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Isolation and Identification of Precocenes and Piperitone from Essential Oils as Specific Inhibitors of Trichothecene Production by *Fusarium graminearum*

ATSUSHI YAGUCHI,[†] TOMOYA YOSHINARI,[†] RIE TSUYUKI,[†] HARUO TAKAHASHI,[‡] TAKASHI NAKAJIMA,[§] YOSHIKO SUGITA-KONISHI,[||] HIROMICHI NAGASAWA,[†] AND SHOHEI SAKUDA*,[†]

Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan, Public Health Laboratory of Chiba Prefecture, 666-2 Nitona-cho, Chuo-ku, Chiba City 260-8715, Japan, National Agricultural Research Center for Kyushu Okinawa Region, Koshi, Kumamoto 861-1192, Japan, and National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Inhibitors of deoxynivalenol production by *Fusarium graminearum* are useful for protecting crops from deoxynivalenol contamination. We isolated precocenes and piperitone from the essential oils of *Matricaria recutita* and *Eucalyptus dives*, respectively, as specific inhibitors of the production of 3-acetyldeoxynivalenol, a biosynthetic precursor of deoxynivalenol. Precocenes I and II and piperitone inhibited 3-acetyldeoxynivalenol production by *F. graminearum* in a liquid culture with IC_{50} values of 16.6, 1.2, and 306 μM, respectively, without inhibiting fungal growth. Precocene II also inhibited deoxynivalenol production by the fungus in a solid culture on rice with an IC_{50} value of 2.0 ppm. Precocene II and piperitone decreased the mRNA levels of *Tri4*, *Tri5*, *Tri6*, and *Tri10* encoding proteins required for deoxynivalenol biosynthesis.

KEYWORDS: Deoxynivalenol; *Matricaria recutita*; precocene; *Fusarium graminearum*; piperitone; *Eucalyptus dives*

INTRODUCTION

Contamination of agricultural products by mycotoxins, which are fungal toxic secondary metabolites, is a worldwide human and livestock health concern that also has the potential to cause drastic economic consequences. Among the mycotoxins, deoxynivalenol (**Figure 1A**), belonging to the trichothecenes, is particularly troublesome because of its contamination of important cereal crops, such as wheat (*1*). *Fusarium graminearum* is a worldwide predominant pathogen that causes Fusarium head blight (FHB) of wheat and other grain cereals and produces deoxynivalenol in infected grain. FHB epidemics reduce crop yield, and deoxynivalenol contamination limits grain utilization. Therefore, developing an effective means of controlling both FHB and deoxynivalenol contamination is crucial.

At present, the use of fungicides is the most effective method for controlling FHB and deoxynivalenol contamination. However, there have been cases where the use of fungicides results in increased levels of deoxynivalenol in the grain (*2*, *3*), and inhibition of fungal growth may sometimes lead to creation and spread of resistant fungal strains (*4*). On the other hand, it is known that deoxynivalenol production is not necessary for the fungal growth or initial infection on wheat, but the spread of FHB is suppressed when all trichothecene production is completely inhibited (*5*). Therefore, specific inhibitors of deoxynivalenol production may be useful in protecting crops from both FHB and deoxynivalenol contamination, without incurring a rapid spread of resistant strains. To date, a few synthetic and natural products have been reported as inhibitors of trichothecene production. Ancymidol (*6*), an inhibitor of cytochrome P450 monooxygenase, and some flavonoids (*7*) have been shown to inhibit the biosynthesis of T-2 toxin, but the inhibitory activity of these compounds is very weak and not specific. 4-Acetyl-2-benzoxazolinone, a constituent of corn, inhibited 3-acetyldeoxynivalenol (**Figure 1A**) production by *Fusarium culmorum* in a liquid culture with the IC_{50} value of 4 μM (*8*) but did not inhibit deoxynivalenol production by *F. graminearum* grown on the maize grain which contained this compound at the concentration of more than 100 μM (*9*). Recently, spiroethers of German chamomile were found to inhibit the enzymatic activity of TRI4, a key cytochrome P450

* Corresponding author. Telephone: 81-3-5831-5133. Fax: 81-3-5841-8022. E-mail: asakuda@mail.ecc.u-tokyo.ac.jp.
[†] University of Tokyo.
[‡] Public Health Laboratory of Chiba Prefecture.
[§] National Agricultural Research Center for Kyushu Okinawa Region.
[||] National Institute of Health Sciences.

