Characterization of the acivicin effects on trichothecene production by *Fusarium graminearum* species complex

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Some *Fusarium* species produce trichothecene mycotoxins under certain growth conditions (Desjardins, 2009; Kimura et al., 2007). *Fusarium graminearum* species complex (O’Donnell et al., 2004) is an important pathogen of wheat, barley, and maize, and can accumulate type B trichothecenes, such as deoxynivalenol (DON) and nivalenol (NIV), in infected grains. To reduce the risks of mycotoxin contamination, various attempts have been undertaken to develop inhibitors of trichothecene biosynthesis. Trichothecene production inhibitors are divided into two major types; those that inhibit biosynthetic enzymes (Desjardins et al., 1988; Hesketh et al., 1993; Nakajima et al., 2013; Takahashi-Ando et al., 2008) and those that inhibit biosynthetic gene expression (Boutigny et al., 2009; Etzerodt et al., 2015; Yaguchi et al., 2009; Yoshinari et al., 2008). Inhibitors of both types have been isolated and characterized (Sakuda et al., 2016), but none have been used in practice in the field.

Acivicin is an inhibitor of glutamine amidotransferase, which constitutes one of the catalytic domains of enzyme complexes involved in various metabolic activities, including the biosynthesis of certain amino acids, nucleotides, and essential nutrients (Massiere and Badet-Denisot, 1998). We previously found that acivicin inhibits trichothecene accumulation by *F. graminearum* JCM 9873, a 15-acetyldeoxynivalenol (15-ADON) chemotype, when cultured in moderately trichothecene-inducing YS_60 medium (Maeda et al., 2014). To determine whether the suppressive effect of acivicin on trichothecene production was specific to the strain JCM 9873, we first investigated its activity by using different *F. graminearum* strains under the same pre-culture and culture conditions (see Table S1 for medium composition). As shown in Fig. 1, the acivicin (LKT Laboratories, Inc., St. Paul, MN; lot number 28422001) used in a previous study (Maeda et al., 2014) also inhibited trichothecene production by MAFF 240560 (strain ZEA1), a 3-acetyldeoxynivalenol (3-ADON)/DON chemotype, and MAFF 240548 (strain NIV2), a NIV chemotype, without significant growth inhibition. The differences in the amount of trichothecene accumulation between control and acivicin-treated mycelia became smaller with a prolonged incubation period, as was the case with strain JCM 9873 (Maeda et al., 2014). At day 6, the difference in the amount of trichothecenes was especially small with strain MAFF 240548, although it was statistically significant. Considering that complete inhibition of trichothecene production without affecting fungal growth is difficult to achieve (Boutigny et al., 2009; Etzerodt et al., 2015; Yaguchi et al., 2009; Yoshinari et al., 2008), the result suggests that acivicin exerts its action to various *F. graminearum* strains under the aforementioned culture conditions.

Trichothecene biosynthesis is triggered by the transcriptional activation of Tri6 encoding a Cys₃His₂ zinc finger...
Acivicin effects on mycotoxin production

Fig. 1. Inhibition of trichothecene production by acivicin in strains (A) MAFF 240560 (strain ZEA1; 3-ADON/DON chemotype) and (B) MAFF 240548 (strain NIV2; NIV chemotype). Difco™ yeast extract lot number 3254027 was used for the preparation of the YS_60 medium. Mycelial dry weight (MDW) at the end of the culture is shown at the bottom of the graph. The concentrations of 3-ADON and DON (not detected during the incubation period) were determined using HPLC as described previously (Maeda et al., 2014). 4-Acetylnivalenol (4-ANIV) and 4,15-diacetylnivalenol (4,15-diANIV) were separated by sequential elution using a PEGASIL ODS column (diameter, 4.6 mm; length 250 mm; Senshu Scientific Co., Tokyo, Japan), with 8 ml of 25% (v/v) acetonitrile/water, 2 ml of a linear gradient of 20–35% acetonitrile, and 10 ml of 35% acetonitrile, at a flow rate of 1.0 ml/min at 40°C. The concentrations of 4-ANIV and 4,15-diANIV were calculated from the peak areas of the chromatogram by applying the calibration curves obtained using 4-ANIV (fusarenon-X; Wako Pure Chemical Industries Ltd., Osaka, Japan) and 4,15-diANIV (purified by preparative HPLC in our laboratory) standards. Statistical analyses were performed using Excel 2004 for Macintosh with the add-in software Statcel 3 (OMS publishing Ltd., Tokorozawa, Japan). Asterisks denote significant differences (*p < 0.05, **p < 0.01) relative to the control without acivicin determined by Welch’s t-test (n = 4).

