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Short Communication

Identification of amino acids negatively affecting *Fusarium* trichothecene biosynthesis

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Abstract

Nitrogen sources of media have a significant impact on the onset of secondary metabolism in fungi. For transcriptional activation of many nitrogen catabolic genes, an AreA transcription factor is indispensable. The facts also hold true for *Fusarium graminearum* that produces trichothecenes, an important group of mycotoxin, in axenic culture. Despite the presence of numerous consensus AreA-binding sites in the promoters of *Tri* genes in the trichothecene cluster core region, the effect of medium amino acids on trichothecene biosynthesis is poorly understood. In this study, we examined the effect of certain amino acids, which were predicted to activate AreA function and increase *Tri* gene transcription, on trichothecene production in liquid culture. By frequent monitoring and adjustments in the pH of the culture medium, including replacement of the spent medium with fresh medium, we demonstrate the suppressive effects of the amino acids, used as the sole nitrogen source, on trichothecene biosynthesis. When the medium pH was maintained at 4.0, Gly, L-Ser, and L-Thr suppressed trichothecene production by *F. graminearum*. Enhanced trichothecene-inducing effects were observed when the medium pH was 3.5, with only L-Thr suppressing trichothecene synthesis.

Keywords: AreA transcription factor; Defined medium; Fed-batch culture; Mycotoxin biosynthesis; Secondary metabolite; Threonine metabolism

Introduction

Trichothecenes are toxic secondary metabolites of *Fusarium* species, including *F. graminearum* species complex, *F. culmorum*, and *F. sporotrichioides* (Kimura et al. 2007; Moretti et al. 2013). Trichothecene production is regulated by a Cys_2His_2 zinc finger transcription factor encoded by *Tri6* (Proctor et al. 1995), located in the core region of the trichothecene gene (*Tri*) cluster. The sequence 5'-HGATAR-3', a consensus binding-site for the zinc finger transcription factor AreA (Todd et al. 2005), is abundant in the *Tri5* and *Tri4/Tri6* bidirectional promoters in the cluster core region (Merhej et al. 2011). In *Aspergillus nidulans*, nitrogen deficiency increases active AreA levels in the nucleus and stimulates synthesis of appropriate catabolic enzymes and permeases in response to the nitrogen status of the cell (Wong et al. 2008). In *F. graminearum*, the ortholog FgAreA (Min et al. 2012) was reported to be indispensable for utilization of L-Asp, L-Cys, L-Gly, L-Glu, L-Ile, L-Leu, L-Lys, L-Thr, L-Trp, L-Tyr, and L-Val (Giese et al. 2013).

We recently found that autoclaved brown rice flour solid medium supplemented with excess L-Thr suppresses trichothecene production by *F. graminearum* (Maeda et al. 2017). Functional AreA is required for utilization of L-Thr (Giese et al. 2013). Therefore, the suppressive effect of L-Thr treatment on trichothecene biosynthesis was unexpected because the presence of numerous AreA-binding sites in the *Tri* gene promoter was rather suggestive of a stimulative effect of this amino acid on trichothecene production. The suppressive effects of L-Thr mixed with rice flour solid medium were detected with statistically significant differences in toxin/ergosterol ratio (the amount of trichothecenes per unit mycelial mass) between L-Thr-treated and control cultures. The absolute amount of trichothecenes that accumulated in the solid medium, however, varied considerably with subtle differences in preparation of the medium and fungal inoculum in each experiment (Maeda et al. 2017). As autoclaving

complex media accelerates formation of some substances, presumably by Maillard reaction, that inhibit trichothecene production (Tanaka et al. 2019), small differences in thermal sterilization of the brown rice flour medium may result in variations in the toxin/ergosterol ratio in each experiment. Although trichothecene biosynthesis inhibition by L-Thr was reproducibly observed when L-Thr-treated and control cultures were compared in a single experiment, wherein the brown rice flour media were simultaneously autoclaved and subsequently inoculated with aliquots of a conidial suspension, biochemical and molecular genetic studies are hindered by methodological difficulties; *i.e.*, isolation of proteins and nucleic acids from the mycelia firmly adhered to the solid medium.

In this work, we sought to determine whether the suppressive effects of certain amino acids against trichothecene biosynthesis are also observed in liquid culture when these amino acids are used as the sole nitrogen source in the medium. To confirm this, we used fed-batch culture method with frequent pH adjustments.

Materials and methods

Strains and media

Strain JCM 9873 of *F. graminearum* that produces 15-acetyldeoxynivalenol (15-ADON) in liquid culture was used in this study. This strain produces marginal amount of deoxynivalenol in liquid culture (Etzerodt et al. 2015). Trichothecene non-inducing YG medium contained 2% (w/v) glucose and 0.5% (w/v) Bacto™ yeast extract (BD Company, Franklin Lakes, NJ, USA). Synthetic liquid medium containing 5 mM of each test amino acid (Table S1) was used to examine the effect of nitrogen source on trichothecene production. For preparation of the agar medium, liquid medium was solidified using 1.0% (w/v) agar. The pH of the medium was 7.2 when K_2HPO_4 was used or 4.2 in the presence of KH_2PO_4 (Table S1). For preparation of the YE-KH_2PO_4 and YE-K_2HPO_4 (agar plate only) media, filter-sterilized stock solution of 2 % (w/v) Bacto™ yeast extract was added at 1/20 the total volume of the synthetic liquid medium in exchange for the 5 mM amino acid.

Disruption of FgAreA

The *FgAreA* deletion mutant (Δ*FgareA*) of JCM 9873 was generated by replacing the entire coding region (FGSG_08634) with an *hph::tk* cassette by double cross-over homologous recombination (see Fig. S1A and legend) using an *FgAreA* disruption vector pΔFgAreA (Fig. S2). The deletion mutants were screened by PCR (Fig. S1B) and further verified by Southern blotting (Fig. S1C).

Culture conditions in synthetic liquid medium

Conidia, prepared as previously described (Etzerodt et al. 2015), were inoculated into YG medium at a final concentration of 4×10^4 conidia mL^{-1}, pre-cultured at 25˚C with reciprocal shaking (125 strokes/min) for 16 h, and used as an inoculum. For evaluation of the trichothecene productivity, 300 µL of the pre-culture was added to 30 mL synthetic liquid medium in a 100-mL Erlenmeyer flask and incubated on a gyratory shaker at 135 rpm and 25˚C. The pH was frequently monitored (every 2−3 h during the incubation period of 24−72 h) using a hand-held pH meter LAQUAtwin B-712 (Horiba Ltd., Kyoto), as previously described (Kitou et al. 2016), and maintained at an initial value by the addition of 0.5 N KOH or 0.5 N HCl. After 36 h of incubation, the mycelia were collected by filtration through Miracloth (Merck Millipore, Billerica, MA), suspended in fresh synthetic medium, and further incubated for 36 h. The culture filtrates at 36 and 72 h were combined, and used for quantification of trichothecenes.

Trichothecene analysis

Trichothecenes in the culture filtrate were extracted with ethyl acetate and quantified by HPLC, as previously described (Etzerodt et al. 2015). The amount of trichothecenes was expressed relative to the fungal mass (mycelial dry weight). The effects of nitrogen source on trichothecene biosynthesis were determined from the ratio of toxin/fungal mass of each culture to that of the L-Gln culture, wherein the media were inoculated with aliquots of a fungal pre-culture. The significance of differences between the control (L-Gln) and each amino acid were analysed by Student's *t*-test ($^{*}P < 0.05$, $^{**}P < 0.01$).

