18_Rev: 5′- CTCTAGAGGATCCCCTGTTGGGGTGGTAGATGCGT -3′

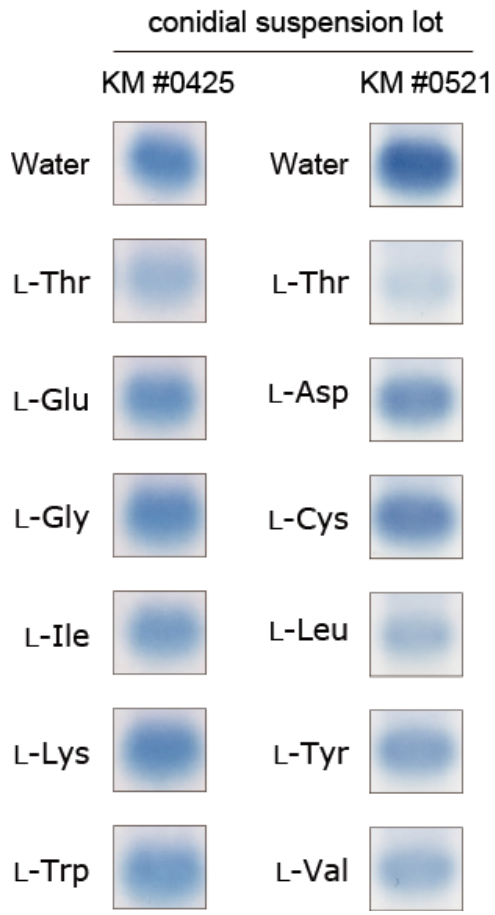
(B)

#11, JCM_Tri10_qRT-F: 5′- GAGGTCGTATGAGGCGACA -3′
#12, JCM_Tri10_qRT-R: 5′- AAGACCTGAAGAGTTCCGTCAT -3′
#13, qPCR-Tri6_P125_L: 5′- TTACATGGAGGCCGAATCTC -3′
#14, qPCR-Tri6_P125_R: 5′- AGACGCAACTCGATCAAAGAG -3′
#15, qRT-Tri5_Fw2: 5′- TGAAAAGGTCAAGGATCAGGA -3′
#16, qRT-Tri5_Rev2: 5′- CCTGCTCAAAGAACTTGCAGA -3′
#17, qRT-Tri4_Fw: 5′- TTGAAAAAGCTCCGAGAGGA -3′
#18, qRT-Tri4_Rev: 5′- AGAATACTGGCCGTCCTTGA -3′
#19, qRT-UBC-Fw: 5′- AAGCGTATCAACAAGGAGTTGAC -3′
#20, qRT-UBC-Rev: 5′- AAACAAATCCTCGCCAACAG -3′

(A) Construction of a single standard DNA containing cDNA fragments of *Tri10* (purple), *Tri6* (green; this study), *Tri5* (orange; this study), *Tri4* (blue), and *Ubc* (reddish brown; this study) genes. Five PCR fragments amplified with two adjacent primer pairs of opposite orientation (#1 − #10; dotted lines representing sequences of adjacent DNA fragments to create the necessary 15 bp overlap) and a *Sma*I-linearized vector pUC18 were assembled using a NEBuilder® HiFi DNA Assembly Cloning kit

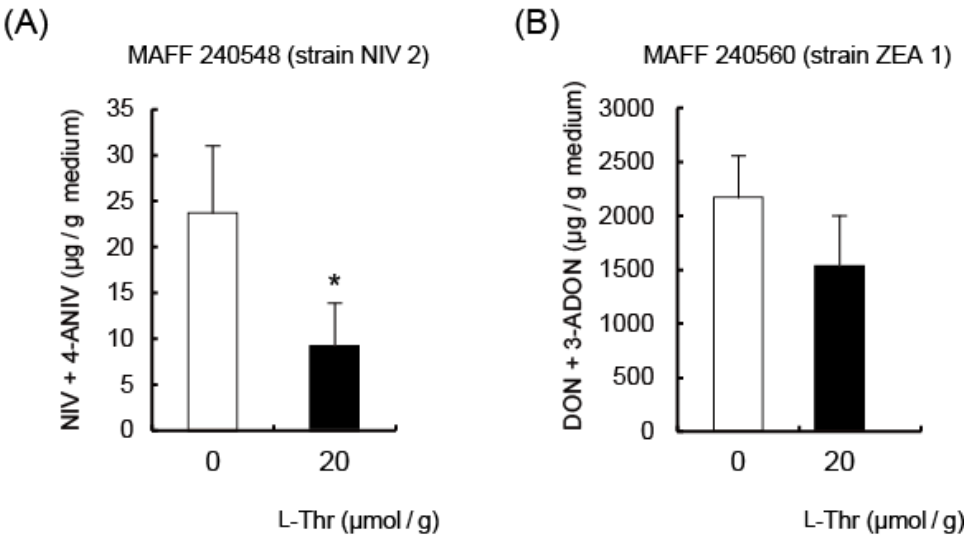
(New England Biolabs Japan Inc., Tokyo). (B) Primers and probes used for quantitative real-time RT-PCR. The primer/probe sets of *Tri6* (#13, #14, and UPL probe #125), *Tri5* (#15, #16, and UPL probe #142), and *Ubc* (#19, #20, and UPL probe #15) were used for amplification of the target *Tri* genes and the endogenous reference *Ubc* gene from both cDNA samples and a single standard DNA.

Fig. S3 Effect of each amino acid added to autoclaved brown rice flour solid medium (final 10 μmol/g medium) on trichothecene production by *F. graminearum* JCM 9873.



The solid medium (5 g) was treated with 500 μl of amino acid solution (100 mM; L-Cys, L-Gly, L-Ile, L-Leu, L-Lys, L-Thr, and L-Val) or suspension (100 mM; L-Asp, L-Glu, L-Trp, and L-Tyr). After 4 days of incubation with *F. graminearum* (conidial suspension lot numbers #KM0425 and #KM0521), trichothecenes were extracted and analyzed as described previously (Etzerodt et al. 2015). Trichothecenes that accumulated in the media were visualized as blue spots on a TLC plate, whose intensities are proportional to their amount. Only the spot of 15-ADON is shown for simplicity of presentation, because the amount of DON was marginal during the incubation period.

Fig. S4 Effects of L-Thr on trichothecene production by different strains of *F. graminearum*.



Strains MAFF 240548 (A) produces a moderate amount of 4-ANIV/NIV, while strain MAFF 240560 (B) produces a large amount of 3-ADON/DON on autoclaved brown rice flour medium. The solid medium was treated with L-Thr (20 μmol/g brown rice flour medium), incubated with the fungal strains for 4 days, and the amount of toxin was quantified by HPLC using a PEGASIL ODS column (diameter, 4.6 mm; length, 250 mm; Senshu Scientific Co., Tokyo, Japan) eluted at a flow rate of 1.0 ml/min at 40 ^{o}C as follows: isocratic elution with 12.5% acetonitrile (0 − 10 min) and 25% acetonitrile (10 − 25 min) for quantification of DON (8.3 min) and 15-ADON (20.0 min); isocratic elution with 12.5% acetonitrile (0 − 8 min) and 25% acetonitrile (8 − 16 min) for quantification of NIV (6.3 min) and 4-ANIV (15.6 min). Asterisk indicates a significant difference [*$P < 0.05$; (A) $P = 0.015$, (B) $P = 0.083$] between L-Thr-treated and mock-treated cultures ($n = 4$; Student's t test).