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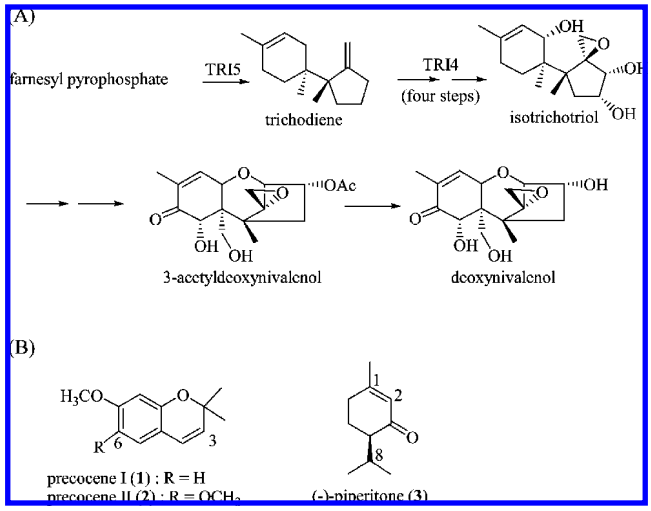

Figure 1. Biosynthetic pathway to (A) deoxynivalenol and (B) structures of precocenes I and II and (−)-piperitone.

monooxygenase involved in the biosynthesis of trichothecenes, and 3-acetyldeoxynivalenol production of *F. graminearum* (*10*).

We have been searching for mycotoxin-production inhibitors among plant essential oils (*10, 11*). Constituents of essential oils have relatively simple structures, and their biosynthetic pathways are not complicated. Therefore, active components involved in essential oils may be attractive as lead compounds for developing practically effective drugs. In addition, the genes responsible for their biosynthesis may be useful for creating transgenic plants which resist deoxynivalenol contamination by in situ production of an active compound.

In this study, we investigated the isolation of precocenes and piperitone from essential oils as specific 3-acetyldeoxynivalenol production inhibitors, and their inhibitory activities against 3-acetyldeoxynivalenol and deoxynivalenol production and expression of genes encoding proteins required for deoxynivalenol biosynthesis.

MATERIALS AND METHODS

***F. graminearum* Culture Conditions and Analysis of 3-Acetyldeoxynivalenol and Deoxynivalenol.** A strain of *F. graminearum* described previously (*10*) was used as a 3-acetyldeoxynivalenol and deoxynivalenol producer. The following liquid culture was used for the bioassay to evaluate 3-acetyldeoxynivalenol production inhibitory activity. SYEP (sucrose 5%, yeast extract 0.1%, polypeptone 0.1%) liquid medium (5 mL) was put into test tubes (1.6 cm × 18 cm), and each test sample (an essential oil for screening) in methanol was added to the medium (the final concentration of methanol was 0.3%). Each tube was inoculated with a spore suspension of the strain (1×10^5 spores/tube) and then incubated with continuous shaking (300 rpm) at 26.5 °C for 7 days. The resulting culture broth was filtered with cheesecloth to obtain mycelia and filtrate. The weight of the mycelia obtained was measured as follows. The mycelia were washed with distilled water (5 mL) and collected into a 1.5 mL microtube. After drying the mycelia at 100 °C for 3 h, mycelial weight was calculated by subtracting the weight of a 1.5 mL microtube without mycelia from the total weight. To analyze the amount of 3-acetyldeoxynivalenol in the culture filtrate, the filtrate (1 mL) was extracted with 200 μL of ethyl acetate, and the ethyl acetate solution was subjected to thin layer chromatography analysis using silica gel 60 F254 plates (Merck, Darmstadt, Germany) and a solvent system of chloroform/methanol (5:1). In the case of LC/MS analysis for quantification, the ethyl acetate solution was evaporated to dryness and the residue obtained was dissolved in 200 μL of 10% acetonitrile in water. The effects on 3-acetyldeoxynivalenol and deoxynivalenol production in a solid culture were evaluated with the following method. Grains of rice (1.0 g) and distilled water (0.3 mL) were placed in a vial (20 mL) and autoclaved. A methanol solution of precocene II, precocene I, or piperitone was added to the vial and mixed well with the rice grains. Each vial was inoculated with a spore suspension (1×10^5 spores/tube) and then incubated statically at 26.5 °C for 7 days. A solution of 50% acetonitrile in water (10 mL) was added to the culture and left for 2 h at room temperature. The mixture was then ground in a mortar with a pestle and centrifuged to obtain the supernatant. After removing acetonitrile from the supernatant by evaporation, the residual solution was applied to a Sep-Pak C_{18} cartridge and the 50% methanol eluate from the cartridge was subjected to LC/MS analysis using a 2695 HPLC system (Waters, Milford, MA) equipped with a 150 mm × 2.0 mm i.d. Capcell-Pak C_{18} column eluted with a gradient of 10−80% acetonitrile in water containing 10 mM ammonium acetate in 20 min followed by an isocratic elution of 80% acetonitrile in water containing 10 mM ammonium acetate. The flow rate was 0.2 mL/min, and the retention times of deoxynivalenol and 3-acetyldeoxynivalenol were 3.9 and 9.4 min, respectively. MS analysis was done with a micromassZQ (Waters) by ESI, in positive ion mode; spray chamber parameters: source temperature, 120 °C; desolvation