Results and discussion

Identification of amino acids that require functional FgAreA for their utilization

Since *F. graminearum* shows significant genetic variations among strains (Boutigny et al. 2009; Ponts et al. 2011), we examined the effect of the *FgAreA* deletion on growth of JCM 9873 by placing a mycelial plug taken from the edge of YG agar culture on synthetic agar media. When each of the 19 proteinogenic amino acids, except for the extremely insoluble L-Tyr, were used as the sole nitrogen source in the synthetic agar media, growth of the Δ*FgareA* mutant was supported only by L-Arg, L-Gln, and L-Pro with KH_2PO_4, and L-Arg, L-Gln, L-Pro, and L-Asn with K_2HPO_4 (Fig. 1). Similar results were obtained when conidia of the mutant were used as the inocula for the agar plate assay (Fig. S3). The results indicate that functional FgAreA is necessary for utilization of most amino acids by JCM 9873. The medium pH may adversely affect uptake of nutrients in the absence of FgAreA function, as growth of the Δ*FgareA* mutant supported by yeast extract also proved to be suppressed to some extent at an acidic pH (Fig. 1; compare YE-KH_2PO_4 and YE-K_2HPO_4). Interestingly, the AreA-dependent nitrogen sources were different in strain PH-1, which is also capable of using L-Ala, L-Phe, and L-Ser in the absence of functional FgAreA (Giese et al. 2013). Thus, there appear to be significant differences in the regulatory mechanism of catabolism of certain amino acids within a single fungal species.

Production of trichothecenes in synthetic medium using a single amino acid as the sole nitrogen source

We compared the trichothecene-inducing activity of each amino acid in synthetic liquid medium using the conventional batch culture method. L-Gln was used as the standard

amino acid for comparison because most fungi, including *F. graminearum*, can metabolize this amino acid without the presence of an active AreA protein. Among the 19 amino acids tested, trichothecene accumulation after 8 days of incubation decreased in synthetic liquid media when Gly, L-Val, L-Ser, or L-Thr were used as the sole nitrogen sources (Fig. 2), although statistical significance of toxin/fungal mass was not detected in these experiments (n = 3). In contrast, use of amino acids, such as L-Phe and L-Trp, led to increased production of 15-ADON. Since the pH of all these media was apparently acidic, both at the beginning (pH 4.2) and the end (pH 3.2–3.6) of the culture, the possibility of alkaline pH preventing the mycotoxin production (Gardiner et al. 2009b) could be excluded while interpreting the results. However, detailed time-course monitoring of the culture pH revealed significant differences in their profiles (Fig. S4); while the pH of L-Val, L-Ser, and L-Thr media gradually decreased, the pH of L-Gln and Gly media rapidly increased first (up to approximately 5.3 and 4.7, respectively, at around 36 h), followed by a sharp decrease to a final pH of 3.4. Other than the L-Gln and Gly media, the pH of the L-Ser medium was the highest pH compared to that of the other media until the late stage of the culture. The pH of L-Val medium was lower than 3.5 after 48 h, which may severely affect metabolic activities of the mycelia. In this way, metabolism of different nitrogen sources significantly influenced the medium pH in a time dependent manner. As the effects of *FgAreA* deletion on trichothecene biosynthesis were evaluated without considering medium pH changes in previous studies (Hou et al. 2015; Min et al. 2012), the results with specific nitrogen sources need to be interpreted with care.

In a previous study (Gardiner et al. 2009a), the effects of nitrogen sources on induction of *Tri5* expression were investigated by using a transgenic *F. graminearum* strain that carried a *Tri5* promoter-*GFP* gene fusion between *Tri6* and *Tri5* in the genome of a parent isolate CS3005 (Gardiner et al. 2014). Phenotype microarray profiling with Biolog's PM3 Microplate™ revealed a strong induction of *Tri5*

expression by agmatine and putrescine, while most amino acids, including L-Gln, L-Phe, and L-Thr, failed to activate *Tri5* transcription. We previously demonstrated that the type of culture vessels considerably affects the final trichothecene yields (Nakajima et al. 2016). Since the Biolog's system is based on a 96-well microtiter plate in testing microbial phenotypes, lack of *Tri5* expression in the L-Gln and L-Phe media (Gardiner et al. 2009a) may be attributed to culture conditions, which are different from those of the present study using a 100-mL Erlenmeyer flask. Alternatively, different genetic backgrounds of fungal strains may be the cause of phenotypic differences, as observed for their responses to oxidative stress in trichothecene biosynthesis (Ponts et al. 2009).

Identification of amino acids that suppress trichothecene biosynthesis by frequent monitoring and adjustments of culture pH

To establish a cause-and-effect relationship between the quality of nitrogen source and the amount of mycotoxin produced in the culture, pH of synthetic media has to be kept constant throughout the culture. In addition, nitrogen source deficit should not occur during evaluation of the influence of different amino acids on trichothecene biosynthesis. Hence, the spent medium was replaced with a fresh one after 36 h of culturing. This time point was chosen based on the result of quantification of residual amines in the L-Gln medium, which revealed that 31.7%, 62.2%, and 98.8% of this nitrogen source was lost in 24 h, 36 h, and 48 h, respectively. We first tried to maintain constant culture pH of the L-Gln medium using a 10 mM malate buffer (pKa_1 = 3.4; pKa_2 = 5.1), but it did not help maintain a constant pH of fungal culture (Fig. S5). Rather, due to the consumption of the organic acid by the fungus, the pH of the culture increased up to the neutral pH range. Hence, we decided to maintain the pH of the culture manually with HCl and KOH and frequently determined the pH using a hand-held pH meter. Functional FgAreA is necessary for the metabolism of L-Phe but

not for L-Gln; therefore these amino acids were included as controls in the experiment. Since metabolism of Gly, L-Val, L-Ser, and L-Thr resulted in lower production of trichothecenes when compared to that of L-Gln by the conventional batch culture method (Fig. 2), the effects of these four amino acids were further examined in detail.

We measured the total amount of trichothecenes (combined amount at 36 and 72 h) in the synthetic liquid culture of *F. graminearum* by frequent monitoring and adjustments of the culture pH, including replacement of the spent medium with fresh medium. In contrast to that in the L-Gln medium, toxin/fungal mass was not detected in Gly, L-Ser and L-Thr media when the pH was maintained at 4.0 for 72 h (Fig. 3). There were no statistically significant differences when L-Val medium was used for the culture. As expected, trichothecene production was increased in L-Phe medium, presumably owing to the functional activation of FgAreA required for the metabolism of this amino acid. When the pH was maintained at pH3.5, suppression of trichothecene production by L-Thr, as well as stimulation by L-Phe, was observed during the incubation period of 72 h. However, the suppression of mycotoxin biosynthesis in the presence of Gly and L-Ser was not detected at this pH (Fig. 3). Thus, competition between stimulation by acidic pH conditions and suppression by specific amino acid metabolism seems to determine the amount of trichothecenes accumulated in the culture. These results highlight the effect of L-Thr metabolism on the suppression of trichothecene biosynthesis.

The liquid YE-KH_2PO_4 medium, which contains 0.1% (w/v) Bacto™ yeast extract in exchange for the 5 mM amino acid in the synthetic liquid medium (Table S1), also induces trichothecene biosynthesis. To see the effect of an extra amino acid added to the yeast extract, the YE-KH_2PO_4 medium was supplemented with 5 mM each of L-Thr and L-Phe. After transferring the pre-culture to the amended YE-KH_2PO_4 medium (30 mL in a 100-mL Erlenmeyer flask), the pH of the fungal culture was maintained at 4.0, including replacement of the spent medium with fresh medium, during the

incubation period of 72 h. When used as a supplement to the complex media at 5 mM, L-Thr also suppressed 15-ADON production while the mycotoxin production was increased by adding L-Phe (Fig. S6). This is similar to the case of the fungal culture on brown rice flour solid media, in which exogenous addition of L-Thr suppressed trichothecene biosynthesis by JCM 9873 (Maeda et al. 2017).

According to available biochemical knowledge, metabolic breakdown of L-Thr proceeds along two main pathways: (1) degradation into Gly and acetyl-CoA, involving a step with either aldol or Claisen-type cleavage, and (2) degradation into propionyl-CoA, an odd-chain fatty acid degradation product, followed by conversion into succinyl-CoA *via* methylmalonyl-CoA. A preliminary microarray analysis of L-Thr-induced gene expression in brown rice flour culture medium (Maeda et al. 2017) indicated increased transcription of a probable threonine aldolase gene (FGSG_12344) that is involved in the former pathway, but not of threonine ammonia-lyase genes that are involved in the latter pathway (GEO accession number GSE115673). Thus, we speculate that Gly and acetyl-CoA were the main products when L-Thr was added as the sole nitrogen source in the medium. In this context, it seems reasonable that when Gly is used as the sole nitrogen source of the medium, its effect on trichothecene synthesis partially resembles the effect of L-Thr when L-Thr is used as the sole nitrogen source in the medium.

