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Enhancement of Trichothecene Production in *Fusarium graminearum* by Cobalt Chloride

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ABSTRACT: The effects of cobalt chloride on the production of trichothecene and ergosterol in *Fusarium graminearum* were examined. Incorporation experiments with ^{13}C-labeled acetate and leucine confirmed that both 3-acetyldeoxynivalenol and ergosterol were biosynthesized via a mevalonate pathway by the fungus, although hydroxymethyl-glutaryl CoA (HMG-CoA) from intact leucine was able to be partially used for ergosterol production. Addition of cobalt chloride at concentrations of 3−30 μM into liquid culture strongly enhanced 3-acetyldeoxynivalenol production by the fungus, whereas the amount of ergosterol and the mycelial weight of the fungus did not change. The mRNA levels of genes encoding trichothecene biosynthetic proteins (TRI4 and TRI6), ergosterol biosynthetic enzymes (ERG3 and ERG25), and enzymes involved in the mevalonate pathway (HMG-CoA synthase (HMGS) and HMG-CoA reductase (HMGR)) were all strongly up-regulated in the presence of cobalt chloride. Precocene II, a specific trichothecene production inhibitor, suppressed the effects of cobalt chloride on *Tri4*, *Tri6*, *HMGS*, and *HMGR*, but did not affect *erg3* and *erg25*. These results indicate that cobalt chloride is useful for investigating regulatory mechanisms of trichothecene and ergosterol production in *F. graminearum*.

KEYWORDS: deoxynivalenol, *Fusarium graminearum*, cobalt chloride, ergosterol, precocene

INTRODUCTION

Fusarium graminearum is a worldwide predominant plant pathogen that causes Fusarium head blight of wheat and other grain cereals and produces trichothecene mycotoxins, mainly deoxynivalenol, in infected grains.[1] Contamination of deoxynivalenol in important cereal crops is a serious human and livestock health concern that also has the potential to cause drastic economic consequences. Therefore, it is absolutely necessary to protect crops from deoxynivalenol contamination. Presently, the use of fungicide is the most effective method for controlling deoxynivalenol contamination. However, inhibition of fungal growth may sometimes lead to the creation and spread of resistant fungal strains.[2] Therefore, developing other effective means of controlling deoxynivalenol contamination is crucial. To determine the optimal target for developing an effective method, it is very important to understand the basic regulatory mechanisms for deoxynivalenol production in the fungus.

Trichothecenes are biosynthesized from farnesyl pyrophosphate, which is produced via the mevalonate pathway (Figure 1). Several *Tri* genes are responsible for trichothecene biosynthesis from farnesyl pyrophosphate;[3] among them, *Tri6* and *Tri10* encode key regulatory proteins for trichothecene biosynthesis.[4−7] In *Fusarium sporotrichioides*, TRI10 is the first key regulator, which positively regulates TRI6 expression, whereas in *F. graminearum*,[4,5] TRI6 may be the main regulator because the *Tri10* deletion mutant was shown to have no obvious effect on TRI6 expression and deoxynivalenol production.[6] In both *Fusarium* species, the expression of *Tri* genes encoding trichothecene biosynthetic enzymes is under the positive control of TRI6. TRI6 also up-regulates the expression of genes encoding enzymes involved in the mevalonate pathway.[7] Upstream events that lead to TRI6 or TRI10 expression have not yet been clarified.

Some environmental and nutritional factors important for trichothecene production are known. Carbon sources,[8] pH,[9] and some amines [10] affect trichothecene production. Mg^{2+} and Mn^{2+} have been shown to suppress trichothecene production, whereas Fe^{2+} and Zn^{2+} have been shown to stimulate it at low and high concentrations, respectively.[11,12] However, the effect of Co^{2+} on trichothecene production in fungi has not been reported.

In the fungus *Cryptococcus neoformans*, $CoCl_2$ affects the regulatory system of ergosterol biosynthesis, similar to that found under low-oxygen conditions.[13,14] It has been shown that $CoCl_2$ induces expression of fungal genes homologously to SREBP (sterol regulatory element-binding protein) and SCAP (SREBP cleavage-activating protein), which leads to further up-regulation of the expression of many enzyme genes involved in ergosterol biosynthesis. Because similar findings were observed in *Schizosaccharomyces pombe*,[13,15] it has been suggested that the effects of $CoCl_2$ on ergosterol biosynthesis regulation are conserved in fungi. In *F. graminearum*, the mevalonate pathway is commonly used for trichothecene and ergosterol biosynthesis, and there is only one set of genes that encode enzymes involved in the mevalonate pathway in the fungus. Therefore, we planned to test the effects of $CoCl_2$ in *F. graminearum* with the expectation that $CoCl_2$ affects the regulatory system of not only ergosterol production but also that of trichothecene production.

