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ORIGINAL ARTICLE

Impact of nitrogen metabolism-associated culture pH changes on regulation of *Fusarium* trichothecene biosynthesis: revision of roles of polyamine agmatine and transcription factor AreA

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Abstract

Fusarium graminearum produces trichothecene mycotoxins in infected grains and axenic liquid culture. A proposed regulatory model of trichothecene biosynthesis was examined in relation to nitrogen utilization. First, we showed that an important factor for the stimulation of trichothecene biosynthesis was not the occurrence of agmatine as a specific inducer molecule, but rather continuous acidification of the liquid culture medium arising from agmatine catabolism. When the pH of the L-Gln synthetic medium was frequently adjusted to the pH of the agmatine culture, trichothecene productivity of the L-Gln culture was equal to that of the agmatine culture. For efficient trichothecene biosynthesis, the culture pH should be lowered at an appropriate time point during the early growth stage. Second, we re-evaluated the role of the nitrogen regulatory GATA transcription factor AreA in trichothecene biosynthesis. Since *Tri6* encodes a transcription factor indispensable for trichothecene biosynthesis, all fifteen AreA-binding consensus sequences in the *Tri6* promoter were mutated. The mutant could catabolize L-Phe as the sole nitrogen source; furthermore, the pH profile of the synthetic L-Phe medium (initial pH 4.2) was the same as that of the wild-type (WT) strain. Under such conditions, the promoter mutant exhibited approximately 72% of the trichothecene productivity compared to the WT strain. Thus, *F. graminearum* AreA (FgAreAp) is dispensable for the functioning of the *Tri6* promoter, but it contributes to the increased production of mycotoxin under mildly acidic conditions to some extent. Further investigations on the culture pH revealed that extremely low pH bypasses the function of FgAreAp.

Keywords Agmatine · Deoxynivalenol · Transcription regulator · *Tri6* · Trichothecene production

Introduction

The ascomycetes fungi, *Fusarium graminearum* species complex and closely related *Fusarium* species, are important pathogens of small cereal grains that cause devastating disease of Fusarium head blight. They produce trichothecene mycotoxins, a group of toxic secondary metabolites with a 12,13-epoxytrichothec-9-ene skeleton (Kimura et al. 2007; McCormick et al. 2013). Molecular mechanisms of *Fusarium* development, plant infection, and mycotoxin production have been the subject of intensive research in recent years. Accumulation of trichothecenes in grains is a serious threat to human and animal health, and development of appropriate means of controlling mycotoxin production represents urgent issues in agricultural and food industries (Desjardins 2009; Kazan and Gardiner 2018). Secondary metabolite biosynthesis, including mycotoxin production, is generally subject to regulation by nutritional factors in producing fungi (Merhej

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et al. 2011). Carbon catabolite regulation applies in certain cases, as exemplified by glucose repression of penicillin production in *Aspergillus nidulans* and *Penicillium chrysogenum* (Espeso and Penalva 1992; Feng et al. 1994; Martin et al. 1999), and of lovastatin production in *Aspergillus terreus* (Hajjaj et al. 2001). Although trichothecene biosynthesis is not repressed by glucose in *F. graminearum* (Jiao et al. 2008; Nakajima et al. 2016), it is regulated by the quality and quantity of nitrogen sources.

Trichothecene biosynthesis requires combined functions of pathway enzymes, a specific efflux pump, and regulatory factors, encoded by trichothecene (*Tri*) genes clustered in the *Fusarium* genome (Kimura et al. 2007; McCormick et al. 2013). *Tri6*, encoding a Cys_2His_2 zinc finger transcription factor, is indispensable for *Tri* gene expression. Numerous consensus binding sequences of a GATA family transcription factor, AreA (FgAreAp; *F. graminearum* AreA protein), are found in the promoters of *Tri6* and other *Tri* genes (other than *Tri10*) located in the core region of the *Tri* gene cluster (Merhej et al. 2011). This led to the hypothesis that *Tri6* is under the positive transcriptional control of FgAreAp, which is functionally activated on poor nitrogen sources and under nitrogen-limiting conditions. Levels of the active form of AreA in the nucleus increase in the absence of readily assimilable nitrogen sources such as ammonia and L-Gln (Wong et al. 2008). In *F. graminearum*, *FgAreA* disruption (Δ*FgareA*) mutants are unable to grow on synthetic media containing most amino acids as the sole nitrogen source, except L-Gln, and a small number of others, depending on the strains and medium pH (Giese et al. 2013; Min et al. 2012; Shiobara et al. 2019). When an FgAreAp function-dependent nutrient, L-Phe, is used as the sole nitrogen source and the culture pH is manually maintained at a constant value during incubation, the fungus accumulates greater amount of trichothecenes than in the synthetic L-Gln medium maintained at the same pH (Shiobara et al. 2019).

Use of agmatine as the sole nitrogen source in liquid culture medium results in higher levels of trichothecene accumulation in *F. graminearum* CS3005 (Gardiner et al. 2009, 2014). Hence, polyamines in the arginine–polyamine biosynthetic pathway of host plants have been proposed to function as inducers of trichothecene biosynthesis (Gardiner et al. 2009; Kazan and Gardiner 2018). Consistent with other reports, strain JCM 9873 also exhibited increased levels of trichothecene accumulation in synthetic agmatine medium compared to the levels of accumulation in L-Gln medium (Kitou et al. 2016a). With longer incubation periods, however, trichothecene production levels in agmatine medium are not strikingly elevated; in spite of the high agmatine concentration of 5 mM used for induction, the amount of trichothecene 15-acetyldeoxynivalenol (15-ADON) generated was twofold–threefold greater than that of the L-Gln culture by day 8 (Kitou et al. 2016a). In contrast to agmatine, the furanocoumarin NPD12617 and its cognate compounds activate trichothecene biosynthesis at μM concentrations on complex media distributed among 24-well plates (Maeda et al. 2018). In addition, regardless of the high sensitivity to sucrose-degrading enzymes, sucrose and related oligosaccharides (e.g., 1-kestose, raffinose) added to the culture have been shown to act as inducer molecules at 100 μM (Nakajima et al. 2016), a concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) widely used as an inducer for expression of the β-galactosidase α-fragment in molecular biology experiments (Sambrook et al. 1989). Thus, although there remains a possibility of an extremely limited amount of potent trichothecene-inducing molecules being synthesized by metabolizing agmatine, the role of polyamines as inducers seems questionable.

In previous studies, the batch culture method was used to evaluate the effects of nitrogen sources on trichothecene biosynthesis induction (Gardiner et al. 2009). In such cases, pH profiles (pH change as a function of time) of the synthetic media should be consistent throughout the culture period to establish a cause-and-effect relationship between catabolizing a specific nitrogen source and activating secondary metabolism. However, changes in pH between the agmatine and L-Gln cultures were not considered (Gardiner et al. 2009; Hou et al. 2015; Min et al. 2012). If pH profiles are maintained the same, the use of nitrogen sources other than agmatine may result in accumulation of similar or even greater amounts of trichothecenes.