Conclusions

Synthetic liquid culture is often used for studying the effect of different nitrogen sources on the induction of trichothecene biosynthesis (Gardiner et al. 2009a). However, changes in the pH of the medium associated with specific amino acid metabolism (*e.g.*, L-Gln or Gly) are often overlooked; rapid and remarkable pH changes occur during the

growth of *F. graminearum*. The time-dependent pH profiles of the amino acids tested as the sole nitrogen sources were considerably different. As the difference in pH affects the regulation of trichothecene biosynthesis, frequent monitoring and adjustments of the culture pH were necessary to precisely evaluate the effect of metabolism of each amino acid. Thus, we distinctly demonstrated that L-Val metabolism was not associated with the suppression of trichothecene biosynthesis, while L-Thr most effectively suppressed the mycotoxin production. Compared to the experiments with autoclaved brown rice flour culture (Maeda et al. 2017) and synthetic liquid culture without pH maintenance (Fig. 2), this fed-batch culture presents a suitable method to study the suppressive effect of L-Thr by biochemical and molecular genetic approaches.

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Author contributions Y.N., K.M., K.K., T.K., and M.K. designed study, T.S., Y.N., K.M., M.A., Y.K., and T.N. performed research, T.S., Y.N., K.M., S.O., T.N. and M.K. analyzed data, and M.K. wrote the paper.

Conflict of interest The authors declare no conflict of interest.

Electronic supplementary material The online version of this article contains supplementary material, which is available to authorized users.

Table S1 Composition of synthetic agar and liquid media

Fig. S1 Generation of the Δ*FgareA* mutant of *F. graminearum* JCM 9873

Fig. S2 Sequence and structure of pΔFgAreA

Fig. S3 Germination and growth of the *FgAreA* deletion mutant Δ*FgareA* #1 on a synthetic agar plate containing one of the proteinogenic amino acids as a nitrogen source

Fig. S4 Time course of pH changes of the fungal culture with various amino acids as the sole nitrogen source

Fig. S5 Time course of pH changes of the fungal culture using 10 mM malate as the buffer

Fig. S6 Effect of amino acid supplementation to the complex medium on trichothecene productivity of *F. graminearum* JCM 9873

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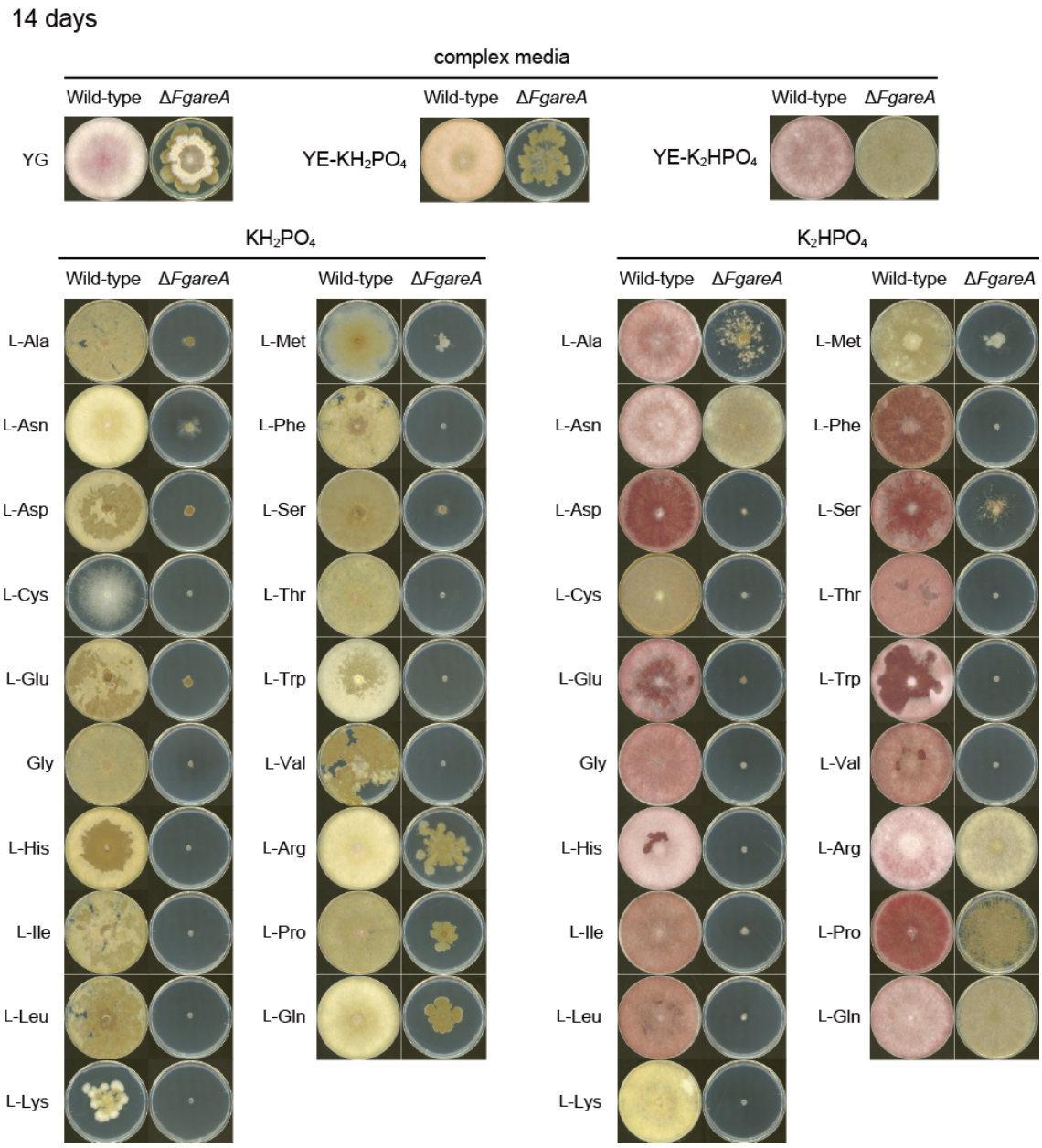

Fig. 1 Growth of the *FgAreA* deletion mutant Δ*FgareA* #1 on agar plates with complex media (panels in the first row) and synthetic media containing one of the proteinogenic amino acids as a nitrogen source (other panels). See Table S1 for medium composition. The type of the phosphate salt used for preparation of the medium affected the pH; pH was 4.2 with KH_2PO_4 and 7.2 with K_2HPO_4. YE-KH_2PO_4 and YE-K_2HPO_4 contain 0.1% (w/v) Bacto™ yeast extract in exchange for the 5 mM amino acid in the synthetic