In *Gibberella fujikuroi*, it has been speculated that ergosterol and gibberellin are biosynthesized from different pools of mevalonate, which originate from leucine and three acetates, respectively.[16] Therefore, we first performed feeding experiments of ^{13}C-labeled acetate and leucine to confirm the origin of mevalonate used for the biosynthesis of ergosterol and trichothecene in *F. graminearum*. Next, we examined the effects of $CoCl_2$ on

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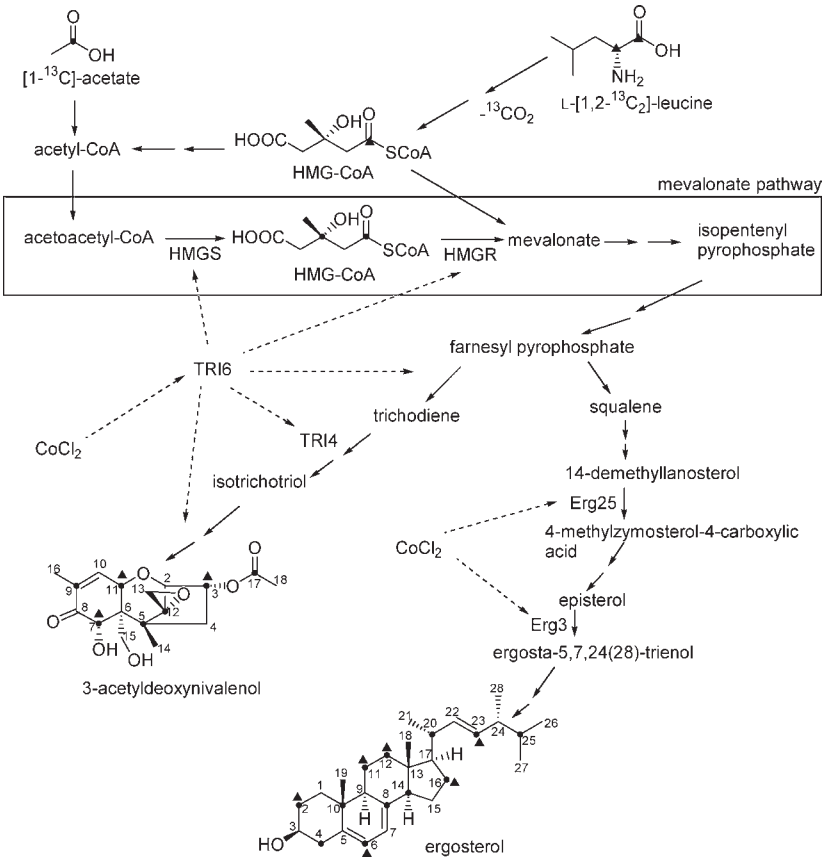

Figure 1. Biosynthesis of ergosterol and 3-acetyldeoxynivalenol and the effects of $CoCl_2$. Carbons that are labeled when 3-acetyldeoxynivalenol and ergosterol are biosynthesized via HMG-CoA from [1-^{13}C]-acetate and intact L-[1,2-$^{13}C_2$]-leucine are marked with ● and ▲, respectively.

ergosterol and trichothecene production and the expression of genes encoding enzymes and regulatory proteins involved in their biosynthesis. Finally, we tested whether or not precocene II, a specific inhibitor of trichothecene production,[17] could suppress the effects of $CoCl_2$.