In this paper, the effects of nutritional and genetic factors on trichothecene biosynthesis regulation were re-evaluated by frequent monitoring and adjustments of the pH of the culture medium (Shiobara et al. 2019). We show that agmatine was not an inducer of trichothecene biosynthesis. The role of FgAreAp in regulation of trichothecene biosynthesis was also re-evaluated; the extent of FgAreAp contribution to trichothecene biosynthesis regulation depended on pH of the cell culture medium.

Materials and methods

Reagents and supplies

KOD-Plus-Neo, Gibson Assembly Master Mix, BigDye Terminator version 3.1 cycle sequencing kit, 2′-deoxy-5-fluorouridine (5-FdU), and standard mycotoxin mixture solution 2 were obtained from TOYOBO (Osaka, Japan), New England Biolabs (Ipswich, MA), Applied Biosystems (Foster City, CA), Carbosynth Ltd (Compton, Berkshire, UK), and Kanto Kagaku Co. (Toyko), respectively. Miracloth™ and Kieselgel F_{254} thin-layer chromatography (TLC) plates (Silicagel 60 F_{254}) were from Merck Millipore (Darmstadt, Germany). PEGASIL ODS SP100 column (diameter, 4.6 mm;

length, 250 mm) was from Senshu Scientific Co. (Tokyo). Other materials were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan).

Fungal strains

F. graminearum strain JCM 9873 (Japan Collection of Microorganisms, Tsukuba, Japan; 15-ADON chemotype) was used as the parental wild-type (WT) strain for genetic manipulations. This strain produces only a very small amount of deoxynivalenol (DON) under the culture conditions used in this study. Strains Δ*FgareA* #1 and #2 are hygromycin B-resistant *FgAreA* null mutants of JCM 9873 reported in a previous study (Shiobara et al. 2019). Self-cloning strains P_{Tri6}/m15 and P_{Tri6}/m0, containing mutated and wild-type *Tri6* promoter sequences, respectively, are derived from a single strain ΔP_{Tri6} #1, a deletion mutant involving the *Tri6* promoter.

Media and culture conditions

Conidia were induced on carboxymethyl cellulose (CMC) medium (Etzerodt et al. 2015), filtered through Miracloth™, suspended in 30% (w/v) glycerol at 1×10^7 conidia/mL, and stored at − 80 °C. For the examination of trichothecene productivity (toxin/fungal mass), the stored conidia were inoculated at a density of 1×10^4 conidia/mL on 50 mL of YG medium (0.5% [w/v] Bacto™ yeast extract, 2% [w/v] glucose) in a 200-mL Erlenmeyer flask and cultured at 25 °C with reciprocal shaking at 125 rpm. After incubation for 16 − 18 h, the pre-culture (300 µL of germinating conidia) was transferred to 30 mL of synthetic medium containing 3% (w/v) sucrose and 5 mM nitrogen source (agmatine, L-Gln, L-Phe, or nitrate) (Gardiner et al. 2009; Kitou et al. 2016b) for trichothecene production. Cell culture was conducted in a 100-mL Erlenmeyer flask and incubated with gyratory shaking at 135 rpm at 25 °C, with or without pH adjustment using a handheld pH meter LAQUAtwin B-712 (Horiba Ltd., Kyoto, Japan). In 24-well plate assays, pre-cultures were inoculated into toxin-non-inducing YG_60 or toxin-inducing YS_60 media (Nakajima et al. 2016).

Northern blot analysis

Total RNA was isolated from mycelia, as described previously (Etzerodt et al. 2015). For northern blot analysis, 10 µg RNA was separated on a 1% agarose gel containing 0.44 M formaldehyde, transferred to a positively charged nylon membrane (Roche Diagnostics GmbH, Manheim, Germany), and hybridized with digoxigenin (DIG)-labeled DNA probes. *Tri4*, *Tri6*, *Tri5*, and *GPD* probes, described in previous studies (Maeda et al. 2018), were hybridized with the target RNA on the membrane by using DIG Easy Hyb (Roche Diagnostics) hybridization solution at 50 °C, and washed as recommended by the manufacturer. Probe-RNA hybrids were detected using a DIG Luminescent Detection kit (Roche Diagnostics).

Construction of vectors for self-cloning of *Tri6* mutant promoter

The first transformation vector, pdPTri6-hph::tk, for positive selection and the second transformation vectors, pCPTri6m15 and pCPTri6m0, for conditional negative selection were constructed as summarized in Fig. S1. An *hph::tk* fusion marker gene (herpes simplex virus thymidine kinase gene *C*-terminally fused to the hygromycin phosphotransferase gene) cassette was used for construction of the vector for self-cloning of the 1,168-bp *Tri6-Tri4* intergenic region (*Tri6* bidirectional promoter).

For the construction of the first transformation vector, pHph::tk–I, containing the *hph::tk* cassette, was used as a vector backbone (Maeda and Ohsato 2017); the upstream and downstream homologous regions of the *Tri6* promoter were amplified from *F. graminearum* genomic DNA by KOD-Plus-Neo (1364 bp and 1313 bp, respectively) and assembled on each side of the *hph::tk* cassette by Gibson assembly (see Fig. S1A for details). The resulting plasmid, pdPTri6-hph::tk, was used to replace the *Tri6* promoter with *hph::tk* via homologous recombination and positive selection with hygromycin B.

To modify the *Tri6* promoter region, the 1168-bp intergenic sequence, with all fifteen 5′-GATA-3′AreA-binding consensus sequences mutated to 5′-GGTG-3′ (Fig. S2), was synthesized and cloned into pTAKN-2 vector by Eurofins Genomics Japan (Tokyo). The mutated *Tri6* promoter was amplified by PCR and connected to the flanking homologous regions (PCR-amplified upstream and downstream regions of the *Tri6* promoter) and *Sma*I-linearized pUC19 vector by Gibson assembly (see Fig. S1B for details). The resulting vector, pCPTri6m15, was used for conditional negative selection with 5-FdU. As a control vector, pCPTri6m0 containing the PCR-amplified wild-type *Tri6* promoter was also used for negative selection.