medium. No phosphate buffer is included in YG agar

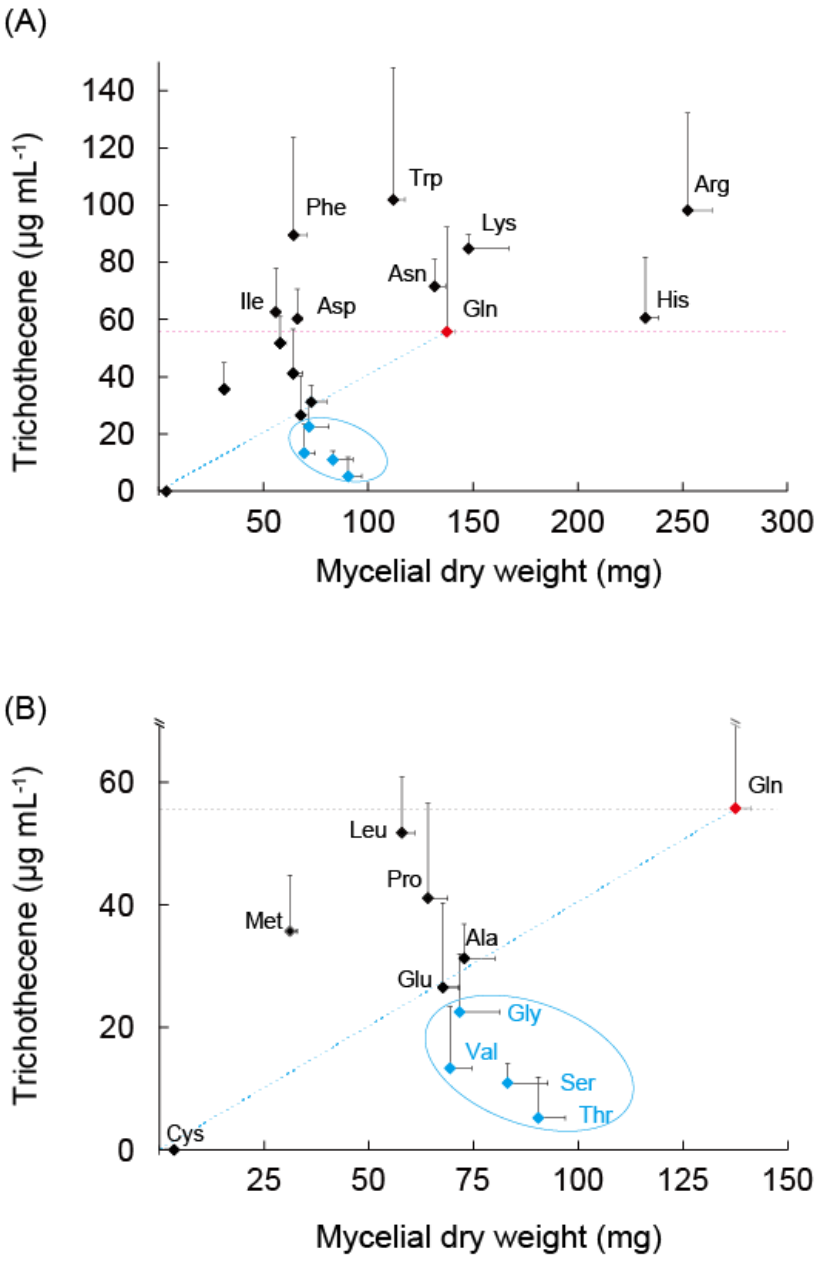

Fig. 2 Trichothecene production by wild-type *F. graminearum* in synthetic liquid medium containing one of the proteinogenic amino acids as the nitrogen source. Strain JCM 9873 was pre-cultured for 16 h; 1% (v/v) of the pre-culture was transferred to 30 mL of the synthetic liquid medium and incubated at 25 ^{o}C with gyratory shaking (135 rpm). Each dot labeled with the name of an amino acid represents the amount of trichothecenes (μg mL^{-1}) and mycelial dry weight (mg) of the culture after 8 days of

incubation (n = 3; standard deviations shown by gray half-bars). A portion of the panel (A) is enlarged in panel (B) for better visualization. As L-Cys did not support growth, the analyses were not performed. The amount of trichothecenes was reproducibly low when L-Ser, L-Thr, and L-Val were used as the sole nitrogen source. See Fig. S4 for time course changes of the culture pH with L-Gln, Gly, L-Val, L-Ser, L-Thr, L-Phe and L-Trp as the sole nitrogen source

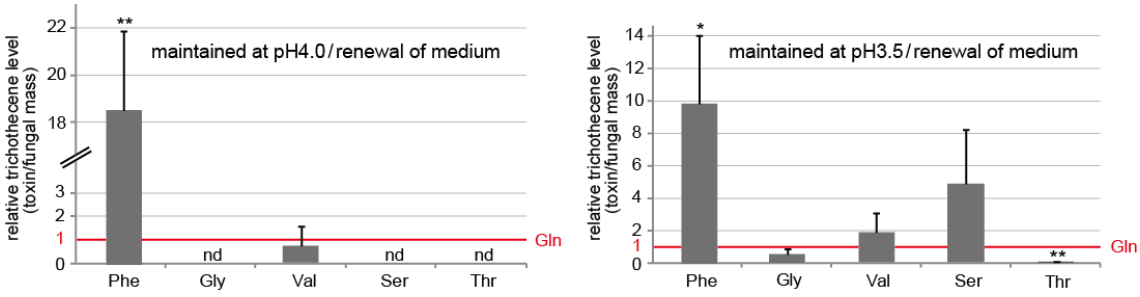

Fig. 3 Effect of amino acid utilization on trichothecene productivity of *F. graminearum* JCM 9873. The fungal cultures were maintained at pH4.0 or pH3.5 by frequently adjusting the pH and renewal of the synthetic liquid media. Asterisks denote significant differences (*$P < 0.05$, **$P < 0.01$) relative to the toxin/fungal mass (μg/mg) in the L-Gln medium, as determined by Student's *t*-test ($n = 3$)

Identification of amino acids negatively affecting *Fusarium* trichothecene biosynthesis by frequent pH adjustments of the liquid culture medium

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Supplementary Methods

Microarray analysis

RNA isolated from fungal cultures on brown rice flour medium after 36 and 48 h of incubation (Maeda et al. 2017) were used for microarray analysis. Double-strand cDNAs were synthesized from total RNA using oligo(dT) primer with a T7 RNA polymerase promoter sequence at its 5′- end (Low Input Quick Amp Labeling Kit; Agilent Technologies (Miwa et al. 2017) from which sequence Cy3-labeled cRNAs were transcribed. After purification of the cRNAs, 600 ng each cRNA was fragmented, and 50 µl of hybridization mixture prepared. A portion (40 µl) of each mixture was used for hybridization with the Agilent 8 X 15k *Fusarium graminearum* custom array with probe sets for 11,675 genes (GEO platform accession number GPL25174). The whole procedure of hybridization and washing was carried out following the manufacturer's instructions. The arrays were scanned at maximum laser intensity in the Cy3 channel using an Agilent microarray scanner (G2565BA; Agilent Technologies). The images were analyzed using Feature Extraction Software (Ver. 10.7.3.1; Agilent Technologies). These data were analyzed further with GeneSpring GX12.0 software (Agilent Technologies) and deposited in GEO database (accession number GSE115673).

Table S1

Composition of synthetic agar and liquid media

Synthetic medium

element	concentration
sucrose	30 g/L
KH_2PO_4 or K_2HPO_4 [1]	1 g/L
KCl	0.5 g/L
$MgSO_4 \cdot 7H_2O$	0.5 g/L
Trace element [2]	0.2 mL/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
L-amino acid [2]	5 mM
agar [1]	10 g/L

[1] K_2HPO_4 and agar used for solid culture only.
[2] filter-sterilized.