MATERIALS AND METHODS

***F. graminearum* Culture Conditions and Analysis of 3-Acetyldeoxynivalenol and Ergosterol.** A Japanese isolate strain, *F. graminearum* MAFF101551, described previously,[18] was used as a 3-acetyldeoxynivalenol producer. A spore suspension of the strain was prepared using carnation leaf agar medium.[19] After cultivation of the strain on the medium at 28 °C for 10 days, the leaf was rinsed in 20% glycerol aqueous solution, and the mixture was filtered with miracloth to obtain a filtrate containing spores, which was stored at −80 °C and used as a spore suspension. 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 autoclaved. Autoclaved aqueous $CoCl_2$ solution (5 μL) and/or methanolic precocene II solution (15 μL) was added to the medium. 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 2−7 days. The resulting culture broth was filtered to obtain the mycelia and filtrate. The mycelia were washed with distilled water (5 mL) and collected into a 1.5 mL microtube. After the mycelia had undergone drying by lyophilization for 2 days, the mycelial weight was calculated by subtracting the weight of the 1.5 mL microtube without mycelia from the total weight. The dried mycelia were ground in a mortar with a pestle in liquid nitrogen and suspended in ethanol (10 mL). A 10% KOH aqueous solution (10 mL) was added to the suspension in a round-bottom flask, and the mixture was heated to reflux for 1 h. After the reaction mixture had been filtered, water (10 mL) was added to the obtained filtrate, and the solution was extracted with *n*-hexane (30 mL × 3). The *n*-hexane layer was evaporated, and the residue was analyzed by HPLC on a 250 mm × 4.6 mm i.d. Capcell-Pak C_{18} column with isocratic elution of 100% methanol at a flow rate of 1 mL/min and detection at 284 nm to quantify the amount of ergosterol (retention time, 8.5 min). To analyze the amount of 3-acetyldeoxynivalenol in the culture filtrate, the filtrate (1 mL) was extracted with 200 μL of ethyl acetate, the ethyl acetate solution was evaporated to dryness, and the obtained residue was dissolved in 200 μL of 10% acetonitrile in water, which was subjected to LC-MS analysis using a 2695 HPLC system (Waters, Milford, MA) equipped with a 150 mm × 2 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. The flow rate was 0.2 mL/min, and the retention time of 3-acetyldeoxynivalenol was 9.4 min. MS analysis was done with a micromassZQ (Waters) by ESI, in positive ion mode; spray chamber parameters were as follows: source temperature, 120 °C; desolvation 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 single-ion recording mode using the extracted ion m/z 339 $(M + H)^+$.

Feeding Experiments of ^{13}C-Labeled Compounds. Sodium [1-^{13}C]-acetate, 99 atom % (Sigma Aldrich, St. Louis, MO), or L-[1,2-$^{13}C_2$]-leucine, 99 atom % (Cambridge Isotope Laboratories, Inc., Andover, MA), was dissolved in distilled water, and the solution was autoclaved or passed through a sterile Millipore filter before administration. The solution (10 μL containing 2.5 mg of sodium [1-^{13}C]-acetate or 2.5 mg of L-[1,2-$^{13}C_2$]-leucine) was added to SYEP liquid medium (5 mL) with or without precocene II (30 μM) in test

tubes (1.6 cm × 18 cm). Each tube was inoculated with a spore suspension of *F. graminearum* and cultured for 7 days under the conditions mentioned above. In the case of later addition of labeled compound, the solution (10 μL containing 2.5 mg of sodium [1-^{13}C]-acetate or 2.5 mg of L-[1,2-$^{13}C_2$]-leucine) was added to the fungal culture in each test tube after 2 days of cultivation, and the culture was continued for an additional 5 days. The resulting culture broth was filtered to obtain the mycelia and filtrate. Labeled ergosterol was isolated from the mycelia according to the procedure for quantification of ergosterol mentioned above except for the use of a different column (250 mm × 10 mm i.d.) and a flow rate (2 mL/min) to afford 0.6, 1.2, 1.2, 1.3, 0.9, and 2.1 mg of labeled ergosterol from each 100 mL of culture broth (20 test tubes) in the feeding experiments of [1-^{13}C]-acetate without precocene II (day 0 addition), L-[1,2-$^{13}C_2$]-leucine without precocene II (day 0 addition), [1-^{13}C]-acetate with precocene II (day 0 addition), L-[1,2-$^{13}C_2$]-leucine with precocene II (day 0 addition), [1-^{13}C]-acetate (day 2 addition), and L-[1,2-$^{13}C_2$]-leucine (day 2 addition), respectively. Labeled 3-acetyldeoxynivalenol was purified from the culture filtrate as follows. The culture filtrate (100 mL) was extracted with ethyl acetate (50 mL × 3), and the ethyl acetate solution was concentrated. The residue was loaded onto a Wakogel C-200 silica gel column (5 g) packed with *n*-hexane and eluted stepwise with *n*-hexane (50 mL), *n*-hexane/ethyl acetate (90:10, v/v, 50 mL), *n*-hexane/ethyl acetate (80:20, v/v, 50 mL), *n*-hexane/ethyl acetate (70:30, v/v, 50 mL), and *n*-hexane/ethyl acetate (60:40, v/v, 50 mL). The *n*-hexane/ethyl acetate (60:40) fraction, which contained 3-acetyldeoxynivalenol as the main component, was concentrated, and the residue (yields of 1.0, 0.8, 2.4, and 1.7 mg from the feeding experiments of [1-^{13}C]-acetate (day 0 addition), L-[1,2-$^{13}C_2$]-leucine (day 0 addition), [1-^{13}C]-acetate (day 2 addition), and L-[1,2-$^{13}C_2$]-leucine (day 2 addition), respectively) was used for ^{13}C NMR analysis. ^{13}C NMR spectra were recorded on a JEOL JMN-A500 spectrometer at 125 MHz in $CDCl_3$.

