Molecular and genetic studies of *Fusarium* trichothecene biosynthesis: toward understanding the transcriptional regulatory mechanisms of trichothecene (*Tri*) genes

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Abstract

To gain insights into the regulatory mechanism of trichothecene biosynthesis, I worked on molecular genetic studies of *Fusarium graminearum* in RIKEN and Nagoya University. First, *Aspergillus nidulans* promoters with different levels of transcription were identified and characterized in *F. graminearum*. Second, subcellular localization of Tri6p, a zinc finger protein essential for trichothecene (*Tri*) gene transcription, was analyzed by using these promoters. When the predicted nuclear localization sequence (NLS) of Tri6 was fused to the *EGFP* (enhanced green fluorescence protein) gene and transcribed under the control of a strong *A. nidulans* TEF1α promoter, the *EGFP* green fluorescence was clearly observed in the nucleus, demonstrating the functionality of the nuclear localization signal. Third, the role of sucrose as an inducer, but not as the carbon source of the medium, in trichothecene production was unambiguously demonstrated. Based on the mode of actions of sucrose and other chemicals that modulate trichothecene production, a new regulatory model of Tri6 expression is now being proposed. Further investigations with molecular and mechanisms of genetic approaches using these tools will clarify the trichothecene biosynthesis regulation.

Introduction

*Fusarium graminearum* is a causal agent of *Fusarium* head blight (FHB) which is occurring worldwide with an increasing incidence. FHB, a devastating disease of wheat and barley, not only brings about a poor harvest of cereal grains of these important crops, but also mycotoxin contamination, due to the ability of the fungus to produce trichothecenes\(^1\). Trichothecene mycotoxins, often contaminating the grains, are the protein synthesis inhibitors of eukaryotes\(^2\). To secure the food safety, elucidation of regulatory mechanism of trichothecene production is important.

*Fusaria* belonging to the *F. graminearum* species complex produce type-B trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV)\(^3,4\). The trichothecene genes, *Tri* genes, are clustered in the genome of *F. graminearum*\(^5\). Among the *Tri* genes, Tri5 encodes a sesquiterpene cyclase that catalyzes cyclization of farnesyl pyrophosphate to trichodiene (TDN)\(^6\). *FgTri4* (*F. graminearum* Tri4) encodes a cytochrome P450 monooxygenase responsible for the four consecutive TDN oxygenation steps\(^7\), and Tri6 and Tri10 are important regulatory proteins that play indispensable roles for expression of pathway *Tri* genes\(^8\). However, the detailed regulatory mechanisms of trichothecene production are not yet elucidated.

Some environmental factors affect *Fusarium* trichothecene biosynthesis. For example, acidic pH strongly stimulates trichothecene production\(^9,10\). Carbon source of the medium is also known as a critical factor for the biosynthesis of trichothecenes; sucrose, but not glucose, was thought to be an important carbon source of the medium for production of trichothecenes in axenic liquid culture\(^11\).

Development of promoter tools for molecular genetic studies of *F. graminearum*

To develop molecular genetic tools for *Fusarium*, promoters of translation elongation factor 1-alpha (*TEF1α*), glyceraldehyde 3-phosphate dehydrogenase (*GPD*), polyubiquitin (*UBI*), and β-tubulin (*TUB*) genes, were cloned from *Aspergillus nidulans*. The activities of
these heterologous promoters were analyzed in *F. graminearum* by using the β-glucuronidase (GUS) gene as a reporter. The promoter-GUS constructs were integrated into the Tri14 coding region, located at the end of the Tri gene cluster, by double crossover homologous recombination (Fig. 1A). All of the four promoter fragments were functional and showed varying levels of transcription in *F. graminearum* \(^{(2)}\). The TEF1α promoter showed by far the highest level of expression, followed by the GPD, UBP, and TUB promoters (Fig. 1B). These promoters are suitable for fine-tuning of the transcription of foreign genes in *F. graminearum*.

**Characterization of Tri6p**

*Tri6* encodes a C\(_2\)H\(_2\) zinc finger transcription factor that is indispensable for transcription of *Tri* genes \(^{(1)}\). Tri6p recognizes the "YNAGGCC" consensus binding sequence in promoters of target genes \(^{(4)}\). To understand the mechanism by which Tri6p functions in *Tri* gene expression, I tried to characterize subcellular localization of Tri6p under trichothecene-inducing (YS_60 medium comprising of 0.1% yeast extract and 175 mM sucrose) and non-inducing (YG medium comprising of 0.5% yeast extract and 111 mM glucose) conditions. For this purpose, an enhanced green fluorescent protein gene (EGFP) was C-terminally fused to the coding region of *Tri6* (*Tri6*:EGFP). When a strong TEF1α promoter was connected to *Tri6*:EGFP and introduced into a *Tri6* disruption mutant, the resulting transformant restored trichothecene production on YS_60 medium, demonstrating the functionality of Tri6p:EGFP as a transcription factor. However, the EGFP fluorescence could not be detected. On trichothecene non-inducing YG medium, the overexpressor did not produce trichothecenes, although accumulation of Tri6p:EGFP fusion protein was greater in comparison with the YS_60 culture (Fig. 2A). Thus, post-translational activation step(s) are required for Tri6p to function as a transcription factor, and this functionalization event does not occur under the culture conditions with the YG medium.