5,000 × Trace elements

element	concentration
citric acid	5 g/100 mL
$MnSO_4$	50 mg/100 mL
$ZnSO_4 \cdot 6H2O$	5 g/100 mL
H_3BO_3	50 mg/100 mL
$Na_2MoO_4 \cdot 2H_2O$	50 mg/100 mL
$CuSO_4 \cdot 5H_2O$	250 mg/100 mL

Stock solutions of L-Asp, L-Glu, and L-Trp were prepared at 15 mM in water. Other highly soluble L-amino acids were dissolved at 50 mM in water. The resulting stock solution of each amino acid (except water-insoluble L-Tyr, which was omitted in this study) was mixed with the appropriate stock solutions containing the rest of the ingredients

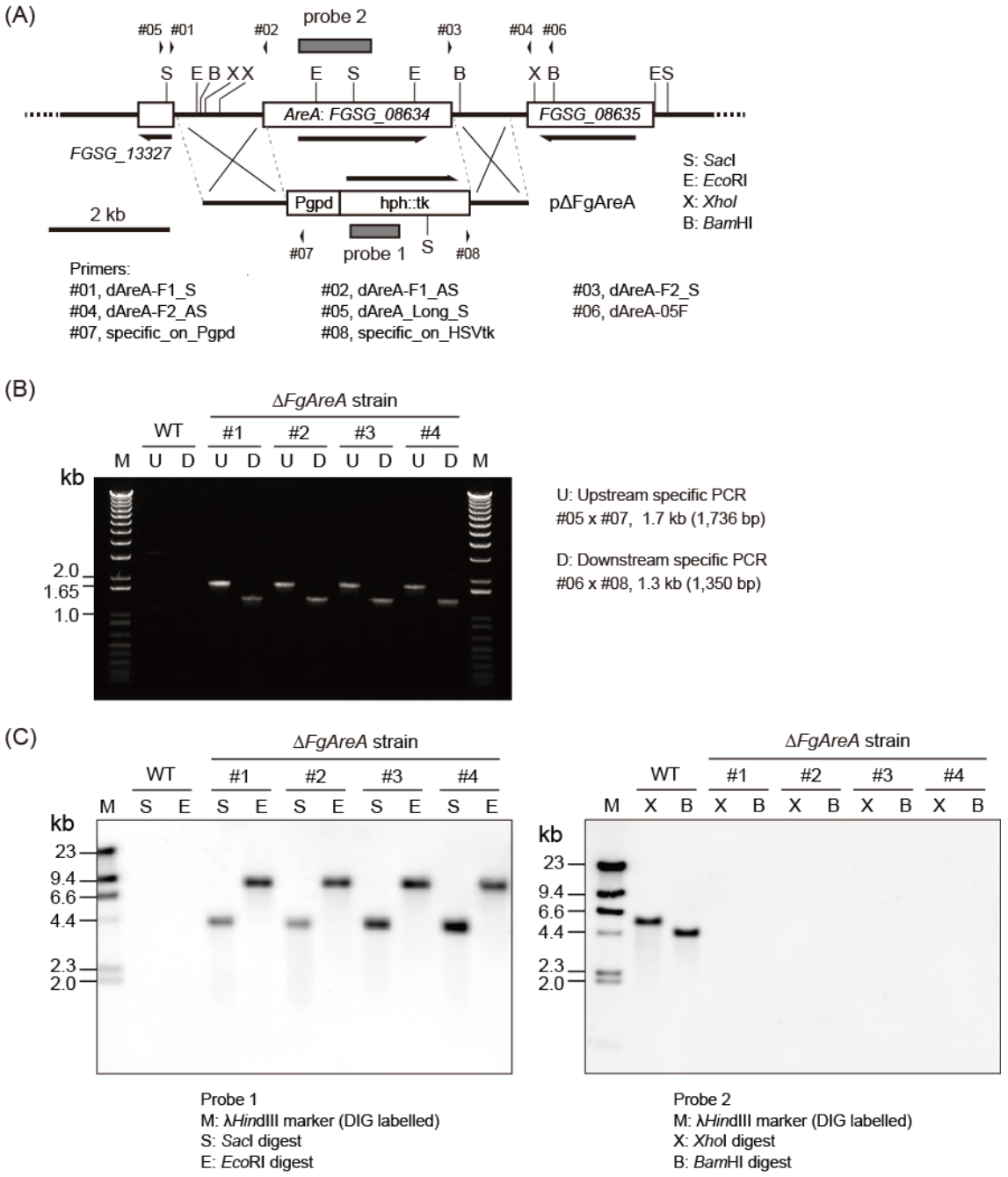
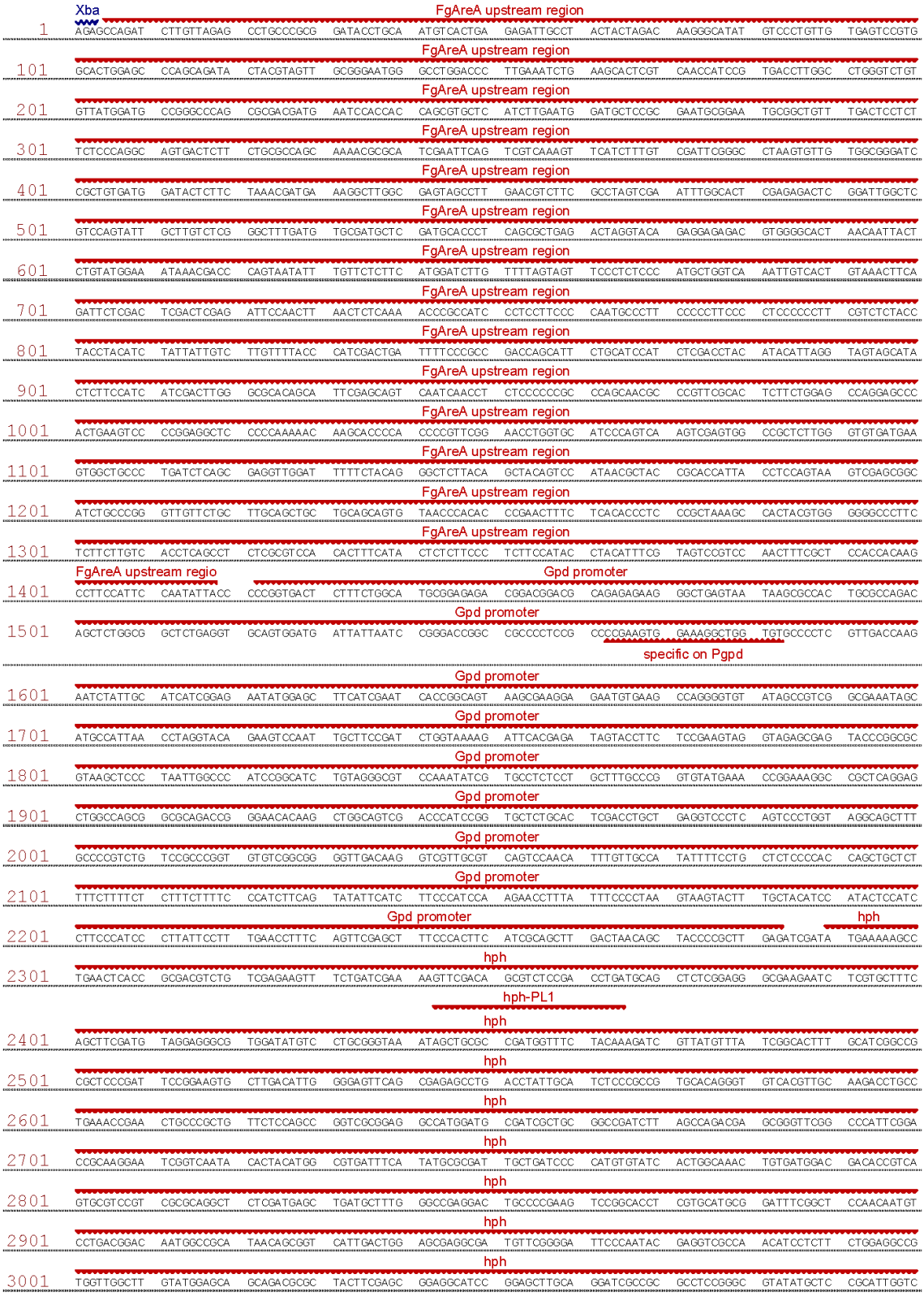


Fig. S1 Generation of the Δ*FgareA* mutant of *F. graminearum* JCM 9873. The Δ*FgareA* mutant #1 was used for the growth assay. (A) A gene disruption vector pΔFgAreA (Fig. S2) was constructed by cloning two PCR fragments upstream and downstream of *FgAreA* into pHph::tk-I (Maeda and Ohsato 2017), a vector that contains a translational fusion between *hph* (hygromycin B phosphotransferase gene) and *HSVtk*