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 3 days with or without $CoCl_2$. The mycelial cake was harvested by filtration and lyophilized. Total RNA was extracted 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′-GCACACCGATCCCAAGAATT-3′ and 5′-TCCATCGCTCAGGCTTGAAC-3′; *Tri6* 5′-CGCCCTTCCCAC-CTTCA-3′ and 5′-CGACTTGCAACTAGGGAATGG-3′; *erg3* 5′-TGATCCACGACGGCGAGTA-3′ and 5′-GCATGCAGCACCGTT-GAC-3′; *erg25* 5′-TGCTTCACCCATCGAAACTG-3′ and 5′-ACAAT-TGGGCATCCAACGA-3′; *HMGS* 5′-AACCCGGTCTGCGTGGTA-3′ and 5′-GGTCGGGCTTGTAGAAATCG-3′; *HMGR* 5′-TGAGAA-GCGAGTCAAAGAGATGAG-3′ and 5′-TCTTGCCGCGCATAGA-CA-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 ± SD. Differences between groups were assessed with one-way ANOVA followed by Dunnett's test. Values of $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Feeding Experiments of [1-^{13}C]-Acetate and L-[1,2-$^{13}C_2$]-Leucine. Incorporation experiments of ^{13}C-labeled acetate to study the biosynthetic origin of ergosterol and trichothecene in *F. graminearum* have been done by Blackwell et al.[20] They showed that both ergosterol and trichothecene are biosynthesized via a mevalonate pathway using crude ^{13}C-labeled samples. We performed feeding experiments of [1-^{13}C]-acetate and L-[1,2-$^{13}C_2$]-leucine to confirm the origin of mevalonate in their biosynthesis. A strain of *F. graminearum* that produces 3-acetyldeoxynivalenol in liquid culture was inoculated into medium, to which a labeled compound was added at the beginning of cultivation. The time course of ergosterol production was parallel to fungal growth, whereas production of 3-acetyldeoxynivalenol began after 2 days of cultivation. After 7 days of cultivation at 26.5 °C, labeled ergosterol and 3-acetyldeoxynivalenol were isolated from the culture broth and their ^{13}C NMR spectra were measured (Table 1). In the feeding of [1-^{13}C]-acetate, high enrichment at the carbons showing the biosynthesis via a mevalonate pathway was observed in the labeled ergosterol. A similar labeling pattern was observed in ergosterol labeled with L-[1,2-$^{13}C_2$]-leucine, but a little higher enrichment was clearly observed at C-2, -6, -12, -16, and -23 than at the other enriched carbons (C-4, -8, -10, -14, -20, and -25) in the molecule. This suggested that mevalonate from three acetate molecules was mainly used for ergosterol biosynthesis in the fungus, but mevalonate via HMG-CoA from intact leucine was also able to be partially used (Figure 1). In the case of 3-acetyldeoxynivalenol, it was shown that mevalonate from three acetates was used for its biosynthesis. It was suggested that HMG-CoA from intact leucine was not used for the biosynthesis of 3-acetyldeoxynivalenol, although it was unclear due to weak enhancement. Because this weak enhancement may be due to later production of 3-acetyldeoxynivalenol than ergosterol, [1-^{13}C]-acetate or L-[1,2-$^{13}C_2$]-leucine was fed to the fungal culture after 2 days of cultivation. The ^{13}C NMR spectra of labeled ergosterol and 3-acetyldeoxynivalenol obtained after a total of 7 days of cultivation showed the same labeling patterns as those observed in the feeding experiments of early addition, although lower and higher incorporations of labeled acetate into the ergosterol and 3-acetyldeoxynivalenol, respectively, than in the cases of early addition were observed. In this later addition experiments, incorporation of HMG-CoA from intact leucine into ergosterol was observed, but its incorporation into 3-acetyldeoxynivalenol was not observed, similarly to the case of early addition mentioned above. Our results showed that mevalonate from three acetates was basically used for the biosynthesis of ergosterol and 3-acetyldeoxynivalenol, although HMG-CoA from intact leucine was able to be partially used for ergosterol production.