Generation of self-cloning strains with all fifteen AreA-binding consensus sequences in the *Tri6* promoter mutated to 5′-GGTG-3′

Self-cloning *F. graminearum* strains containing mutated and wild-type *Tri6* promoter were created by a two-step transformation process, which allowed assignment of the trichothecene production phenotype to the mutation. The parental WT strain, JCM 9873, was first transformed with *Eco*RI-linearized pdPTri6-hph::tk by the polyethylene glycol (PEG)-$CaCl_2$ method (Kimura et al. 1995) and selected with

150 µg/mL of hygromycin B. Transformants containing the *hph::tk* cassette in place of the *Tri6* promoter were screened by PCR, and candidate strains were confirmed for the loss of the *Tri6* promoter by Southern blot analysis (Fig. S3A). One of the deletion strains, ΔP_{Tri6} #1, was used as a host for the second transformation with *Eco*RI-linearized pCPTri6m15 (containing mutated promoter) and pCPTri6m0 (containing wild-type promoter). The transformed protoplasts were mixed with regeneration agar (0.5% [w/v] Bacto™ yeast extract, 0.8 M glucose, 0.5% [w/v] agar), poured into plastic petri dishes, and cultured overnight at 25 °C. For counter selection, top agar (1.5% [w/v] agar) containing 5 µM 5-FdU was layered on top of the protoplast-regenerated plates. After three to five days of incubation, 5-FdU-resistant colonies were picked and homologous recombinants were screened by PCR (Fig. S3B). The *Tri6* promoter sequences of the candidate strains were confirmed by sequencing with BigDye Terminator version 3.1 cycle sequencing kits.

Analytical methods

Fungal metabolites were extracted from the culture supernatants with equal amounts of ethyl acetate and dried under a gentle stream of nitrogen. For TLC analysis, samples reconstituted in ethanol were spotted on TLC plates and developed with ethyl acetate/toluene (3:1) as the solvent. 15-ADON was visualized with 4-(*p*-nitrobenzyl) pyridine as the coloring reagent (Etzerodt et al. 2015). For HPLC analysis, samples reconstituted in water/acetonitrile (75:25) were loaded onto a PEGASIL ODS SP100 column, and isocratic elution performed with water/acetonitrile (75:25) at a flow rate of 1.0 mL/min monitored at 220 nm. The toxin concentration was calculated from the peak area using a calibration curve obtained with the 15-ADON and DON standards (10–100 µg/mL). Trichothecene productivity (µg toxin/mg fungal mass) was calculated by dividing the amount of toxin by mycelia dry weight.

Results

Polyamines do not induce trichothecene biosynthesis when added at µM concentrations to complex medium

We first compared trichothecene biosynthesis-inducing activities of polyamines with those of known activators using cultures grown on a complex medium in 24-well plate assays (Nakajima et al. 2014). When added to YG_60 medium containing glucose and yeast extract (Fig. 1), sucrose and NPD12617 were effective in inducing trichothecene biosynthesis at µM concentrations (100 µM and 5 µM, respectively), as previously reported (Maeda et al. 2018; Nakajima et al. 2016). However, agmatine and putrescine had no effects on trichothecene induction. These results unambiguously demonstrated that polyamines themselves do not induce trichothecene biosynthesis when added at µM concentrations to a complex medium. As the yeast extract contains various amino acids as major nitrogen sources, polyamine catabolism may not have been activated in YG_60 culture. To exclude the possibility of transformed products of polyamines working as trichothecene biosynthesis inducers, it is necessary to examine the effect of catabolizing agmatine as the sole nitrogen source present in the medium.

Tri genes are strongly induced in L-Gln culture, in which medium pH is frequently adjusted to the pH of agmatine culture

Figure 2 shows trichothecene production and pH profile of *F. graminearum* cultured on synthetic media containing 5 mM each of L-Gln or agmatine as the sole nitrogen source. The initial pH of both cultures (pH 4.2) remained at nearly the same level for 24 h after inoculation of the YG pre-culture to the synthetic media. After this time point, the pH of the agmatine culture gradually decreased and reached < pH 2.5. In contrast, the pH of the L-Gln culture first increased, then sharply decreased, and finally reached a plateau at approximately pH 3.5, as previously observed (Shiobara et al. 2019). Such a difference in pH profiles raised the possibility that the higher pH of the L-Gln culture affected the final trichothecene yield. We thus monitored pH levels in the agmatine culture as a reference, and the pH of the L-Gln culture was frequently adjusted to the pH of the agmatine culture. As speculated, expression of *Tri6* and earlier pathway genes, *Tri5* and *Tri4*, under such culture conditions was as high as that observed in the agmatine culture, but expression of these *Tri* genes was much lower in L-Gln culture without pH adjustments (Fig. 2; inset). These results suggested that L-Gln may also be used as a sole nitrogen source in culture medium for effective fermentation of trichothecenes, if the pH profile of the culture is manually maintained at levels similar to that in the agmatine culture medium.

Trichothecene productivity of *F. graminearum* grown on synthetic L-Gln medium is comparable to that on agmatine medium

To prove that agmatine does not specifically stimulate trichothecene production, we repeated the above pH adjustment experiments so that the amount of trichothecene could be evaluated statistically. In each experiment, media containing nitrate, consumption of which *F. graminearum* requires the active form of FgAreAp, were also included for comparison. The culture pH of the L-Gln and nitrate media was not adjusted (L-Gln (−) and