(herpes simplex virus thymidine kinase gene) under the control of *A. nidulans GPD* promoter (Nakajima et al. 2014). Briefly, the following four DNA fragments were joined together using a NEBuilder® HiFi DNA Assembly Cloning kit; upstream region of *FgAreA* amplified by PCR with primers dAreA-F1_S (5′-AGGTCGACTCTAGAGCCAGATCTTGTTAGAGCCTG-3′) and dAreA-F1_AS (5′-AAGAGTCACCGGGGGTAATATTGGAATGGAAGGCTTGT-3′), downstream region of *FgAreA* amplified by PCR with primers dAreA-F2_S (5′-GGGAGGCTAACTGACTTGTAAGGGGACTTGTGTGC-3′) and dAreA-F2_AS (5′-CGAGCTCGGTACCCCAGGCAGGAGTTTGATACGG-3′), and two DNA fragments generated by digesting pHph::tk-I with *Bam*HI and *Xho*I (15 bp overlaps underlined). *F. graminearum* was transformed with *Hin*dIII-linearized pΔFgAreA by the protoplast/polyethyleneglycol method and 30 μg/ml of hygromycin B selection as described previously (Tokai et al. 2007). (B) Identification of the *FgAreA* gene disruptant by PCR screening. Four transformants (#1 − #4) that failed to grow on synthetic agar medium containing nitrate as a nitrogen source were selected, their genomic DNA was isolated, and the gene disruption event was confirmed by PCR. The region encompassing the upstream region of *FgAreA* and *GPD* promoter was amplified with primers dAreA_Long_S (5′-GCAATAGGAAGACAGACAAGG-3′) and specific_on_Pgpd (5′-ACACCAGCCTTTCCACTTCGG-3′), yielding an expected amplicon size of 1,736 bp; the region encompassing the downstream region of *FgAreA* and *hph::tk* was amplified with primers dAreA-05F (5′-AGCTAGCCCTCGGAAACGCAT -3′) and specific_on_HSVtk (5′-GGCTCCATACCGACGATATGC-3′), yielding an expected amplicon size of 1,350 bp. (C) Southern blot analysis of the disruptants. Probes were synthesized using a PCR DIG Probe Synthesis kit and detected using a DIG Luminescent Detection kit (Roche Applied Science). Primers hph-PR1 (5′-GGGGCGTCGGTTTCCACTATCG-3′) and hph-PL1

(5′AGCTGCGCCGATGGTTTCTACAA-3′) were used to prepare the *hph* probe (probe 1), and primers FgAre1_Seq1F_590 (5′-ATCCCATGAATCTCGACGACT-3′) and FgAre1_Seq4R_1778 (5′-CACCTCTGAAAGACTGCGATT-3′) for the *FgAreA* probe (probe 2). When the genomic blot was hybridized with the *hph* probe, expected band sizes (3.9 kb *Sac* I and 7.5 kb *Eco*RI bands) were detected from the *FgAreA* deletion strains (#1 − #4), but no signal from the wild-type strain (upper panel). For the *FgAreA* probe, no signals were detected on the blot for the deletion strains (#1 − #4) while the expected band sizes (5.2 kb *Xho*I and 4.3 kb *Bam*HI bands) were observed for the wild-type strain (lower panel)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101 GCACTGGAGC CCAGCAGATA CTACGTAGTT GCGGGAATGG GCCTGGACCC TTGAAATCTG AAGCACTCGT CAACCATCCG TGACCTTGGC CTGGGTCTGT
201 GTTATGGATG CCGGGCCCAG CGCGACGATG AATCCACCAC CAGCGTGCTC ATCTTGAATG GATGCTCCGC GAATGCGGAA TGCGGCTGTT TGACTCCTCT
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1301 TCTTCTTGTC ACCTCAGCCT CTCGCGTCCA CACTTTCATA CTCTCTTCCC TCTTCCATAC CTACATTTCG TAGTCCGTCC AACTTTCGCT CCACCACAAG
FgAreA upstream regio
Gpd promoter
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Gpd promoter
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specific on Pgpd
Gpd promoter
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Gpd promoter
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Gpd promoter
hph
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hph
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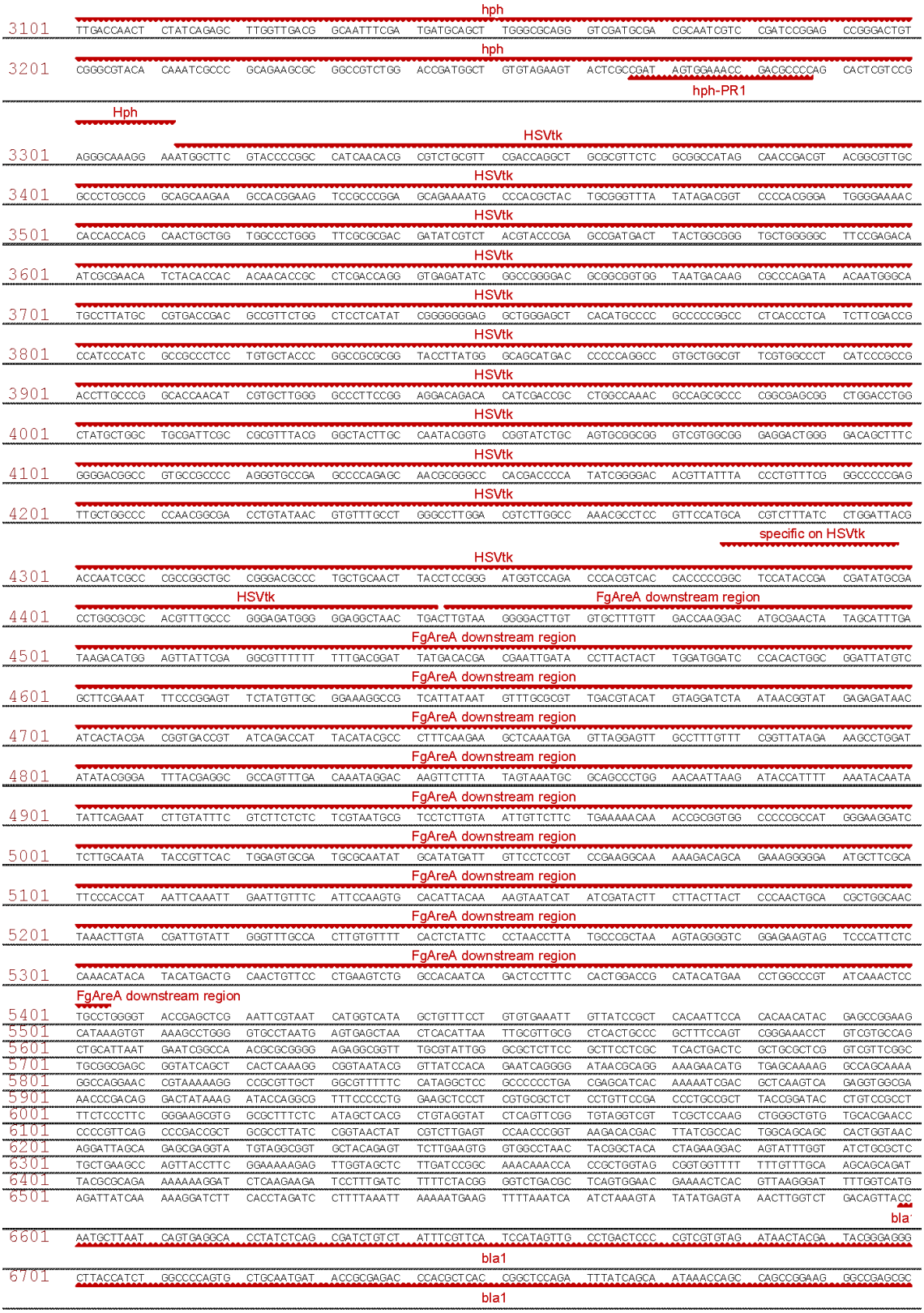
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Hph
HSVtk
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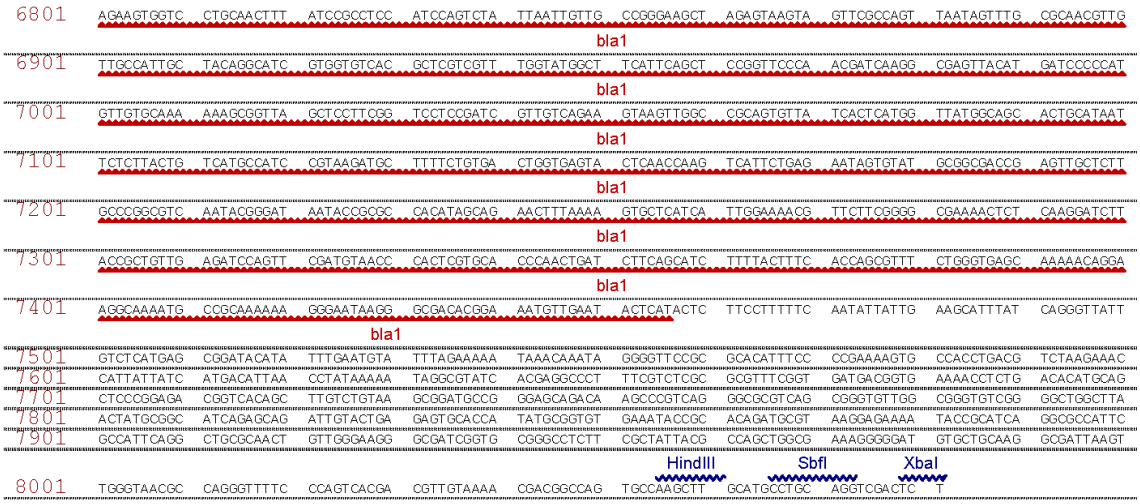