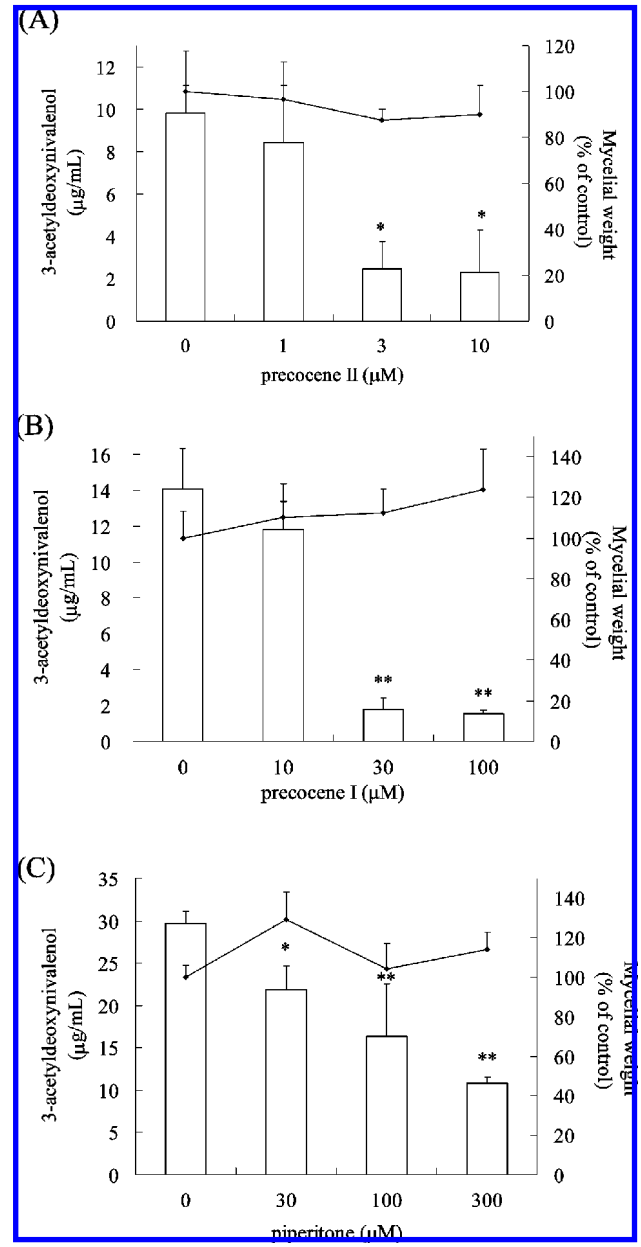

Figure 2. Effects of (A) precocenes II and (B) I and (C) piperitone on 3-acetyldeoxynivalenol production and fungal growth: white bars, 3-acetyldeoxynivalenol; ●, mycelial weight; $n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, vs control.