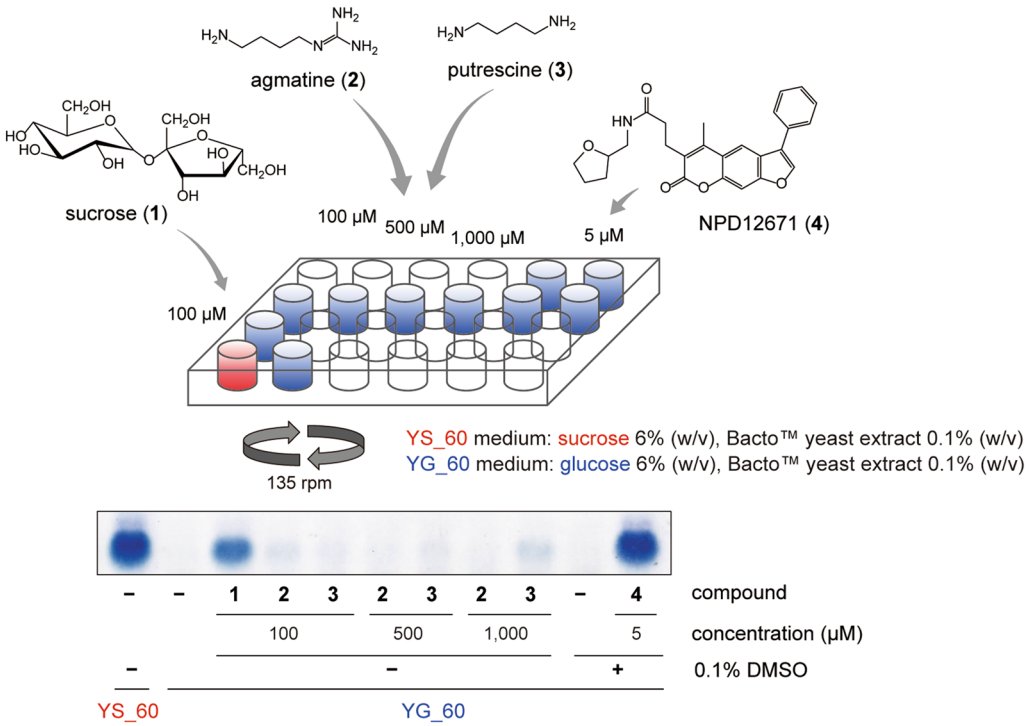

Fig. 1 Effects of small molecule chemicals on trichothecene biosynthesis induction as examined using 24-well plate assays. One milliliter aliquots of germinating conidia in trichothecene-non-inducing YG_60 medium (1% [v/v] inoculation of YG pre-culture) were distributed to wells in a 24-well plate and supplemented with sucrose (**1**), agmatine (**2**), putrescine (**3**), and NPD12617 (**4**) at concentrations indicated in the figure. Dimethylsulfoxide (DMSO) was used as a solvent for **4**. Toxin synthesis-inducing YS_60 medium was used as a reference for comparison. After 3 days of incubation with gyratory shaking at 135 rpm and 25 °C, metabolites from 0.5 mL of the cultures were analyzed by TLC. The spots of 15-ADON visualized by the NBP/TEPA color reaction are shown

NO_3^- (−), respectively) or frequently adjusted to the pH value of the reference agmatine culture by adding 0.5 N HCl solution (L-Gln (+) and NO_3^- (+), respectively). The culture was fermented for 6 days, during which period the pH decreased to pH 2.5 and below. In triplicate experiments, there were no statistically significant differences in the productivity of 15-ADON/DON (µg toxin/mg fungal mass) between the agmatine culture and the L-Gln (+) culture (Fig. 3). These results demonstrated that the constant reduction in pH, which is characteristic of culture in agmatine medium, triggered production of trichothecenes. In this context, agmatine, including its metabolized products, is not a specific inducer molecule of trichothecene biosynthesis in the strict sense of the term. In addition to comparison with the L-Gln (+) culture, no statistically significant differences were detected when trichothecene production in agmatine culture was compared to NO_3^- (+) culture. These results suggested that the gradual pH decrease observed naturally for culture in agmatine determines the productivity of trichothecene synthesis.

Timing of acidification of fungal culture is important for induction of trichothecene biosynthesis

To examine whether the timing of acidification of the culture medium is important for trichothecene production, the nitrate culture pH was abruptly shifted to 2.5 after incubation for 0, 24, 48, 72, and 96 h by adding HCl (Fig. 4a). Trichothecene was extracted from the culture medium at 48, 120, 168, and 240 h of incubation and visualized by TLC. While the pH of the nitrate culture remained constant after shifting to 2.5, the final trichothecene yield differed considerably depending on the time point of the pH shift (Fig. 4a). The total amount of 15-ADON (including a small amount of DON) in the nitrate medium was highest when the pH was shifted after 24 h of incubation, and trichothecene could not be detected with the pH shift later than 48 h.

To establish a more accurate relationship between the timing of medium acidification and the amount of trichothecenes synthesized, the culture pH was abruptly shifted to 2.5 after 0, 16, 20, and 24 h of incubation in

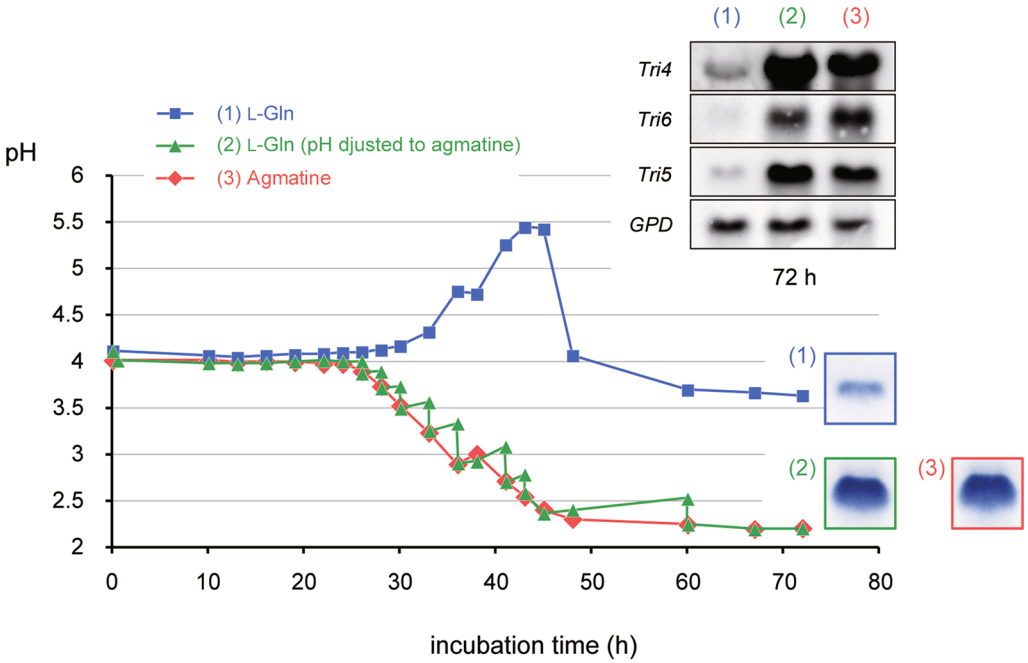

Fig. 2 Effects on trichothecene biosynthesis of frequently adjusting the pH of L-Gln cultures to that of agmatine cultures. The pH of the L-Gln medium (1) was frequently monitored and manually adjusted (2) to that of the agmatine medium (3). Total RNA was isolated from *F. graminearum* at 72 h and used for northern blot analysis with *Tri* and *GPD* probes. Metabolites from 0.5 mL of each culture were analyzed by TLC after 72 h of incubation

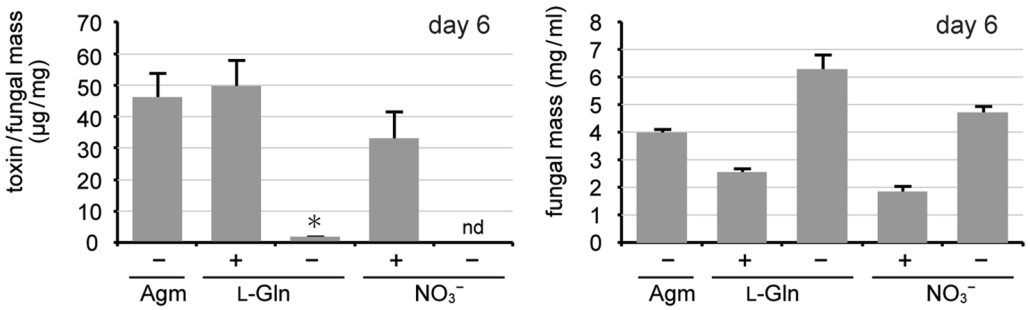

Fig. 3 Comparison of trichothecene productivity of *F. graminearum* on L-Gln and nitrate media with [L-Gln (+), NO_3^- (+)] or without [L-Gln (−), NO_3^- (−)] adjustments of the culture pH to that of the agmatine medium [Agm (−)]. The pH of the L-Gln (+) and NO_3^- (+) culture was adjusted every 2–3 h, as demonstrated in Fig. 2, throughout the incubation period. Values of toxin/fungal mass (15-ADON plus DON per mycelial dry weight) for three independent experiments, each using independent pre-cultures as an inoculum, are shown. An asterisk indicates a significant difference from the control Agm (−) culture (*$P < 0.05$, ANOVA followed by Dunnett's multiple comparison test). The value of the NO_3^- (−) culture was excluded from the statistical analysis, as trichothecene was not detected (nd). Values of fungal mass are also shown as a reference for growth