Effects of $CoCl_2$ on Production of Ergosterol and 3-Acetyldeoxynivalenol and on Transcription of Genes Encoding Enzymes and Regulatory Protein Involved in Their Biosynthesis. *F. graminearum* was cultured in medium containing 0−100 μM $CoCl_2$ for 7 days, and the mycelial weight and amounts of ergosterol and 3-deoxynivalenol in each culture were measured. Fungal mycelial weight and the amount of ergosterol were not affected by $CoCl_2$ at concentrations of <30 μM, but they decreased with concentrations of >50 μM (Figure 2A). In contrast, production of 3-acetyldeoxynivalenol was strongly enhanced by the addition of $CoCl_2$ at concentrations of 3−30 μM (Figure 2B).

Table 1. ^{13}C Abundances in Ergosterol and 3-Acetyldeoxynivalenol Obtained from Feeding Experiments with ^{13}C Precursors

		relative ^{13}C abundance[a]					
		[1-^{13}C]-acetate		[1-^{13}C]-acetate + precocene II[b]	L-[1,2-$^{13}C_2$]-leucine		L-[1,2-$^{13}C_2$]-leucine + precocene II[b]
carbon no.	δ_C	day 0[c]	day 2[d]	day 0[c]	day 0[c]	day 2[d]	day 0[c]
ergosterol							
1	38.4	1.2	1.2	1.4	1.1	0.9	1.1
2	32.0	10.6	5.1	14.0	3.6	2.6	4.1
3	70.4	1.0	0.7	1.1	0.9	1.1	1.0
4	40.8	10.2	5.0	11.9	2.7	2.1	3.0
5	139.8	1.1	1.3	1.1	0.9	1.1	0.8
6	119.6	9.8	5.2	12.7	3.5	2.7	3.7
7	116.3	0.7	1.6	1.1	0.9	1.0	1.1
8	141.3	10.1	4.2	12.7	2.6	1.7	2.9
9	46.2	0.8	1.0	1.1	1.1	1.0	1.1
10	37.0	9.6	6.3	11.9	3.2	2.3	3.1
11[e], 21[e]	21.1	5.4	2.1	7.2	2.0	1.9	2.1
12	39.1	10.7	4.8	12.9	3.3	2.7	3.7
13, 24	42.8	0.9	1.3	1.2	0.9	1.0	0.9
14	54.5	9.3	3.7	12.0	2.7	2.0	2.8
15	23.0	1.0	0.7	1.3	1.1	0.9	0.9
16	28.3	9.0	5.0	11.7	3.4	2.6	3.7
17	55.7	1.0	1.3	1.0	1.0	1.0	1.0
18	12.0	0.9	1.1	1.4	1.0	1.0	1.0
19	16.3	1.2	1.0	1.2	1.0	1.0	0.9
20	40.4	10.4	5.4	11.0	2.9	2.0	3.1
22	135.6	0.8	1.1	1.1	1.1	1.0	1.2
23	131.9	10.3	5.9	13.3	3.5	2.8	3.7
25	33.1	8.6	6.6	12.0	2.4	2.1	2.8
26	19.9	0.8	1.1	1.0	0.9	1.0	1.1
27	19.6	1.1	1.0	1.2	0.9	1.1	0.8
28	17.6	0.9	0.6	1.6	1.0	1.1	1.1
3-acetyldeoxynivalenol							
2	79.0	1.0	0.8		1.0	1.0	
3	71.2	2.1	5.5		2.2	1.9	
4	40.4	1.1	0.8		1.0	0.9	
5	45.8	1.9	5.4		1.7	1.6	
6	52.0	1.0	0.9		1.0	1.0	
7	70.1	2.2	6.3		2.1	1.9	
8	199.8	0.6	1.0		0.8	1.0	
9	135.9	2.1	4.4		1.9	2.0	
10	138.4	1.1	0.8		1.2	1.0	
11	74.4	2.1	5.9		2.1	2.0	
12	65.1	1.8	6.0		1.6	2.2	
13	47.5	1.1	0.8		1.2	1.0	
14	14.1	1.2	1.0		1.1	1.0	
15	62.4	0.9	0.9		1.1	0.9	
16	15.3	0.9	0.9		1.0	0.9	
17	170.4	1.5	5.7		1.9	1.6	
18	21.0	1.0	1.0		1.0	1.0	