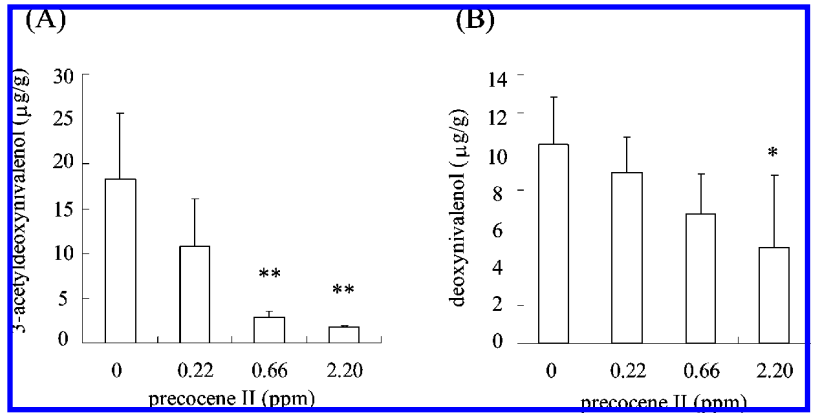

Figure 3. Effects of precocenes II on (A) 3-acetyldeoxynivalenol and (B) deoxynivalenol production when grown on the rice medium. One ppm is defined as the concentration when one microgram of precocene II is topically applied on one gram of the rice medium. $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$, vs control.

temperature, 350 °C; cone, 30 V; desolvation gas, 600 L/h; cone gas, 50 L/h; capillary voltage, 2800 V. MS ions were monitored in a single-ion recording mode using the extracted ions *m*/*z* 297 $(M + H)^+$ for deoxynivalenol and *m*/*z* 339 $(M + H)^+$ for 3-acetyldeoxynivalenol. Standard deoxynivalenol and 3-acetyldeoxynivalenol were purchased from Sigma-Aldrich (St. Louis, MO).

Isolation of Precocenes and (−)-Piperitone. Wakogel C-200 silica gel (50 g) (Wako, Osaka, Japan) was packed in a column with *n*-hexane. The essential oil of *Matricaria recutita* (German chamomile) (2 g) (Naturas Psychos, Japan) was loaded on the column and eluted stepwise with *n*-hexane (800 mL) and *n*-hexane/ethyl acetate (95:5, v/v, 800 mL). The *n*-hexane/ethyl acetate (95:5) fraction was evaporated, and the residue was loaded on a Wakogel C-200 silica gel (10 g) column packed with *n*-hexane and eluted with *n*-hexane/ethyl acetate (95:5, v/v, 800 mL). Fractions corresponding to the eluate from 160 to 240 mL (64.2 mg) and those from 400 to 640 mL (75.6 mg) were further purified by HPLC on a 250 mm × 20 mm i.d. Capcell-Pak C_{18} column with isocratic elution of 60% acetonitrile in water at a flow rate of 5 mL/min and detection at 225 nm to obtain precocene I (retention time: 50.5 min; yield: 2.0 mg) and precocene II (retention time: 34.4 min; yield: 7.9 mg), respectively. Precocene I (**1**): FAB-MS (3-nitrobenzyl alcohol matrix) *m*/*z* 191 $(M + H)^+$; δ_H ($CDCl_3$, 600 MHz): 6.88 (1H, dd, $J = 10$ Hz, H-5), 6.40 (1H, d, $J = 10$ Hz, H-6), 6.37 (1H, s, H-8), 6.27 (1H, d, $J = 10$ Hz, H-3), 5.47 (1H, d, $J = 10$ Hz, H-2), 3.77 (3H, s, OCH_3), 1.42 (6H, s, CH_3). Precocene II (**2**): FAB-MS (3-nitrobenzyl alcohol matrix) *m*/*z* 221 $(M + H)^+$; δ_H ($CDCl_3$, 600 MHz): 6.52 (1H, s, H-5), 6.40 (1H, s, H-8), 6.23 (1H, d, $J = 10$ Hz, H-3), 5.46 (1H, d, $J = 10$ Hz, H-2), 3.82 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 1.40 (6H, s, CH_3).

The essential oil of *Eucalyptus dives* ct piperitone (200 mg) (Naturas Psychos) was loaded on a Wakogel C-200 silica gel column (10 g) packed with *n*-hexane and eluted stepwise with *n*-hexane (160 mL) and *n*-hexane/ethyl acetate (95:5, v/v, 160 mL). The *n*-hexane/ethyl acetate (95:5) fraction was further purified by HPLC under the same conditions as used in the isolation of precocenes to obtain (−)-piperitone (retention time: 29.6 min; yield: 37.6 mg). Piperitone (**3**): FAB-MS (3-nitrobenzyl alcohol matrix) *m*/*z* 153 $(M + H)^+$; $[\alpha]_D^{27.4}$ −18.8 (*c* 0.313, $CHCl_3$) [authentic sample (purity >94.0%, Tokyo Chemical Industry, Tokyo, Japan) −14.6 (*c* 0.504, $CHCl_3$)]; δ_H ($CDCl_3$, 600 MHz): 5.81 (1H, s, H-2), 2.22−2.38 (3H, m), 1.94−2.03 (2H, m), 1.91 (3H, s, H-7), 1.76−1.83 (1H, m), 0.92 (3H, d, $J = 7$ Hz, CH_3), 0.84 (3H, d, $J = 7$ Hz, CH_3); δ_C ($CDCl_3$, 150 MHz): 201.4 (C3), 161.2 (C1), 126.9 (C2), 51.7 (C4), 30.4 (C6), 25.9 (C8), 24.2 (C7), 23.0 (C5), 20.8 (CH_3), 18.6 (CH_3).