the nitrate medium. The culture was incubated for a total of 240 h, and the amount of 15-ADON/DON was quantified. When the pH shift was applied at or before 20 h of incubation, the nitrate culture exhibited higher trichothecene productivity (μg toxin/mg fungal mass) than the control agmatine culture used as a reference for comparison (Fig. 4b; left panel). Trichothecene productivity statistically exceeded that of the agmatine culture when the nitrate medium was adjusted to pH 2.5 at the beginning of the culture ($t = 0$), although the fungal mass was considerably lower (Fig. 4b; right panel).

Culture pH profiles explain the inability of Δ*FgareA* mutant to produce trichothecene in L-Gln culture

To understand the effects of the *FgAreA* deletion on secondary metabolism, the pH levels of Δ*FgareA* mutant cultures were monitored throughout the incubation period (Fig. 5a).

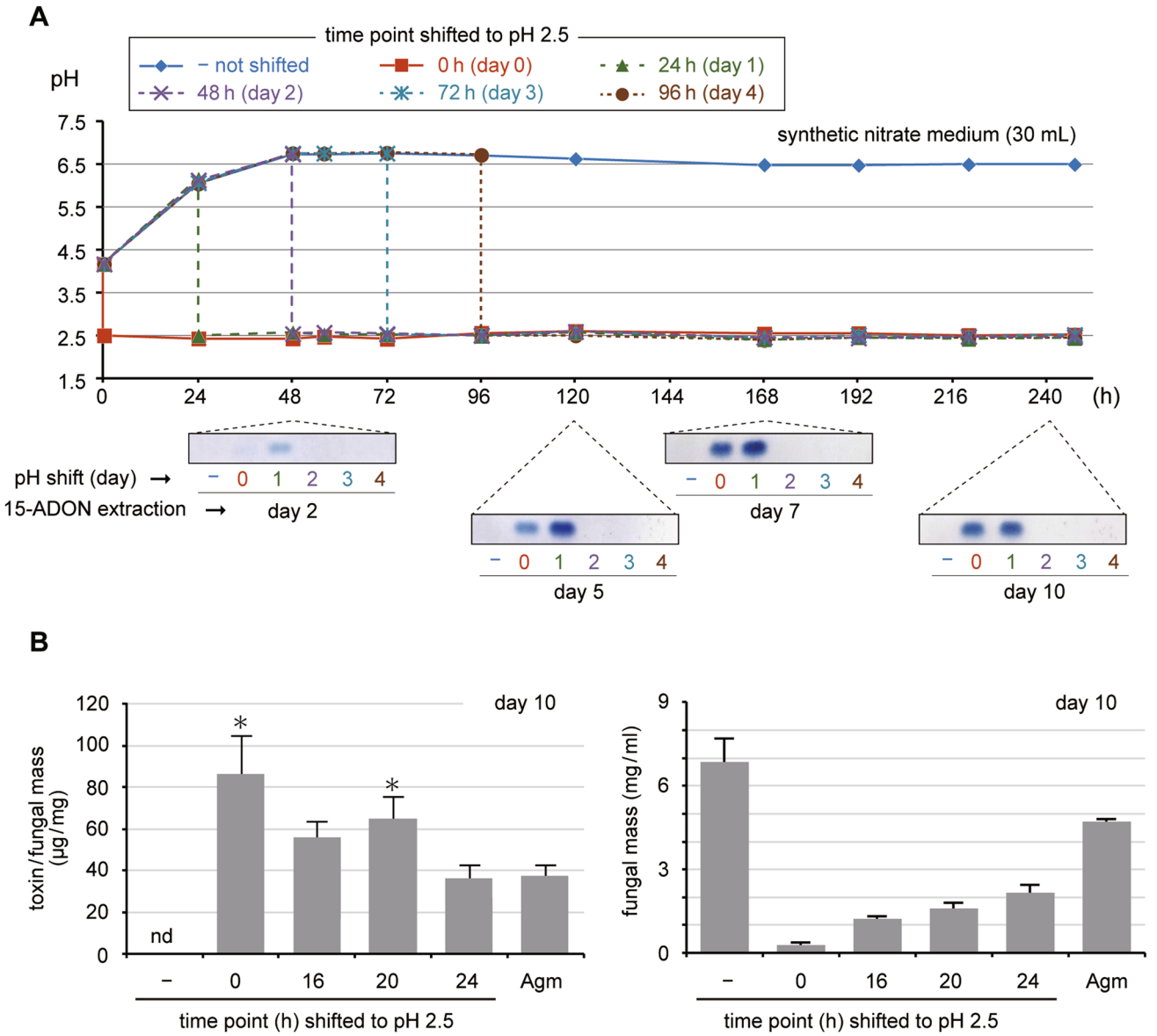

Fig. 4 Effect of timing of abrupt pH shift of *F. graminearum* culture to pH 2.5 on trichothecene production. The agmatine culture (Agm) was used for comparison. **a** Adjustments of the pH of the fungal culture using HCl. The pH of the nitrate medium was shifted to pH 2.5 at the beginning of culture (day 0) and after incubation for 24, 48, 72, and 96 h (days 1, 2, 3, and 4, respectively). As a negative control, the pH of the culture was not adjusted (−). The fungal metabolites included in the culture (0.5 mL) at days 2, 5, 7, and 10 were extracted with ethyl acetate and analyzed by TLC. **b** Trichothecene (15-ADON/DON) productivity (toxin/fungal mass) of the WT culture when the pH shift was applied at earlier time points. The culture was shifted to pH 2.5 at the beginning of incubation (0 h) and after continued culture for 16, 20, and 24 h. As a negative control, the pH of the nitrate culture was not adjusted (−). The amount of trichothecene was quantified 10 days after inoculation. For the evaluation of trichothecene productivity, an agmatine culture was analyzed in parallel for each experiment. The experiment was performed in triplicate, each replicate using the same pre-culture as an inoculum. An asterisk indicates a significant difference from the control agmatine culture at the 5% level by Dunnett's multiple comparison test ($n=3$)

