

A screening system for inhibitors of trichothecene biosynthesis: hydroxylation of trichodiene as a target

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Abstract *Fusarium Tri4* encodes a key cytochrome P450 monooxygenase for hydroxylation of trichodiene early in the biosynthesis of trichothecenes. In this study, we established a system for screening for inhibitors of trichothecene biosynthesis using transgenic *Saccharomyces cerevisiae* expressing *Tri4*. For easy evaluation of the TRI4 activity, trichodiene-11-one was used as a substrate and the formation of 2 α -hydroxytrichodiene-11-one was monitored by HPLC. Using this system, TRI4 proved to be inhibited by various flavones and furanocoumarins. We also found

that a catechin-containing commercial beverage product, Catechin Supplement 300 (CS300), inhibited TRI4 activity, at a concentration which did not significantly affect the growth of the transgenic yeast. At an early stage of culture, both flavone and CS300 exhibited a toxin-inhibitory activity against *Fusarium graminearum*. However, inhibition of trichothecene production was not observed with longer incubation periods at minimum concentrations necessary to inhibit >50% of the TRI4 activity, presumably due to the metabolism by the fungus. The results suggest that this yeast screening system with TRI4 is useful for the rapid identification of lead compounds for the design of trichothecene biosynthesis inhibitors that are resistant to modification by the fungus.

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Introduction

Trichothecenes are toxic sesquiterpene secondary metabolites of *Fusarium* and other species. The trichothecene biosynthetic pathway starts with a cyclization of farnesyl pyrophosphate (FPP) to give trichodiene (TDN), followed by four consecutive oxygenations, a second cyclization, and modifications of the resulting trichothecene skeleton (oxygenations,

acetylations, and esterifications) (Kimura et al. 2007). Production of these toxins in agricultural commodities causes a variety of mycotoxicoses in humans and animals, a worldwide problem in recent years.

Naturally occurring cytochrome P450 monooxygenase (CYP) inhibitors of plant origin, flavonoids and furanocoumarins, were shown to inhibit the biosynthesis of T-2 toxin by *Fusarium sporotrichioides* (Desjardins et al. 1988). Although the necessary concentrations of flavones reported were high enough (i.e. >2 mM) to cause significant growth inhibition as well, the trichothecene precursor, TDN, accumulated in the flavone-treated cultures. Thus, a CYP responsible for the oxygenations of TDN appeared to be a good target of inhibitors, but such a biosynthetic gene was not identified at that time. At present, all the trichothecene (*Tri*) genes necessary for T-2 toxin biosynthesis have been identified in *F. sporotrichioides* and *Tri4* proved to encode a key CYP responsible for four consecutive additions of molecular oxygen to TDN (McCormick et al. 2006; Tokai et al. 2007).

A screening system with a specific target molecule is useful for the development of inhibitors of toxin biosynthesis. In this study, we established a protocol for the screening of trichothecene biosynthesis inhibitors using transgenic *Saccharomyces cerevisiae* expressing *Tri4*. By monitoring the conversion of a TDN analogue trichodiene-11-one to its 2 α -hydroxy derivative, we evaluated the inhibitory effects of flavones and furanocoumarins on trichothecene biosynthesis in detail. We also found inhibitory activity in a catechin-containing commercial beverage product. The utility of this yeast screening system for the development of toxin biosynthesis inhibitors is discussed.

Materials and methods

Strains, culture medium and reagents

Strains INVSc1 of *Saccharomyces cerevisiae* (Invitrogen, Carlsbad, CA) and MAFF 111233 of *Fusarium graminearum* lineage 6 (*Fusarium asiaticum*) were used in this study. In addition to flavonoids and furanocoumarins (Sigma-Aldrich), the following catechin-containing commercial beverage products were tested for the TRI4 assay; Kuro Oolong Tea (KO) and