[a] Peak height ratio of ^{13}C enriched to natural abundance. [b] 30 μM. Production of 3-acetyldeoxynivalenol was completely inhibited. [c] Labeled compound and precocene II were added at the beginning of the cultivation. [d] Labeled compound was added after 2 days of cultivation. [e] Peak overlapping and broadening.

Next, the effects of $CoCl_2$ on the mRNA levels of *Tri4* (encoding a trichothecene biosynthetic enzyme), *Tri6* (encoding a regulatory protein involved in trichothecene biosynthesis), *erg3* and *erg25* (encoding ergosterol biosynthetic enzymes), and *HMGS* and *HMGR* (encoding hydroxymethyl-glutaryl CoA synthase and hydroxymethyl-glutaryl CoA reductase, respectively), which

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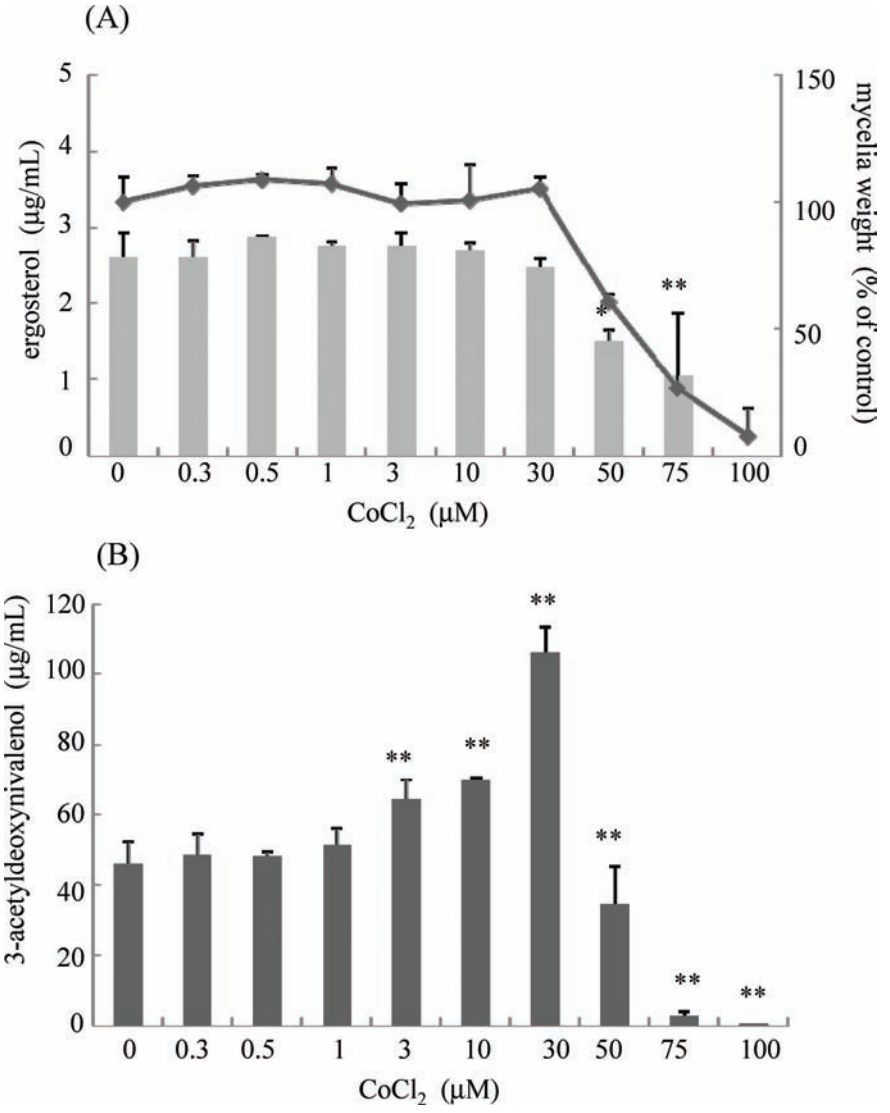