After germination, immature mycelia of the Δ*FgareA* mutants were inoculated into L-Gln medium. It was noted that the pH changes as a function of time were quite different from the changes observed for the WT strain. While the pH of the WT culture first increased to approximately pH 5.3, sharply decreased below pH 4, and ultimately reached a final pH of approximately 3.5, the Δ*FgareA* mutants displayed a higher pH peak after incubation for a longer period ($t=62$ h); after which the peak gradually decreased to approximately pH 4 (Fig. 5a; compare plots 1, 2, and 3). To eliminate the effects of pH on trichothecene synthesis, we tried to shift the pH peak of the Δ*FgareA* mutants to an earlier time point by increasing the inoculum size by 16-fold. Although the timing of pH increase was synchronized by

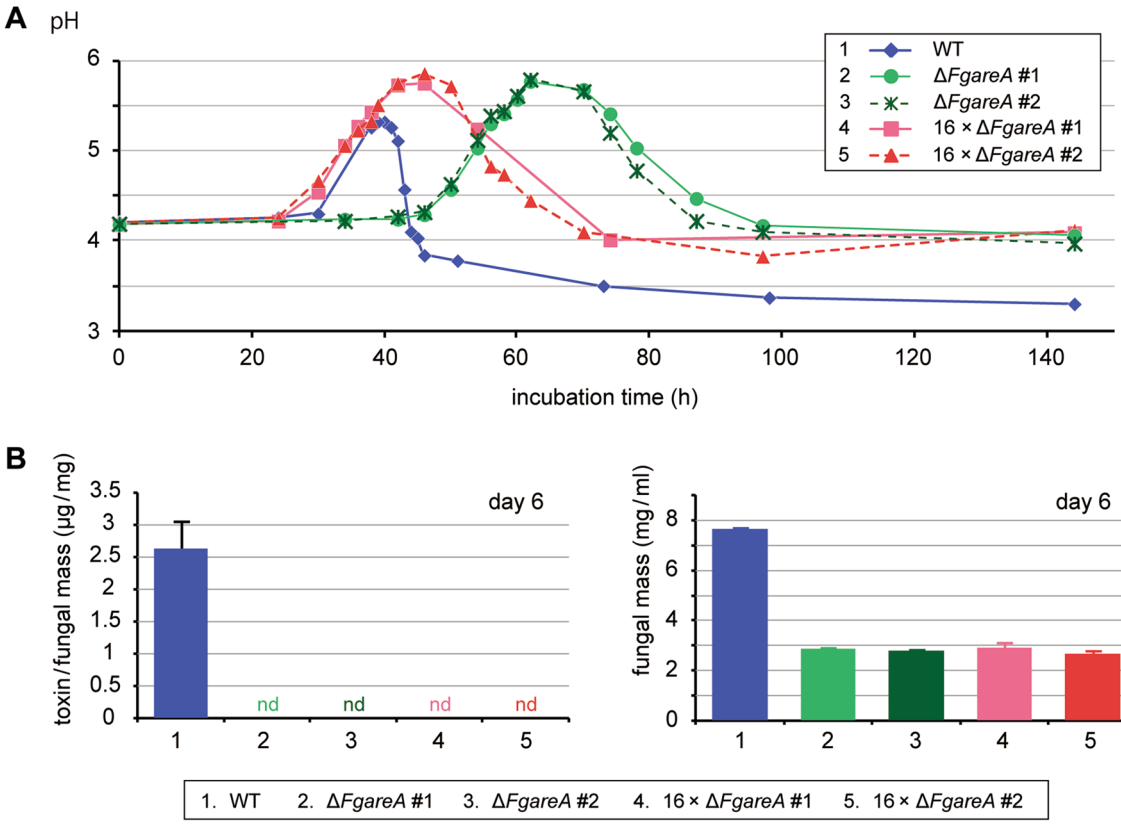

Fig. 5 Effect of *FgAreA* deletion on trichothecene productivity by *F. graminearum* grown on L-Gln medium. **a** pH profiles of the WT and Δ*FgareA* mutant cultures. WT (plot 1), Δ*FgareA* #1 (plot 2), and Δ*FgareA* #2(plot 3) strains were cultured on L-Gln medium, as described in "Materials and methods". As the growth rates of the mutants were considerably slower than that of the WT, the inoculum size was increased by a factor of 16 with Δ*FgareA* #1 (plot 4) and Δ*FgareA* #2 (plot 5). **b** Trichothecene (15-ADON/DON) productivity (toxin/fungal mass) of the WT and Δ*FgareA* mutant cultures on L-Gln medium. Fungal mass and metabolites of each culture were quantified 6 days after inoculation. The experiment was performed in triplicate, each replicate using the same pre-culture as an inoculum

increasing the inoculum size (Fig. 5a; plots 4 and 5), the overall culture pH profile values were higher than those of the WT strain throughout the incubation period. Most notably, the final pH of the culture remained at approximately pH 4. Consistent with previous reports (Min et al. 2012), trichothecene was not detected in the culture medium of the Δ*FgareA* mutants (Fig. 5b).

Effects of mutating all fifteen AreA-binding consensus sequences in the *Tri6* promoter on trichothecene biosynthesis

Although the Δ*FgareA* mutant could grow on L-Gln medium, its metabolic conditions were considerably different from that of the WT strain, as reflected by the culture pH profiles and fungal mass yield. In order for the mutation not to affect growth of the fungus, regulatory genes involved in primary metabolism (i.e., *FgAreA*) must remain intact, whereas the target *cis*-elements involved in regulation of secondary metabolism (i.e., the *Tri6* promoter) should be mutated. For this purpose, we generated an AreA-binding site mutant, in which all fifteen AreA-binding consensus sequences in the *Tri6* promoter, 5′-GATA-3′, were mutated to 5′-GGTG-3′ (Fig. 6a), as described in "Materials and methods" (Figs. S1 and S2). We examined the trichothecene productivity of three self-cloning strains derived from a ΔP_{Tri6} #1 *hph::tk* transformant; P_{Tri6}/m15 #1 and #2 (*hph::tk* replaced with the mutated *Tri6* promoter) and P_{Tri6}/m0 #1 (*hph::tk* replaced with the wild-type *Tri6* promoter). When cultured on trichothecene synthesis-inducing YS_60 medium distributed among the wells of 24-well plates (Nakajima et al. 2016), P_{Tri6}/m15 #1 and #2 produced 15-ADON/DON in an amount similar to that of the WT and P_{Tri6}/m0 strains (Fig. 6b). In addition, the promoter mutant strains responded to a known trichothecene production activator, NPD12671 (**4**), and accumulated a greater quantity of 15-ADON/DON, as was the case with the WT and P_{Tri6}/m0 strains (Fig. 6b). These results suggested that the mutated *Tri6* promoter functions properly in fungal cell culture utilizing YS_60 complex medium.