Analysis of Culture Broth To Detect Intermediates. *F. graminearum* was cultured in a test tube under the conditions described above with or without precocene II (30 μM) or piperitone (1000 μM). The culture broth obtained (5 × 10 mL) was separated to culture filtrate and mycelia. The culture filtrate was extracted with ethyl acetate (50 mL), and the ethyl acetate layer was dried and concentrated. Mycelia were extracted with methanol (30 mL) and filtered to obtain a methanol solution, which was concentrated to remove methanol. After water (50 mL) was added, the mixture was extracted with ethyl acetate (50 mL) and the ethyl acetate layer was dried and concentrated. The concentrated ethyl acetate solution obtained from the culture filtrate or mycelia was analyzed by TLC to detect intermediates with an epoxide group (*12*), which include all intermediates except for trichodiene, and was then loaded on a Wakogel C-200 silica gel (5 g) column packed with *n*-hexane and eluted stepwise with *n*-hexane (50 mL) and ethyl acetate (50 mL). Each of the *n*-hexane and ethyl acetate eluates was concentrated at 30 °C to a small volume and dried up under N_2 gas. By the procedure mentioned above, we prepared 12 samples, *n*-hexane and ethyl acetate fractions from extracts of culture filtrate or mycelia of control, precocene II, or piperitone culture. Each sample obtained was dissolved in $CDCl_3$ and subjected to ^1H NMR analysis. Proton signals of 3-acetyldeoxynivalenol were clearly observed in the spectrum of the ethyl acetate eluate fraction from the extracts of the control culture filtrate, but they were not observed in the spectra of precocene II or piperitone samples. Significant signals characteristic to intermediates such as trichodiene (*13*) were not observed in the spectra of precocene II or piperitone samples.

RT-PCR Analysis of Tri Genes. A spore suspension of *F. graminearum* (1×10^5 spores/tube) was inoculated into each test tube containing 5 mL of SYEP liquid medium, which was incubated with continuous shaking (300 rpm) at 26.5 °C for 2, 3, 4, or 6 days with or without precocene II or piperitone. The mycelial cake was harvested by filtration and lyophilized. Total RNA was extracted by using a TRIzol plus RNA Purification Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA was prepared using the SuperScript III First Strand Synthesis System (Invitrogen) with random hexamer primers, according to the protocol. The cDNA derived from 0.005 μg of total RNA was used as a template. Real-time quantitative RT-PCR was carried out using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA), in a final volume of 25 μL for each reaction, and an ABI PRISM 7300 thermal cycler (Applied Biosystems). Two-step PCR conditions were as follows: after an initial incubation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min were performed. The PCR primers for each gene were as follows: *Tri4* 5′-GCACAC-CGATCCCAAGAATT-3′ and 5′-TCCATCGCTCAGGCTTGAAC-3′:*Tri5* 5′-AATCGTTGGCATGGTTGTATACAG-3′ and 5′-AGAT-AGATCCGCCATGCACTCT-3′:*Tri6* 5′-CGCCCTTCCCACCTTCA-3′ and 5′-CGACTTGCAACTAGGGAATGG-3′:*Tri10* 5′-TGGGTG-GGCTCGACAAGA-3′ and 5′-AAGGGCAACGACGGTACCT-3′: *β-tubulin* (control gene) 5′-CCTGACCTGCTCTGCCATCT-3′ and 5′-TGGTCCTCAACCTCCTTCATG-3′.

The amount of each mRNA was normalized to the amount of *β-tubulin* mRNA in each sample.

Data Analysis. Data are presented as the mean ± S. D. Differences between groups were assessed with one-way ANOVA followed by Dunnett's test. Values of $p < 0.05$ were considered significant.