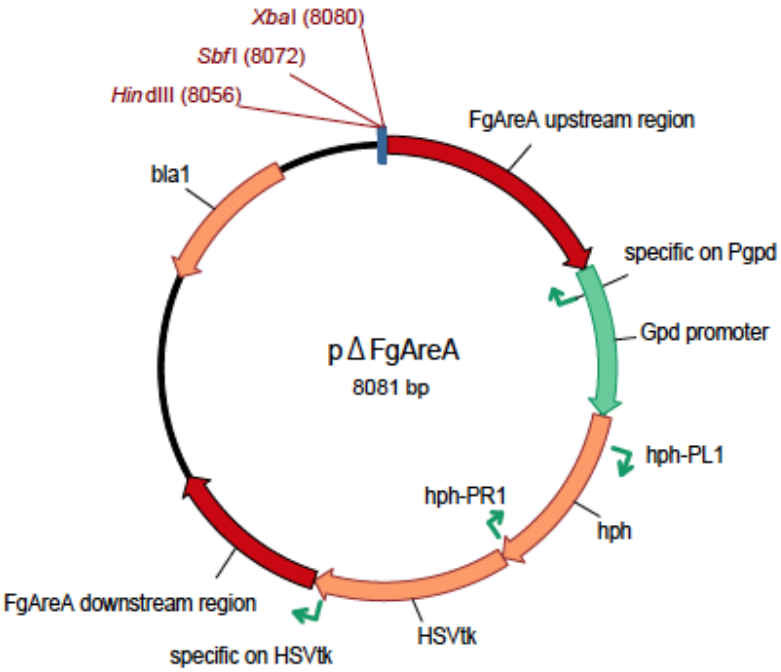

Fig. S2 Sequence and structure of pΔFgAreA

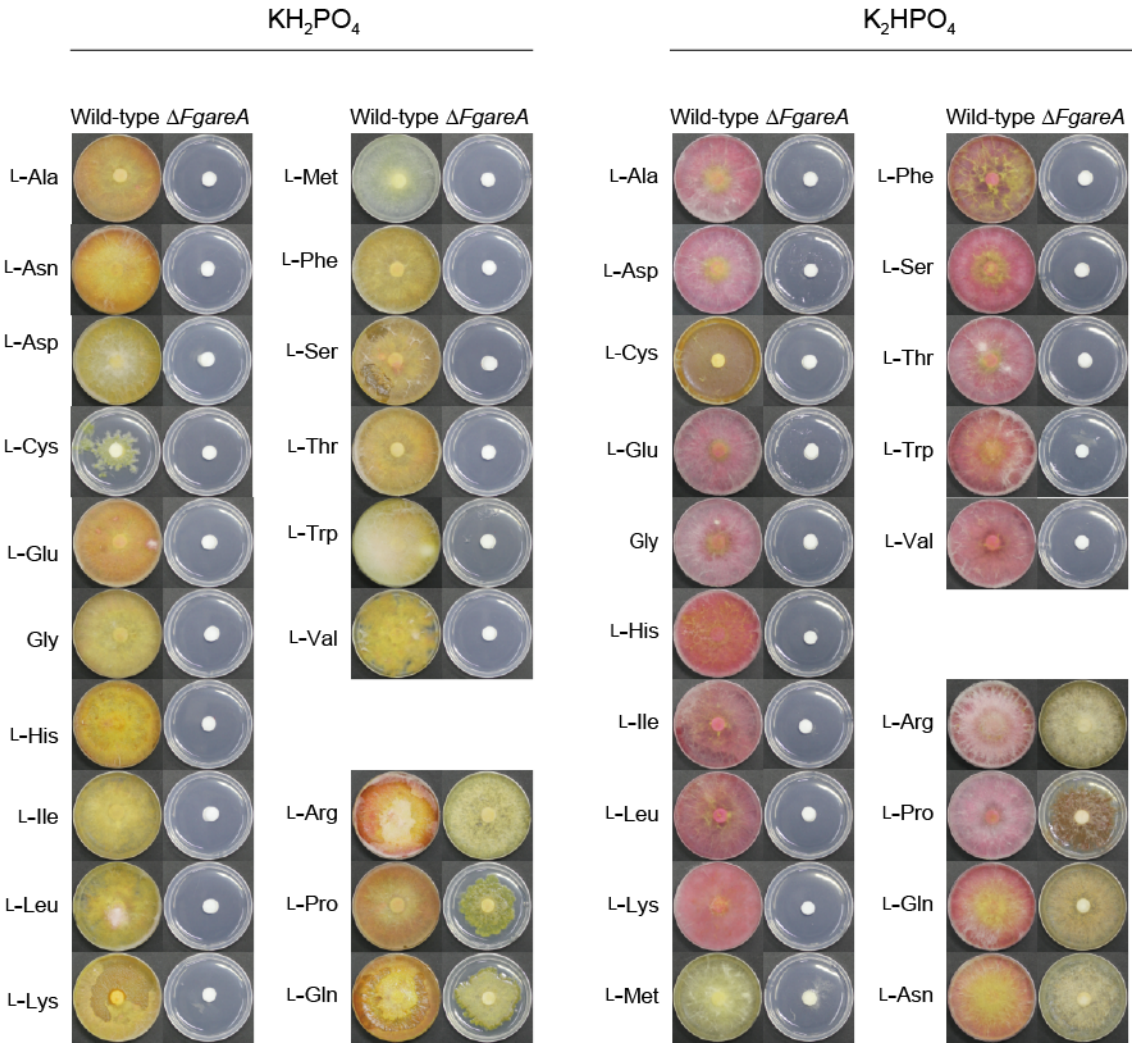

Fig. S3 Germination and growth of the *FgAreA* deletion mutant Δ*FgareA* #1 on a synthetic agar plate containing one of the proteinogenic amino acids as a nitrogen source. See Table S1 for medium composition. The type of the phosphate salt used for preparation of the medium affected the pH; pH was 4.2 with KH_2PO_4 and 7.2 with K_2HPO_4. The conidia (1×10^5), applied on a paper disc (Advantech 49005010; diameter, 8 mm), were placed on the center of synthetic agar plates (diameter, 55 mm) and incubated at 25°C for 2 weeks