Tri6p contains a predicted nuclear localization sequence (NLS) in the zinc finger (ZF) domain. To verify functionality of the predicted NLS, a DNA fragment corresponding to the ZF domain was fused to EGFP (*Tri6*ZF::EGFP) and expressed under the control of the *PTEF* promoter in the *Tri6* deletion mutant. The transgenic strain showed a clear EGFP fluorescence in the nucleus in both YG and YS_60 medium (Fig. 2B). Thus, the predicted NLS functions as a nuclear transport signal in the absence of Tri6p activation domain \(^{(5)}\).

**Sucrose and related oligosaccharides as inducer molecules of trichothecene biosynthesis**

Although sucrose was widely used as the carbon source of the medium for trichothecene production \(^{(1)}\), \(^{(11)}\), \(^{(16)}\), \(^{(17)}\), it seems questionable that catabolism of sucrose, but not of glucose and fructose, specifically stimulates trichothecene synthesis. To examine whether sucrose is a trichothecene-inducing small molecule that is effective at μM concentrations, the *F. graminearum* pre-culture was transferred to YG_60 liquid medium (comprising of 0.1% yeast extract and 333 mM glucose) supplemented with low doses of sucrose. Under the optimized culture condition with a 24-well culture plate, 100 μM of sucrose in the YG_60 medium induced trichothecene production. To gain further insight into the role of sucrose in inducing *Tri* gene expression, time course of *Tri6* and *Tri5* expressions were examined in the presence and absence of this disaccharide. When YG_60 medium was supplemented with 100 μM of sucrose, the concentrations of *Tri6* and *Tri5* transcripts increased after 36 h of incubation. However, expressions of these transcripts decreased by day 2, and became marginal with longer incubation periods, presumably due to the cleavage of sucrose into glucose and fructose. These results indicate that sucrose catabolism is not a prerequisite for trichothecene production and that a small amount of sucrose is sufficient for the induction.

In addition to sucrose, 1-kestose increased the amount of trichothecene when added to the YG_60 culture at 100 μM (Fig 3). Thus, fructo-oligosaccharides, β-(2→1)-linked polymers of D-fructose containing an
Fig. 2  Structure and functionality of Tri6p fusion protein
(A) Characterization of Tri6p::EGFP in mycelia grown on YG and YS_60 medium. Accumulation of fusion protein in fungal cells was analyzed by western blot with anti-GFP antibody. (B) Function of predicted NLS located in ZF domain of Tri6p. EGFP fluorescence of DAPI-stained hyphae demonstrated that Tri6p_ZF::EGFP was localized to nucleus.

Fig. 3  Structures and activities of trichothecene production-inducing oligosaccharides
Each oligosaccharide was added at a concentration of 100 μM to the glucose-based YG_60 medium.

α-(1→2)-linked glucose unit, also work as an inducer of trichothecene biosynthesis. In addition, raffinose and xylosucrose, whose single fructosyl residue is linked to melibiose and xylose, respectively, by an α-(1→2)-glycosidic bond, also induced trichothecenes at 100 μM in YG_60 culture. These results unambiguously demonstrate that the minimum structure of sugars necessary to act as a trichothecene inducer molecule contains α-(1→2) (glucosyl/xylosyl)-fructosyl linkages, and that carbon source of the media may be glucose if only the inducing sugars are included at a low concentration\(^{18}\).

Mechanism of Tri6 expression
From the RIKEN NPDepo chemical library, our group identified small molecule compounds that modulate trichothecene biosynthesis without affecting fungal growth when supplemented at concentrations of 1–10 μM\(^{19}\). The representative chemicals analyzed so far include NPD12671, a synthetic furanocoumarin, and dihydroartemisinin (DHA), an anti-malarial artemisinin analog, which activated and repressed Tri6 transcription, respectively (Fig. 4). Ethyl acetate-extractable Maillard reaction products that are present in yeast extract-based medium proved to inhibit trichothecene production\(^{20}\). By generating various transgenic strains, such as gene and promoter mutants of the nitrogen regulatory AreA\(^{21,22}\), overexpressor strains of Tri6 and Tri10, and disruption and suppressor mutants of PKA, studies on the mode of actions of these trichothecene production modulators gave invaluable keys in understanding the regulatory mechanism of Tri6 expression. Based on our results, pervious Tri6 transcription regulation models\(^{23}\) will be revised in the near future\(^{24}\).

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Fig. 4 A model of transcriptional regulation of Tri6

NPD12671 and DHA modulate Tri6p-independent initial activation of Tri6 expression. Sucrose and DHA appear to influence Tri6p-dependent activation of Tri6 expression.

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