Distinct from the Δ*FgareA* mutant, the *Tri6* promoter mutant was able to use all the nitrogen sources catabolizable

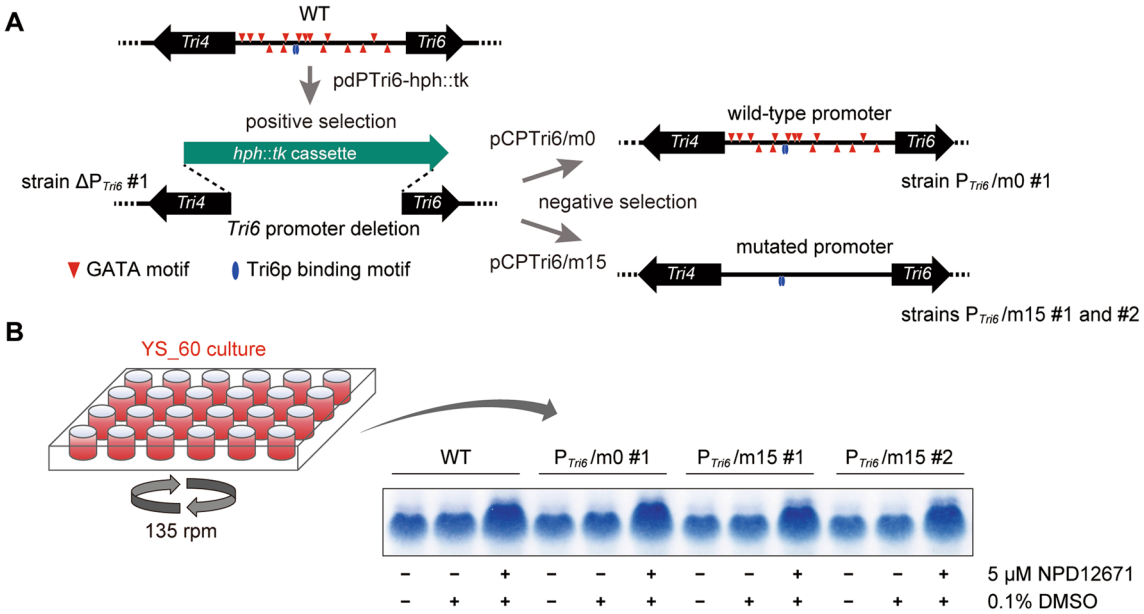

Fig. 6 Effects of mutating the consensus AreA-binding sequences in the *Tri6* promoter of *F. graminearum*. **a** Strategy of generating self-cloning strains P_{Tri6}/m0 and P_{Tri6}/m15, containing wild-type and mutated *Tri6* promoters, respectively. P_{Tri6}/m0 and P_{Tri6}/m15 strains were obtained by negative selection of a single transformant, ΔP_{Tri6} #1, following transformation with plasmids pCPTri6m0 and pCPTri6m15, respectively. **b** Trichothecene biosynthesis as examined using 24-well plate assays. The WT and self-cloning strains were cultured on 1 mL of toxin synthesis-inducing YS_60 medium in the presence or absence of **4**. The culture was incubated for 3 days with gyratory shaking. Each TLC lane contains metabolites from 0.5 mL of the culture extracted with ethyl acetate. DMSO was used as a solvent for **4**

by the WT strain. This enables assessing the effects of using L-Phe, which the Δ*FgareA* mutant cannot grow with as the sole nitrogen source, on trichothecene biosynthesis. When the promoter mutant strains P_{Tri6}/m15 #1 and #2 were cultured on synthetic L-Phe medium, the pH profile and fungal mass were similar to those of the WT and P_{Tri6}/m0 strains (Fig. 7a). However, the mutants exhibited approximately 72% (average of 68% and 76%) the productivity of 15-ADON/DON compared to the WT and wild-type self-cloning strains (Fig. 7b). In this manner, the AreA-binding

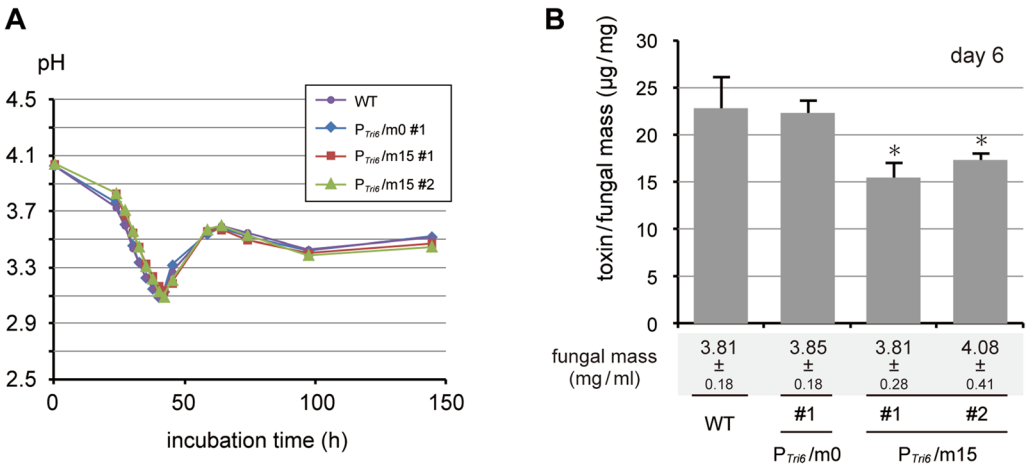

Fig. 7 pH profiles and trichothecene (15-ADON/DON) productivity of the WT and *Tri6* promoter self-cloning strains P_{Tri6}/m0 and P_{Tri6}/m15 grown on L-Phe medium. **a** The pH of the L-Phe culture. The WT and self-cloning strains exhibited the same pH profile. **b** Fungal mass and metabolites of each culture were quantified 6 days after inoculation. The experiment was performed in triplicate, each replicate using the same pre-culture as an inoculum. Asterisks indicate significant differences from the control WT culture at the 5% level by Dunnett's multiple comparison test ($n = 3$)

consensus sequences on the *Tri6* promoter contributed to trichothecene production, but were not essential under the culture conditions using L-Phe as the sole nitrogen source.

Evaluation of role of FgAreAp in trichothecene biosynthesis under extremely acidic conditions

As trichothecene production was strongly induced when the culture was shifted to pH 2.5 (Fig. 4), we next questioned to what extent FgAreAp contributes to induction of trichothecene biosynthesis. Pre-cultures of the WT and self-cloning strains were inoculated into synthetic L-Phe medium maintained at pH 2.5, and the amounts of trichothecenes generated were analyzed after 6 days of incubation. In contrast to cultures in L-Phe medium without pH adjustment, there were no significant differences in the toxin/fungal mass values among the four strains (Fig. 8a); the WT parent strain, wild-type *Tri6* promoter self-cloning strain P_{Tri6}/m0 #1, and mutated *Tri6* promoter strains P_{Tri6}/m15 #1 and #2, produced 89 ± 8.8, 97 ± 1.3, 79 ± 3.1, and 77 ± 4.5 μg/mg fungal mass of 15-ADON/DON, respectively, in the liquid culture. Trichothecene productivity of the Δ*FgareA* strain was also comparable to that of WT when cultured on L-Gln medium maintained at pH 2.5 (Fig. 8b), supporting the theory that the contribution of FgAreAp to induction of trichothecene biosynthesis is marginal at extremely low pH.