Flavan Tea (F) from Suntory (Osaka, Japan), Healthya Green Tea (HG) and Healthya Water (HW) from Kao Corporation (Tokyo, Japan), Nama-cha (N) from Kirin Holdings Company, Ltd. (Tokyo, Japan), Benifuuki Green Tea (BG) from Asahi Beer (Tokyo, Japan), and Catechin Supplement 300 (CS300) from Ito EN, Ltd. (Tokyo, Japan). For evaluation of the inhibitory activity of flavone and CS300 against trichothecene production, *F. graminearum* was grown in SYEP medium (6% w/v sucrose, 0.1% yeast extract and 0.1% peptone). Strain MAFF 111233 produces 4,15-diacetylvalenol (4,15-diANIV) and 4-acetylvalenol (4-ANIV) in SYEP medium. Stock solutions of inhibitors (flavonoids and furanocoumarins) and trichodiene-11-one were dissolved in dimethylsulfoxide and ethanol, respectively.

Quantification of the TRI4 activity using the transgenic yeasts

For determination of the TRI4 activity, we monitored the 2 α -hydroxylation of trichodiene-11-one (Tokai et al. 2005) by transgenic yeasts expressing a codon-optimized synthetic *Tri4* (see Supplementary material). The transgenic yeasts were incubated in induction medium (without blasticidin S) in siliconized L-shaped culture tubes at 28°C (Tokai et al. 2005). At an OD₆₀₀ of 2–3, 1/10 volume of 20% (w/v) glucose was added to repress the *GALI* promoter and the resulting yeast cells were used as a whole-cell biocatalyst. The TRI4 assay was initiated by adding an inhibitor (flavonoids and furanocoumarins) to the cells, or mixing the cells with an equal amount of a catechin-containing commercial beverage product buffered with 50 mM sodium acetate, pH 4.6. In either case, trichodiene-11-one was then added (final 25 μ g/ml), and the reaction mixture was incubated for 6 h at 28°C. The oxygenated product, 2 α -trichodiene-11-one, in the culture (5 ml) was extracted with an equal volume of hexane, evaporated in vacuo, and dissolved in ethanol (250 μ l). Aliquots (10 μ l) were analyzed on a C-4 reversed phase column VP-304-1251 (diameter, 4.6 mm; length, 250 mm; Senshu Scientific Co., Tokyo, Japan) in an HPLC system (SCL-10A; Shimadzu, Kyoto, Japan) at 40°C. The transformed product was eluted with 30% (w/v) acetonitrile at 1 ml/min, and its relative quantity was determined from the peak area monitored at 254 nm.

Quantification of *Fusarium* trichothecenes in liquid culture

F. graminearum was grown in the presence or absence of candidate compounds in 200 ml SYEP medium in a 500 ml Erlenmeyer flask. After incubation with shaking (120 rpm/min) at 25°C, 15 ml culture was sampled and used for the toxin analysis with an HPLC system as described previously (Ochiai et al. 2007). Ergosterol was extracted by shaking the mycelia with 9 ml chloroform/methanol (2:1 v/v) and quantified by HPLC as described previously (Ochiai et al. 2007).

Results and discussion

Development of a screening system for trichothecene biosynthesis inhibitors

We first developed a system for screening for inhibitors of trichothecene biosynthesis as described in “Materials and methods”. At concentrations used for the assay (up to 300 μ M), TRI4 activity was examined in the presence of flavonoids and furanocoumarins (Fig. 1), which did not exert deleterious effects on yeast growth during the incubation period. The transgenic yeast culture could be used as a whole-cell biocatalyst representing the TRI4 oxygenation system.

Consistent with the inhibitory effect of flavones on *Fusarium* trichothecene biosynthesis (Desjardins et al. 1988), unsubstituted flavone was the most potent inhibitor of TRI4 (Fig. 1a). At 100 μ M, flavone inhibited 68% of the 2 α -hydroxylation activity, which appears to be much more efficient than the toxin-inhibitory activity previously reported in *F. sporotrichioides*. Substitutions of the flavone rings with hydroxyl, methyl, or methoxy groups reduced inhibitor activity to different degrees depending on their positions (Fig. 1a). Other flavones, such as 4'-methoxyflavone, 6-methoxyflavone, 5-hydroxyflavone, and 7-hydroxyflavone, were poor inhibitors of TRI4 (<20% inhibition at 200 μ M). Reduction of the 2,3-double bond to flavanone resulted in little loss of the TRI4-inhibitory activity (34 and 56% inhibition at 50 and 100 μ M, respectively), as reported for the toxin-inhibitory activity of flavanone in *F. sporotrichioides* (Desjardins et al. 1988). The furanocoumarins, bergapten (5-methoxypsoralen) and xanthotoxin (8-methoxypsoralen), also inhibited TRI4 as efficiently