Figure 2. Effects of $CoCl_2$ on ergosterol production and fungal growth (A) and 3-acetyldeoxynivalenol production (B). *F. graminearum* was cultured in SYEP liquid medium with $CoCl_2$ for 7 days. The mycelial weight (◆), ergosterol amount (gray bar), and 3-acetyldeoxynivalenol amount (black bar) were analyzed. Data are presented as the mean ± SD ($n = 3$). Differences were assessed by one-way ANOVA, followed by the Dunnett test. *, $P < 0.05$, **, $P < 0.01$, versus the control.

are enzymes involved in the mevalonate pathway, were examined (Figure 1). *F. graminearum* was cultured in a medium with 30 μM $CoCl_2$ for 3 days. Total RNA was extracted from the mycelia, and the mRNA level was analyzed by quantitative RT-PCR. Cobalt chloride dramatically enhanced the mRNA levels of all these genes as shown in Figure 3. The up-regulation of *erg3* and *erg25* through the action of the SREBP-like regulatory protein Sre1 in the presence of $CoCl_2$ has been observed in *C. neoformans*,[13] suggesting that a similar regulatory system might be present in *F. graminearum*.

Effects of Precocene II on the Action of $CoCl_2$. Precocene II inhibits 3-acetyldeoxynivalenol production of *F. graminearum* in liquid culture with an IC_{50} value of 1.2 μM, without affecting fungal growth.[17] It suppresses transcription of *Tri6* and *Tri10*, which leads to inhibition of expression of trichothecene biosynthetic enzymes and production of 3-acetyldeoxynivalenol. The target molecule of precocene II has not yet been identified. Next, we used precocene II to investigate the mode of action of $CoCl_2$ on trichothecene production.

We confirmed that precocene II (30 μM) did not affect ergosterol production of *F. graminearum* (data not shown). Feeding experiments of [1-^{13}C]-acetate and L-[1,2-$^{13}C_2$]-leucine in the presence of precocene II (30 μM) indicated that the origin of mevalonate used for ergosterol biosynthesis was the same as in culture without precocene II (Table 1). The fungus was cultured in medium containing both $CoCl_2$ (30 μM) and precocene II (0−300 μM) for 5 days. The mycelial weight of the fungus was not affected by the co-addition (Figure 4). The 3-acetyldeoxynivalenol production, which was much promoted by $CoCl_2$ as mentioned above, was inhibited by the co-addition of precocene II in a dose-dependent manner, and complete inhibition was observed with 300 μM precocene II (Figure 4). This precocene II concentration (300 μM) needed for complete inhibition of 3-acetyldeoxynivalenol was much higher than that (30 μM) in the case of culture without $CoCl_2$.

When *F. graminearum* was cultured with both $CoCl_2$ (30 μM) and precocene II (30 μM) for 3 days, the mRNA levels of *Tri6*, *Tri4*, *HMGS*, and *HMGR* were much lower than those found