Discussion

In this study, we stressed the importance of the culture pH in assessing the roles of nutritional and genetic factors in trichothecene biosynthesis. When included as the sole nitrogen source at 5 mM in the synthetic medium, agmatine was a more suitable nutrient than L-Gln for production of large quantities of trichothecene. However, on frequent pH monitoring and adjustment so that the pH profile of the L-Gln culture could be superimposed on that of the agmatine culture, the trichothecene productivity was found to be comparable (Fig. 2). In another experiment using the toxin-non-inducing nitrate medium, the abrupt medium acidification to pH 2.5 led to activation of trichothecene biosynthesis (Fig. 4). The pH shift was effective only when it was applied during an early growth stage in the nitrate culture. Thus, the gradual culture pH decrease to pH 2.5 and below, as observed naturally for the agmatine culture (Fig. 2), represents a compromise between fungal mass generation (i.e., preference for neutral to slightly acidic condition) and trichothecene productivity (i.e., preference for extremely low pH at an early growth stage), and appears to be an optimal condition for accumulation of a large amount of trichothecene in liquid culture medium. In consistent with this theory, agmatine failed to induce trichothecene production when the liquid cultures were maintained at pH 4, pH 5.5, and pH 7 (Fig. S4). Together with the inability of agmatine and putrescine to activate trichothecene biosynthesis in YG_60 medium even at 1000 μM (Fig. 1), which is far beyond the concentrations necessary for the induction by sucrose and NPD12671, polyamines are not suitable to be defined as inducers.

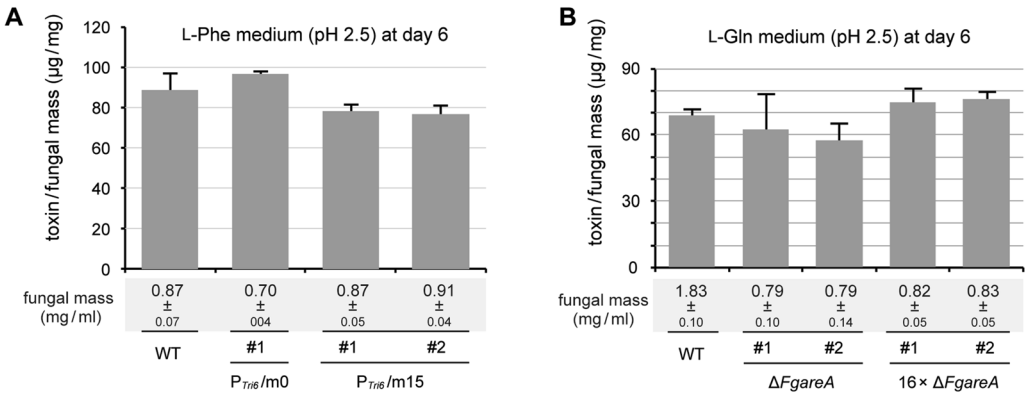

Fig. 8 Trichothecene (15-ADON/DON) productivity under extremely acidic conditions. The experiment was performed in triplicate, each replicate using the same pre-culture as an inoculum. **a** The effect of mutating all fifteen AreA-binding consensus sequences in the *Tri6* promoter on trichothecene productivity. The WT and *Tri6* promoter self-cloning strains were grown on L-Phe medium and constantly maintained at pH 2.5. **b** The effect of mutating *FgAreA* on trichothecene productivity. The WT and Δ*FgareA* mutant strains (the same inoculum size as that of WT and inoculum size increased by a factor of 16) were grown on L-Gln medium and constantly maintained at pH 2.5. In either experiment, metabolites of each culture were quantified 6 days after the inoculation. No significant differences in toxin/fungal mass values were detected between the control WT and self-cloning and/or mutant strains at the 5% level by Dunnett's multiple comparison test ($n = 3$)

We also re-examined the role of FgAreAp in activation of trichothecene biosynthesis. As L-Gln was the only amino acid able to be catabolized by the Δ*FgareA* mutant of JCM 9873, synthetic L-Gln medium was used to evaluate the role of FgAreAp in induction of trichothecene biosynthesis. However, the growth rate of the Δ*FgareA* mutant was considerably lower, and the pH profile of the L-Gln culture could not be superimposed on that of the WT by increasing the inoculum size (Fig. 5a). In order for the mutation not to affect the growth of the fungus, we used self-cloning strains with all fifteen AreA-binding consensus sequences in the *Tri6* promoter mutated. The possibility of transformation-mediated illegitimate mutations affecting trichothecene production was assessed by analyzing two independent *Tri6* promoter mutants, P_{Tri6}/m15 #1 and #2, and a wild-type *Tri6* promoter strain, P_{Tri6}/m0 #1. The trichothecene levels and regulatory patterns of these self-cloning strains on YS_60 complex medium were the same as those of the parental WT strain, thus excluding the aforementioned possibility. This result also suggested that FgAreAp did not contribute to trichothecene biosynthesis activation when the fungus was grown on YS_60 with the 24-well plate culture method. Next, trichothecene productivity of the promoter mutants was investigated on synthetic L-Phe medium. In contrast to the Δ*FgareA* mutant, the promoter mutants P_{Tri6}/m15 #1 and #2 could grow on L-Phe as the sole nitrogen source, as did the WT and P_{Tri6}/m0 #1 strains, which allowed evaluation of the contribution of fully activated FgAreAp to trichothecene biosynthesis. The fact that trichothecene productivity of the promoter mutant was reduced to some extent (Fig. 7b) clearly demonstrates the importance, but also dispensability, of FgAreAp for trichothecene production.

Although pH profiles are critical factors in the regulation of trichothecene biosynthesis, previous studies did not consider the importance of pH changes associated with nitrogen metabolism, and interpreted the overall differences between the WT and Δ*FgareA* mutant cultures as being directly attributable to the binding of FgAreAp to its target sequences. In this context, the contribution of the AreA-binding consensus sequences demonstrated in this study represent the first circumstantial evidence for the involvement of FgAreAp in regulation of *Tri* gene expression.

In conclusion, we questioned previous understanding of the roles of agmatine and FgAreAp in activation of trichothecene biosynthesis. From our results, nitrogen metabolism-associated culture pH changes were shown to considerably affect the onset of secondary metabolism. While agmatine was not an inducer of trichothecene biosynthesis in the strict sense of the term, *FgAreA* was suggested to be involved in trichothecene biosynthesis under certain conditions. However, extremely low culture medium pH had the greatest impact on trichothecene productivity beyond the nitrogen source and FgAreAp transcription factor contributions (Fig. 8).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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