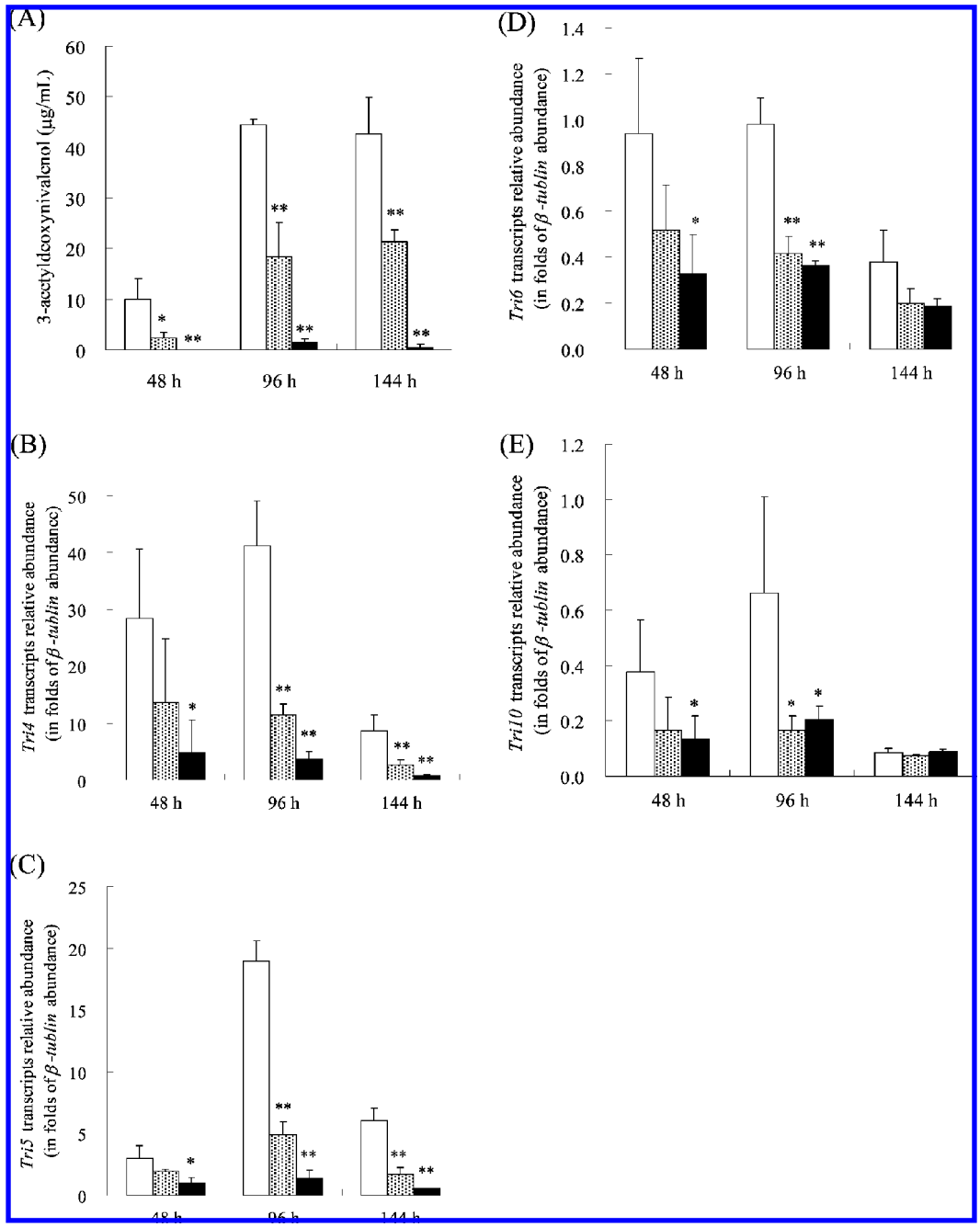

Figure 4. Effects of precocene II on (A) 3-acetyldeoxynivalenol production and the transcription of (B) *Tri4*, (C) *Tri5*, (D) *Tri6*, and (E) *Tri10*: white bars, control; gray bars, 3 μM of precocene II; black bars, 30 μM of precocene II; $n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$, vs control.