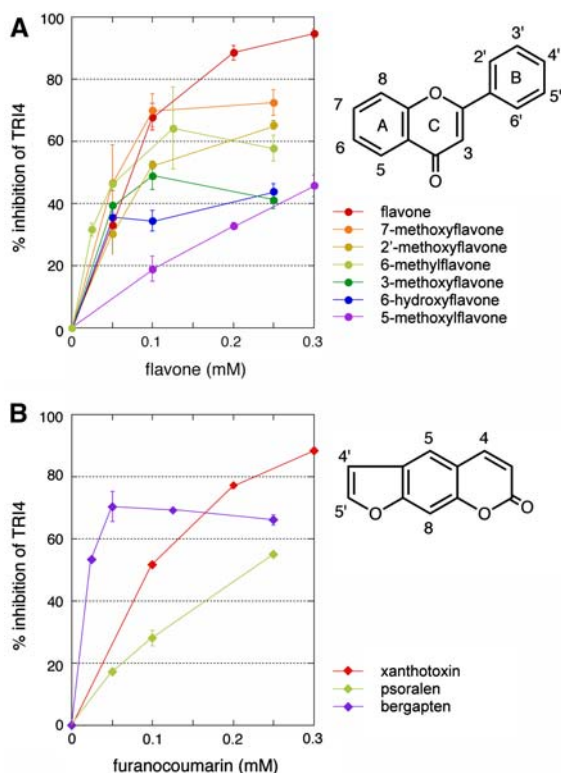


Fig. 1 Effects of flavones (a) and furanocoumarins (b) on the biosynthesis of trichothecenes using recombinant *S. cerevisiae* expressing *Tri4*. Conversion of trichodiene-11-one to its 2 α -hydroxy derivative was monitored at 254 nm in the presence of flavones and furanocoumarins. Inhibition (%) was calculated by measuring the amount of 2 α -hydroxytrichodiene-11-one in the presence (flavones and furanocoumarins; up to 300 μ M) and absence of candidate compounds. Bars indicate standard deviations

as unsubstituted flavone, but psoralen, an unsubstituted furanocoumarin, showed a slightly lower level of activity (Fig. 1b).

The above results provide clear evidence that TRI4 is the target site of inhibition by flavones and furanocoumarins. Because of the easy handling of yeasts with short incubation periods, this system may contribute to the initial screening for TRI4 inhibitors of natural or synthetic chemical libraries.

Inhibition of TRI4 by a catechin-containing commercial beverage product

Catechins, major polyphenol constituents of green tea, are flavonoids called flavan-3-ols and are inhibitors of CYPs (Wang et al. 1988). They are known as

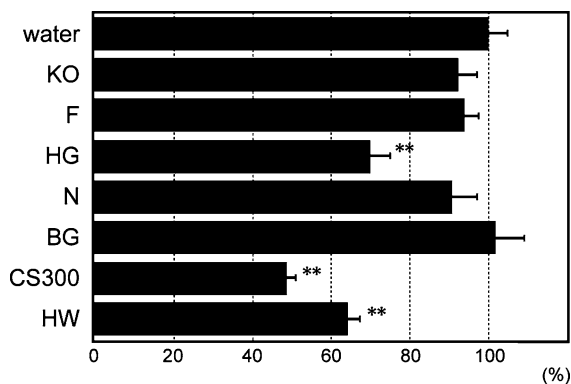


Fig. 2 Effects of commercial beverage products that contain catechin on TRI4 activity (for abbreviations, see “Materials and methods”). Conversion of trichodiene-11-one to its 2 α -hydroxy derivative was monitored at 254 nm with half the concentration of the beverage product buffered with sodium acetate (pH 4.6). Activity (%) is represented as the amount of the product, 2 α -hydroxytrichodiene-11-one, relative to the amount in the control (water). Bars indicate standard deviations. The significance of differences between the control (water) and each product was analyzed with Student’s *t*-test: **P* < 0.05, ***P* < 0.01