Fig. 2. Effect of acivicin on the expression of Tri6, Tri5, and Tri4. F. graminearum JCM 9873 (conidial suspension lot number #0120) was pre-cultured on a synthetic pre-culture medium and transferred to a YS_60 main culture medium (Difco™ yeast extract lot number 421675). The mycelia were collected at different time points (36, 48, 60, and 72 h) for RNA extraction. Single-stranded cDNA synthesis was performed as described previously (Etzerodt et al., 2015). Quantitative RT-PCR was carried out using the LightCycler 1.5 Instrument (Roche Diagnostics Japan, Tokyo) with LightCycler® TaqMan® Master (Roche). Expression of the β-tubulin (Tub) gene was used as a reference. Specific primers and a Universal ProbeLibrary (UPL) probe (Roche) were designed at the manufacturer’s design center. The specific primers qPCR-Tri6_P125_L and qPCR-Tri6_P125_R, and a UPL probe #125, were used for quantification of Tri6, the specific primers qRT-Tri5_Fw2 and qRT-Tri5_Rev2, and a UPL probe #142, for Tri5, the specific primers qRT-Tri4_Fw and qRT-Tri4_Rev, and a UPL probe #75, for Tri4, and the specific primers qRT-b-tub2_Fw and qRT-b-tub2_Rev, and a UPL probe #77, for Tub (see Fig. S1). Expression levels of Tri6, Tri5, and Tri4 were normalized to the expression level of Tub, which was used as an endogenous reference in the same RNA sample by dividing the target Tri gene cDNA copy number by the Tub cDNA copy number. A single standard DNA (Ohno et al., 2012) containing cDNA fragments of Tri10, Tri6, Tri5, Tri4, and a β-tubulin gene (FGSG_09530; Tub) (Fig. S1) was used as a reference to determine the number of cDNA molecules in the reverse-transcribed samples. Acivicin suppressed expression of these Tri genes at all time points examined (**p < 0.01 as determined by a Student’s t-test; n = 3).

transcription factor (Proctor et al., 1995). Important trichothecene pathway and regulatory genes (Tri genes) including Tri6 are clustered in the genome of F. graminearum (Kimura et al., 2007). Suppression of Tri6 expression is associated with the lack of expression of
pathway Tri genes such as Tri5, a key gene encoding trichodiene synthase required for the first cyclization (Hohn and Beremand, 1989), and Tri4, another key gene encoding a cytochrome P450 monooxygenase required for the four consecutive oxygenation steps (McCormick et al., 2006; Tokai et al., 2007). Small-molecule compounds that negatively affect activation of Tri6 inhibit trichothecene production. To determine whether the drug inhibits Tri gene expression, we examined the effect of a non-toxic level of acivicin (25 μM) on expressions of Tri6, Tri5, and Tri4 using F. graminearum strain JCM 9873. After transfer of the pre-culture to YS_60 medium (Table S1) with or without acivicin (25 μM), the main culture was distributed to wells in a 24-well plate, incubated at 25°C with gyratory shaking (135 rpm), and total RNAs were isolated from the mycelia collected from 3 wells at different time points. Real-time quantitative reverse-transcription (RT)-PCR analyses were carried out using locked nucleic acid probes. Since trichothecene productivity is sometimes affected by small differences in the physiological conditions of the conidia that are specific to each conidial suspension lot, the experiment was repeated two times using different conidial suspension lots as inocula, which yielded similar results (Fig. S2). A representative result using conidial suspension lot number #0120 with two replicates (n = 1 + 2 = 3) is shown in Fig. 2; the inhibition of mycotoxin production was mediated through transcriptional suppression of Tri5 and Tri4, caused by suppression of Tri6 expression. These results support our previous hypothesis that a non-growth-inhibitory level of acivicin negatively affects the conditioning of physiological factors necessary for triggering expression of the master regulator gene.