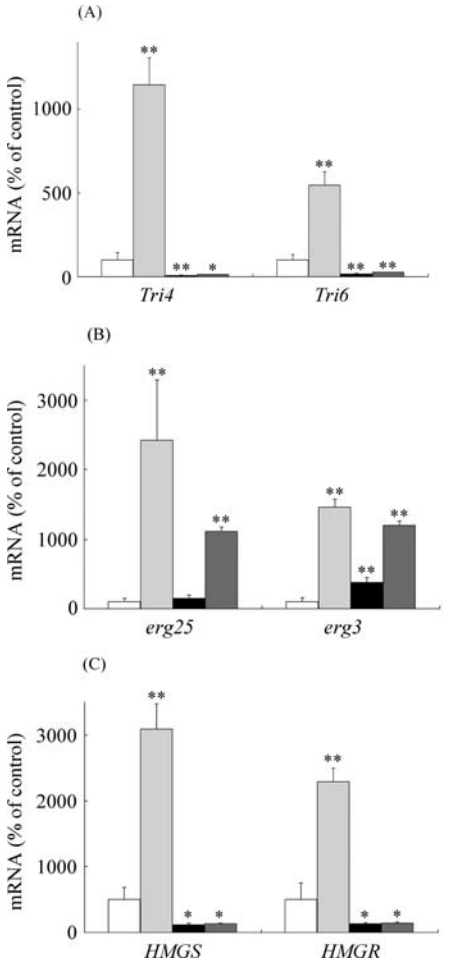

Figure 3. Effects of precocene II and $CoCl_2$ on the transcription of genes encoding trichothecene biosynthesis enzymes (A), ergosterol biosynthesis enzymes (B), and enzymes involved in the mevalonate pathway (C). Total RNA was prepared from *F. graminearum* cultured in SYEP liquid medium for 3 days. Transcription of each gene was analyzed by real-time quantitative PCR (white bar, control; light gray bar, $CoCl_2$; black bar, precocene II; dark gray bar, $CoCl_2$ and precocene II). The amount of each mRNA was normalized to the amount of β-tubulin mRNA in each sample. Data are the mean ± SD (n = 3). Differences between the amounts of mRNA were assessed by one-way ANOVA, followed by the Dunnett test. ∗, $P < 0.05$, ∗∗, $P < 0.01$, versus the control.

with only $CoCl_2$ addition (Figure 3). Furthermore, the levels observed by the co-addition were lower than the control level and near the level in the case of sole addition of precocene II. Reduction of the mRNA levels of *Tri* genes by the addition of $CoCl_2$ (30 μM) and precocene II (30 μM) was more remarkable than that of 3-acetyldeoxynivalenol production amount (Figure 4), which was the same as observed in the case of sole addition of precocene II [17]. On the other hand, the mRNA levels of *erg3* and *erg25*, which were much enhanced by $CoCl_2$ addition, decreased somewhat by the co-addition of precocene II. However, these mRNA levels maintained a much higher level than that of the control (Figure 3).

Transcription of *HMGS* and *HMGR* is known to be up-regulated by TRI6 in *F. graminearum*[6] or by Sre1 in the presence of $CoCl_2$ in *C. neoformans*,[13] similar to the cases of *erg3* and *erg25*. Our results suggested that strong up-regulation of *Tri6* by $CoCl_2$ may lead to high mRNA levels of *HMGS* and *HMGR* because

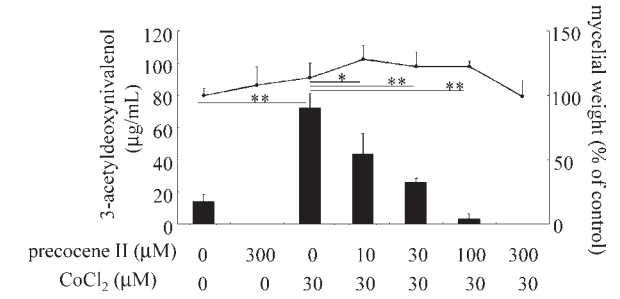

Figure 4. Effects of precocene II and $CoCl_2$ on 3-acetyldeoxynivalenol and fungal growth. *F. graminearum* was cultured in SYEP liquid medium for 5 days. The amount of 3-acetyldeoxynivalenol (black bar) and the mycelial weight (●) were analyzed. Data are presented as the mean ± SD (n = 3). Differences were assessed by one-way ANOVA, followed by the Dunnett test. ∗, $P < 0.05$, ∗∗, $P < 0.01$, versus the control.

their transcription was lowered by co-addition of $CoCl_2$ and precocene II to the same level as that in the case of sole addition of precocene II. It is not clear if a regulatory system leading to up-regulation of *erg25* and *erg3* in the presence of $CoCl_2$ is related to up-regulation of *Tri6*. It may be possible to speculate the presence of a regulatory system that up-regulates transcription of both genes responsible for ergosterol and trichothecene biosynthesis in the presence of $CoCl_2$.

In conclusion, $CoCl_2$ clearly enhanced trichothecene production through activation of *Tri6* transcription in *F. graminearum*. The mode of action of $CoCl_2$ may afford a clue to clarify the regulatory mechanism of trichothecene production. Basic information on the regulatory mechanism is very important in the development of effective methods for controlling deoxynivalenol contamination.

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