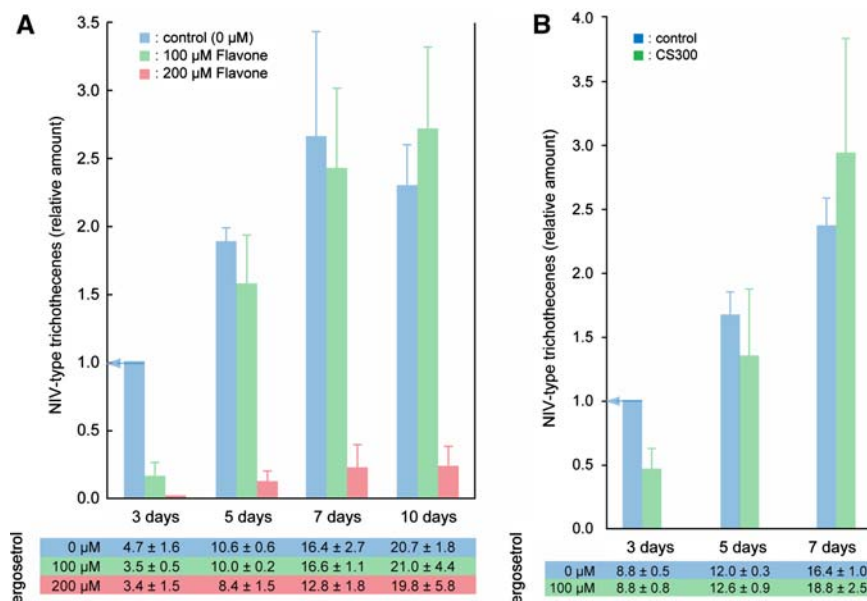
cancer preventive agents (Bushman 1998) and are considered non-harmful and to promote human health. We therefore searched for toxin-inhibitory activity in catechin-containing commercial beverage products using the assay. As shown in Fig. 2, HG, HW, and CS300 showed significant inhibition of

TRI4 at half the concentration used for consumption as a beverage.

Effects of candidate compounds on *Fusarium* toxin production

We next examined whether unsubstituted flavone and CS300 inhibit toxin production by *F. graminearum*. Three independent experiments were carried out and the trichothecene content was expressed as a ratio to the control (27.2, 30.7, and 21.2 μ g trichothecene per ml culture 3 days after inoculation). As shown in Fig. 3a, 100 μ M flavone failed to inhibit trichothecene production at longer incubation periods (>5 days) although it efficiently inhibited TRI4 (inhibition > 60%) at this concentration (Fig. 1a). Since flavone was not recovered from the fungal culture (Desjardins et al. 1988), the reduction of toxin-inhibitory activity is likely to be due to metabolism by the fungus during the long incubation period. At a higher concentration (200 μ M), flavone effectively decreased the amount of trichothecenes at day 10 without significant changes in fungal biomass (dry weight). Amendment with CS300 (half the concentration used for consumption) also resulted in a toxin profile similar to the case with 100 μ M flavone, whose inhibitory effect was observed only at an early stage of culture (day 3). The screening system described here is useful to develop specific

Fig. 3 Effects of candidate compounds on *Fusarium* toxin production. The fungal inoculum (1 ml in YG medium) was added to SYEP medium (100 ml) supplemented with 0, 100, or 200 μ M flavone (a), or half the concentration of CS300 (b), and incubated with gyratory shaking at 25°C. Colored bars indicate the relative molar amounts of 4-acetylvalenol/4,15-diacetylvalenol (NIVs) in proportion to the control at day 3 in each experiment. Ergosterol content (μ g/g medium) is shown at the bottom of the graph



inhibitors of trichothecene biosynthesis without missing a promising lead compound, which may contribute to the development of inhibitors resistant to modification by the fungus.

Conclusion

A system for screening for inhibitors of trichothecene biosynthesis was established by using *TRI4* as a target. This system may be used for the rapid identification of a lead compound for the design of toxin biosynthesis inhibitors.

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