In the previous trichothecene production assay with acivicin, strain JCM 9873 was pre-cultured in a synthetic pre-culture medium (Table S1) with an initial inoculum density of 1 ¥ 10⁴ conidia/ml. After 16 h of pre-incubation (reaching the exponential growth phase), 1% (v/v) of the pre-culture was transferred to YS_60 medium. Because media compositions affect antibiotic titers (Clavaud et al., 2012; Doern et al., 1986; Elefanti et al., 2013), we sought to clarify whether the inhibitory activity of acivicin against

![Effects of acivicin on trichothecene production by different combinations of pre- and main culture media.](image)
trichothecene production is significantly affected by culture conditions: we explored trichothecene assays with 25 μM of acivicin using different combinations of pre-culture and main culture media. Acivicin obtained from a different manufacturer (Enzo Life Sciences, Farmingdale, NY; lot number 05011401) was used. When JCM 9873 was pre-cultured in the synthetic pre-culture medium (Table S1) and transferred to a YS_60 medium as in the previous study (Maeda et al., 2014), a statistically significant decrease in the amount of 15-ADON was observed with 25 μM of acivicin added to the main culture (Fig. 3A). Fungal growth was not affected at this concentration of acivicin, as was not the case in the previous study (Maeda et al., 2014). The acivicin effect was somewhat dependent on the lot of Difco™ yeast extract (BD Company, Franklin Lakes, NJ) used for preparation of the YS_60 medium in the main culture (Fig. S3). When the main culture was performed using a strongly trichothecene-inducing synthetic induction medium (Table S1) containing agmatine as a source of nitrogen, the suppressive effect of acivicin was markedly enhanced (Fig. 3B). In contrast, the mycotoxin inhibitory effect of acivicin was completely abrogated by using complex media for both the pre-culture (YG medium; Table S1) and main-culture (YS_60 medium) (Fig. 3C). The inhibitory activity of acivicin on trichothecene production was recovered by changing the main culture medium from a moderately trichothecene-inducing YS_60 medium to a strongly trichothecene-inducing synthetic induction medium (Fig. 3D).

In comparison with complex media containing yeast extracts, synthetic pre-culture and synthetic induction media (Table S1) do not contain any amino acids or nucleotides. The presence of such nutrients in the pre-culture medium compromised the mycotoxin inhibitory activity of acivicin in the main YS_60 culture (compare Figs. 3A and 3C), while their absence in the main culture enhanced such an activity (compare Figs. 3A and 3B; Figs. 3C and 3D). Thus, the deficiency of intracellular nutrients caused by a non-growth-inhibitory level of acivicin may lead to the disabled conditioning of some signals that are necessary to trigger the initial Tri6 expression. Transcriptome and metabolome analyses of F. graminearum treated with the acivicin may give some clues to understand the affected factors necessary for transcriptional activation of Tri6.

In conclusion, we found that acivicin inhibits trichothecene production by F. graminearum through transcriptional repression of Tri6. Detailed investigation of the culture system used for the trichothecene assay revealed that changes in the primary metabolism of the fungus caused by pre-culture and main culture nutritional conditions significantly affected the inhibitory activity of acivicin against mycotoxin production. Compared to the nutritions of complex media used in this study, the flowering spikelets of small cereal grains, where the pathogen causes an initial infection, contain a marginal amount of readily metabolizable nitrogen source, as is the case with the synthetic induction medium (Strange et al., 1974). Acivicin deserves to be tested for its ability to control wheat scab.

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Supplementary Materials

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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