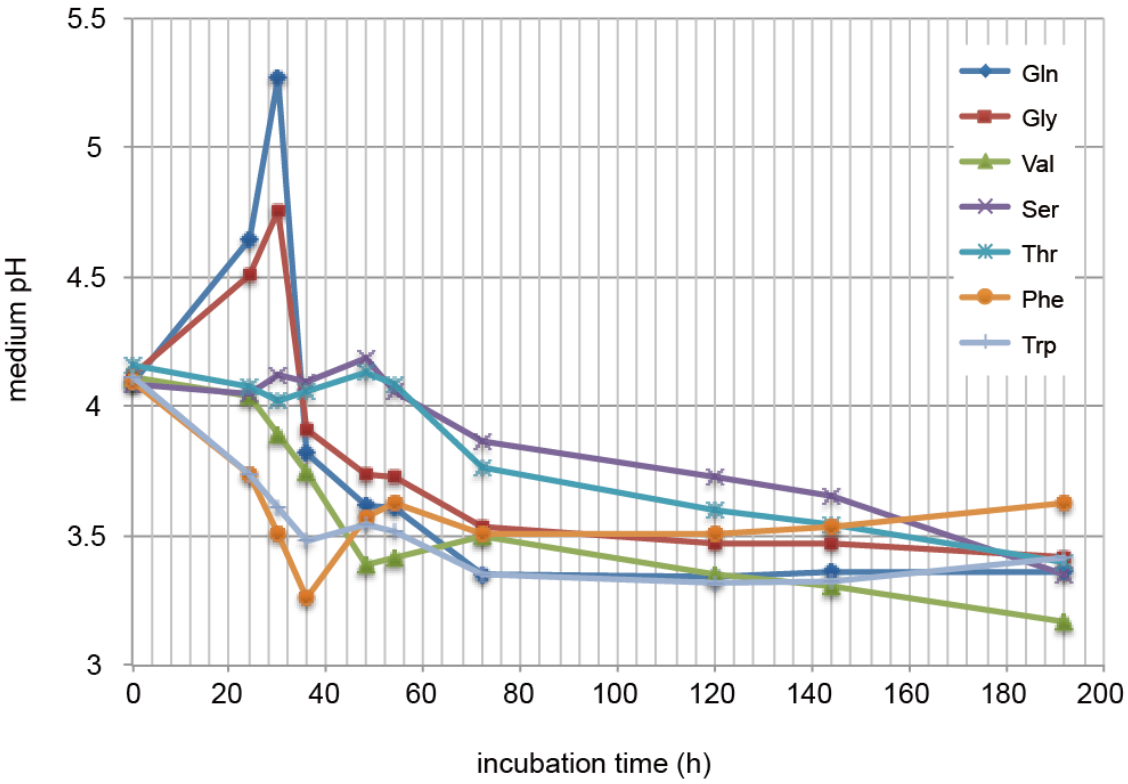

Fig. S4 Time course of pH changes of the fungal culture with various amino acids as the sole nitrogen source. After inoculation of *F. graminearum* pre-culture into the synthetic media supplemented with L-Gln, Gly, L-Val, L-Ser, L-Thr, L-Phe and L-Trp media, the pH wad periodically monitored using a hand-held pH meter LAQUAtwin B-712

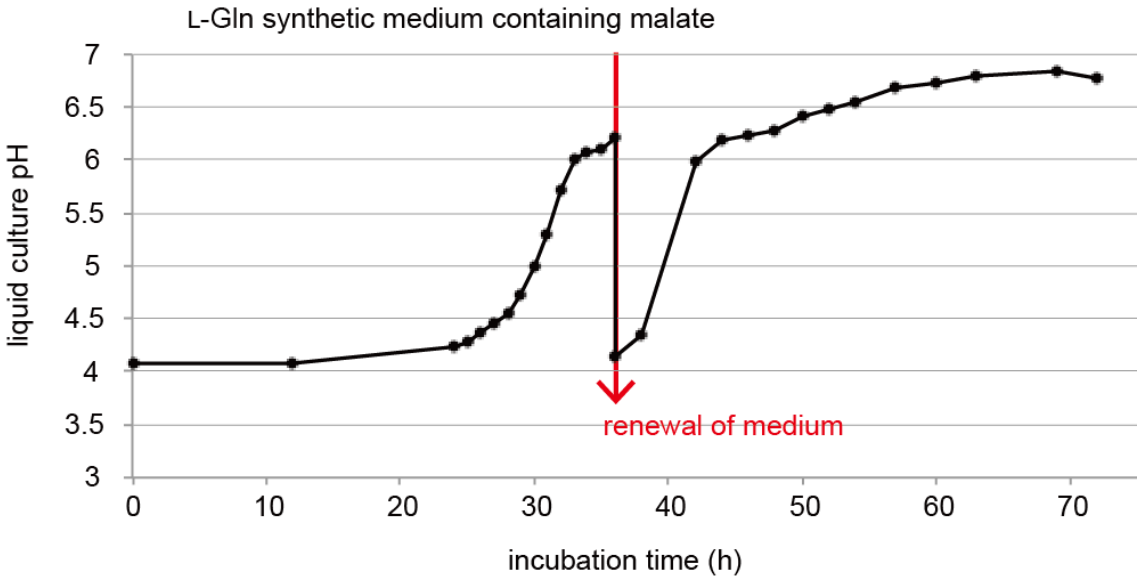

Fig. S5 Time course of pH changes of the fungal culture using 10 mM malate as the buffer. The synthetic L-Gln medium was inoculated with *F. graminearum* pre-culture and the pH was periodically monitored using a hand-held pH meter LAQUAtwin B-712. After 36 h of incubation, the mycelia were transferred to a new L-Gln medium containing 10 mM malate, and further incubated for additional 36 h

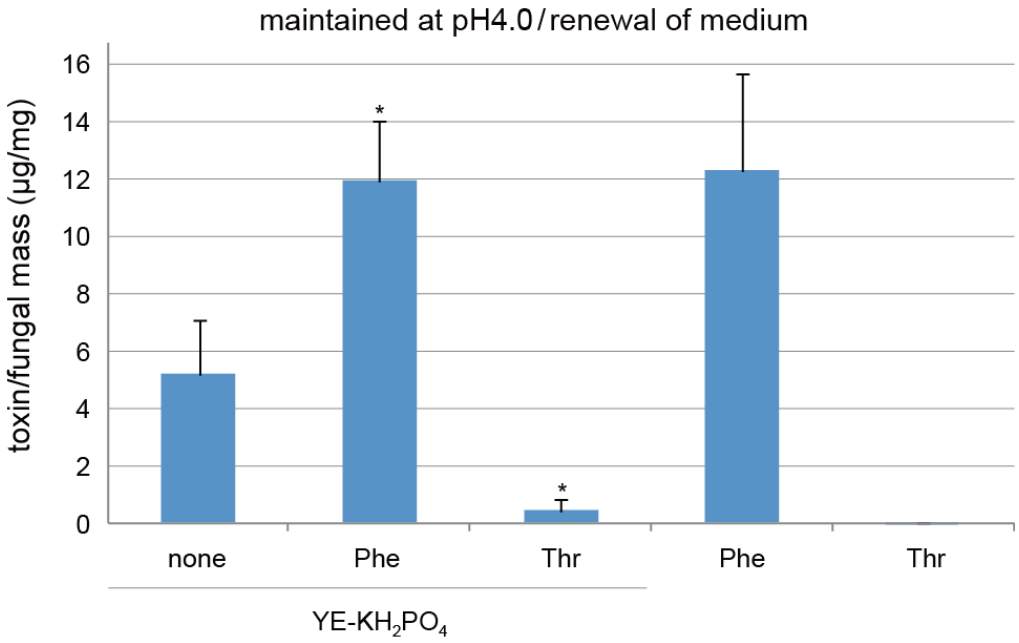

Fig. S6 Effect of amino acid supplementation to the complex medium on trichothecene productivity of *F. graminearum* JCM 9873. YE-KH_2PO_4 medium (none) was supplemented with of L-Phe or L-Thr (5 mM each). The fungal culture was maintained at pH4.0 by frequently adjusting the pH and renewal of the liquid media. For reference, toxin/fungal mass (μg/mg) of culture in the related synthetic medium was also indicated. An asterisk denote significant differences (*$P < 0.05$) relative to the toxin/fungal mass in the YE-KH_2PO_4 medium (none), as determined by Student's *t*-test ($n = 3$)

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