RESULTS AND DISCUSSION

Screening and Isolation of 3-Acetyldeoxynivalenol Production Inhibitors. The strain of *F. graminearum* used in this study produces deoxynivalenol and 3-acetyldeoxynivalenol, a biosynthetic precursor of deoxynivalenol (**Figure 1A**), in a solid culture, but produces only 3-acetyldeoxynivalenol in a liquid culture. Since production of 3-acetyldeoxynivalenol in the liquid culture was more constant than that of deoxynivalenol and 3-acetyldeoxynivalenol in the solid culture, a bioassay search for inhibitors of 3-acetyldeoxynivalenol production in the liquid culture was used for our screening. During the course of the screening in 110 commercially available essential oils, inhibitory activity toward 3-acetyldeoxynivalenol production was found in the essential oils of *Matricaria recutita* and *Eucalyptus dives*. The main active components were purified from the oils by a silica gel column chromatography and reverse-phase HPLC. The compounds isolated from *M. recutita* were identified as precocenes I (**1**) and II (**2**) (**Figure 1B**), and that from *E. dives* was identified as (−)-piperitone (**3**) (**Figure 1B**).

3-Acetyldeoxynivalenol and Deoxynivalenol Production−Inhibitory Activity of Precocenes and Piperitone. Precocene II strongly and dose-dependently inhibited 3-acetyldeoxynivalenol production in the liquid culture with an IC_{50} value of 1.2 μM, but the mycelial weight of the fungus was not significantly affected (**Figure 2A**). The inhibitory activity of precocene I (IC_{50} =16.6 μM) on 3-acetyldeoxynivalenol production was weaker than that of precocene II (**Figure 2B**). Piperitone inhibited 3-acetyldeoxynivalenol production much more weakly than precocenes (IC_{50} =306 μM), but it showed no growth inhibitory activity toward the fungus, similarly to the case of the precocenes (**Figure 2C**).

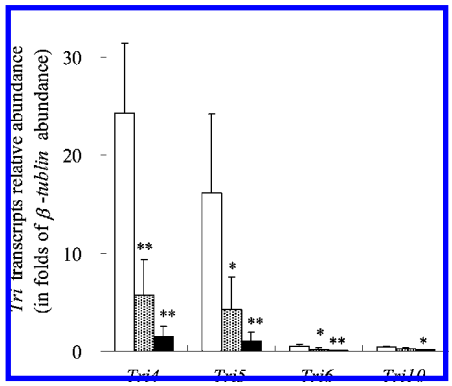

Figure 5. Effects of piperitone on the transcription of genes required for deoxynivalenol production: white bars, control; gray bars, 300 μM of piperitone; black bars, 1000 μM of piperitone; $n = 3$; $^*P < 0.05$, $^{**}P < 0.01$, vs control.

Next, the effects of precocene II on 3-acetyldeoxynivalenol and deoxynivalenol production by the *F. graminearum* strain were examined in a solid culture on rice. Precocene II was topically applied on the rice before inoculating with fungus. After cultivation of the fungus on the rice, the amounts of 3-acetyldeoxynivalenol and deoxynivalenol produced in the culture were measured by LC/MS. Although 3-acetyldeoxynivalenol or deoxynivalenol production in the rice medium was not constant compared to the case of 3-acetyldeoxynivalenol production in the liquid culture, each compound was produced at the concentration around 10−20 μg/g in the rice culture when cultured for 7 days. Precocene II strongly reduced 3-acetyldeoxynivalenol production with an IC_{50} value of 0.36 ppm (**Figure 3A**) and moderately reduced deoxynivalenol production (IC_{50} = 2.0 ppm) (**Figure 3B**). Precocene I and piperitone also inhibited 3-acetyldeoxynivalenol production in the rice medium with IC_{50} values of 1.3 and 61.2 ppm, respectively, but weakly reduced deoxynivalenol production by 20% and 25% at 5.7 and 152 ppm, respectively.

It is known that deacetylation of 3-acetyldeoxynivalenol affords deoxynivalenol and that the reverse reaction catalyzed by Tri101 also occurs to protect the producing fungus from deoxynivalenol poisoning (*14*). On the other hand, Tri101 is also known as an essential enzyme responsible for an early step commonly involved in the trichothecene biosynthetic pathway (*15*). Therefore, unbalanced reduction in 3-acetyldeoxynivalenol and deoxynivalenol production caused by precocenes or piperitone cannot be explained by reduction of the enzymatic activity of Tri101. This phenomenon might suggest the presence of an unknown mechanism which regulates the conversion from deoxynivalenol to 3-acetyldeoxynivalenol according to culture conditions.

Effects of Precocene II and Piperitone on the Transcription of Genes Encoding Proteins Required for Deoxynivalenol Biosynthesis. We analyzed extracts of the culture filtrate or mycelia by TLC and NMR but have not detected the accumulation of any biosynthetic intermediates in them when 3-acetyldeoxynivalenol production by the fungus was inhibited by precocene II or piperitone (data not shown). This suggested that these compounds might not directly inhibit an enzyme involved in the DON biosynthetic pathway. To clarify the mode of action of precocene II and piperitone, we examined the effects of precocene II or piperitone on the transcription of *Tri4*, *Tri 5*, *Tri6*, and *Tri10*, which encode proteins involved in deoxynivalenol biosynthesis. TRI5 and TRI4 are enzymes that catalyze the conversion of farnesyl pyrophosphate to trichodiene, and trichodiene to isotrichotriol (**Figure 1A**), respectively, in deoxynivalenol biosynthesis (*14*), whereas TRI6 and TRI10 are regulatory proteins necessary for the initiation of deoxynivalenol biosynthesis. In the known cascade for regulation of deoxynivalenol biosynthesis, TRI10 is the first key regulator (*16*), which positively regulates expression of TRI6, the second key regulator. The expression of genes that encode deoxynivalenol biosynthetic enzymes, including TRI4 and TRI5, is under the positive control of TRI6 (*17*). Upstream events, including those that regulate TRI10 expression, have not yet been clarified. The *F. graminearum* strain was cultured in a liquid medium with or without precocene II at the concentration of 3 or 30 μM for 48, 96, or 144 h. Reduction of 3-acetyldeoxynivalenol production by precocene II was observed at all cultivation times (**Figure 4A**). We extracted total RNA from mycelia and analyzed the mRNA level of each gene by quantitative RT-PCR. We used β-tubulin mRNA as an endogenous reference because its copy number is not influenced by precocene II or piperitone. When precocene II was added to the culture, the mRNA levels of *Tri4* (**Figure 4B**) and *Tri5* (**Figure 4C**) at all cultivation times and *Tri6* (**Figure 4D**) and *Tri10* (**Figure 4E**) at 48 and 96 h significantly decreased dose-dependently. Similarly, the transcription of each *Tri* gene was clearly suppressed significantly when the strain was cultured with piperitone at the concentration of 300 or 1000 μM for 72 h (**Figure 5**). These results suggest that precocene II and piperitone may affect an early step in the DON biosynthetic pathway that precedes TRI10 expression.

Compounds **1** and **2** were found as constituents of essential oils of *Ageratum* species and were named ageratochromene (*18*) and 6-demethoxyageratochromene (*19*), respectively. They were later rediscovered as compounds with specific antijuvenile hormone activity against insects and renamed precocenes I and II for their novel bioactivity (*20*). Precocenes show allatocidal activity (*21*), but their molecular target for the activity is still unclear. Weak antifungal activity of compound **2** toward *Pyricularia oryzae* was reported (*22*). In this study, we first showed that precocenes can inhibit trichothecene production of *F. graminearum* without affecting the fungal growth. The order of activity (precocene II > precocene I) toward 3-acetyldeoxynivalenol production was the same as that in the case toward insects (*23*). Precocenes are not used as insecticides practically but are very important probes to study insect metamorphosis. Hepatotoxicity of precocenes is known at high doses (*24*). Despite their simple structures, the biosynthetic pathway of precocenes (*25*) and the genes involved in their biosynthesis have not yet been established.

Piperitone is a major constituent of the essential oil of *E. dives* (*26*) and is used in fragrances (*27*). Piperitone has been reported to have insecticidal and weak antifungal activities (*28*). Because its inhibitory activity toward 3-acetyldeoxynivalenol production was relatively weak, piperitone itself may not be a practical agent for preventing deoxynivalenol contamination. However, its typical *p*-menthane structure may be useful not only for further screening among related compounds but also for creating a transgenic plant producing piperitone or a more effective compound with a similar structure because many genes encoding enzymes involved in biosynthesis of monoterpenes with a *p*-menthane skeleton have been identified (*29*).

We showed that molecular targets of precocene II and piperitone may be present in an unknown early regulatory step in deoxynivalenol biosynthesis. Therefore, further study on the mode of action of precocene II or piperitone may lead not only to the development of more effective deoxynivalenol production

inhibitors but also to a better understanding of the regulatory mechanism involved in fungal deoxynivalenol production.

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