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Effects of a bile acid elicitor, cholic acid, on the biosynthesis of diterpenoid phytoalexins in suspension-cultured rice cells

Takafumi Shimizu [a,1], Yusuke Jikumaru [a,1], Atsushi Okada [a], Kazunori Okada [a], Jinichiro Koga [b], Kenji Umemura [c], Eiichi Minami [d], Naoto Shibuya [e], Morifumi Hasegawa [f], Osamu Kodama [f], Hideaki Nojiri [a], Hisakazu Yamane [a,*]

[a] *Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b] *Food and Health R&D Laboratories, Meiji Seika Kaisha Ltd., Sakado, Saitama 350-0289, Japan*
[c] *Agricultural and Veterinary Research Laboratories, Meiji Seika Kaisha Ltd., Kohoku-ku, Yokohama 222-8567, Japan*
[d] *Department of Biochemistry, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba 305-8602, Japan*
[e] *Department of Life Sciences, School of Agriculture, Meiji University, Kawasaki, Kanagawa 214-8571, Japan*
[f] *College of Agriculture, Ibaraki University, 3-21-1 Chuo, Ami, Ibaraki 300-0393, Japan*

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Abstract

An elicitor of rice defense responses was recently isolated from human feces and was identified as cholic acid (CA). Pathogen infection in rice leaves induces phytocassanes and momilactones, both of which are major diterpenoid phytoalexins in rice, whereas CA mainly induces phytocassanes. We established a high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry protocol for the rapid and accurate quantification of phytocassanes and momilactones. Using this method, we showed that CA preferentially induced the formation of phytocassanes in suspension-cultured rice cells, while a fungal chitin oligosaccharide elicitor induced that of both phytocassanes and momilactones. We further investigated the effects of CA on the expression of diterpene cyclase genes involved in phytoalexin biosynthesis. CA induced the transcription of the genes *OsCPS2* (*OsCyc2*) and *OsKSL7* (*OsDTC1*), which are involved in phytocassane biosynthesis, to a greater extent than the genes *OsCPS4* (*OsCyc1*) and *OsKSL4*, which are involved in momilactone biosynthesis. *OsCPS2* was particularly strongly induced, suggesting that it is one of the main mechanisms by which CA induces high levels of phytocassanes.

Keywords: Index *Oryza sativa*; Gramineae; Rice suspension cells; Phytoalexin; Phytocassane; Diterpene cyclase gene; Cholic acid; HPLC–MS/MS

1. Introduction

When plants are attacked by pathogenic microorganisms, they respond with a variety of defense reactions, including the production of secondary metabolites called phytoalexins, which serve as plant antibiotics. Four structurally distinct types of polycyclic diterpenoid phytoalexins have been identified from the leaves of rice (*Oryza sativa*) that were either infected with the rice leaf blast pathogen, *Magnaporthe grisea*, or exposed to ultraviolet (UV) irradiation; these include phytocassanes A–E (**1–5**); oryzalexins A–F (**6–11**); momilactones A (**12**) and B (**13**); and oryzalexin S (**14**) (Fig. 1) (Cho et al., 2004; Koga et al., 1995, 1997; Peters, 2006). These phytoalexins are all biosynthesized from a common substrate, geranylgeranyl diphosphate (GGDP) (**15**), via a two-step sequential cyclization to give *ent*-cassa-12,15-diene (**16**), *ent*-sandaracopimaradiene (**17**), 9βH-pimara-7,15-diene (**18**), or stemar-13-ene (**19**), followed by several oxidation steps (see Fig. 2). All of the diterpene cyclase genes involved in these reactions have

* Corresponding author. Tel.: +81 3 5841 3067; fax: +81 3 5841 8030.
E-mail address: ayamane@mail.ecc.u-tokyo.ac.jp (H. Yamane).
[1] These authors contributed equally to this work.

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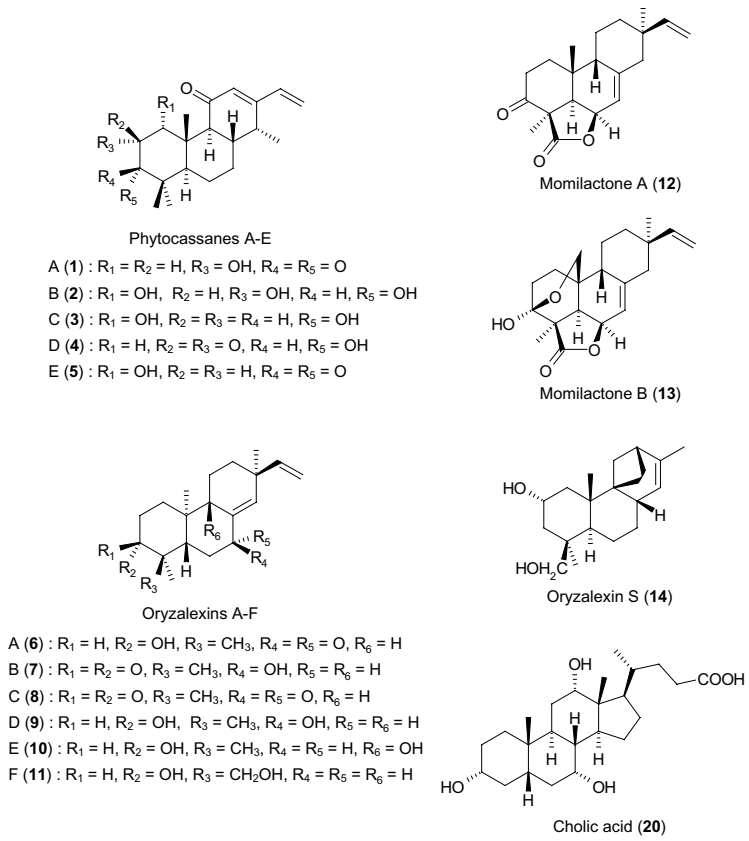

Fig. 1. Chemical structures of rice diterpenoid phytoalexins (phytocassanes A–E (**1–5**), olyzalexins A–F (**6–11**), momilactones A (**12**) and B (**13**), and olyzalexin S (**14**)) and the bile acid elicitor cholic acid (CA, **20**).

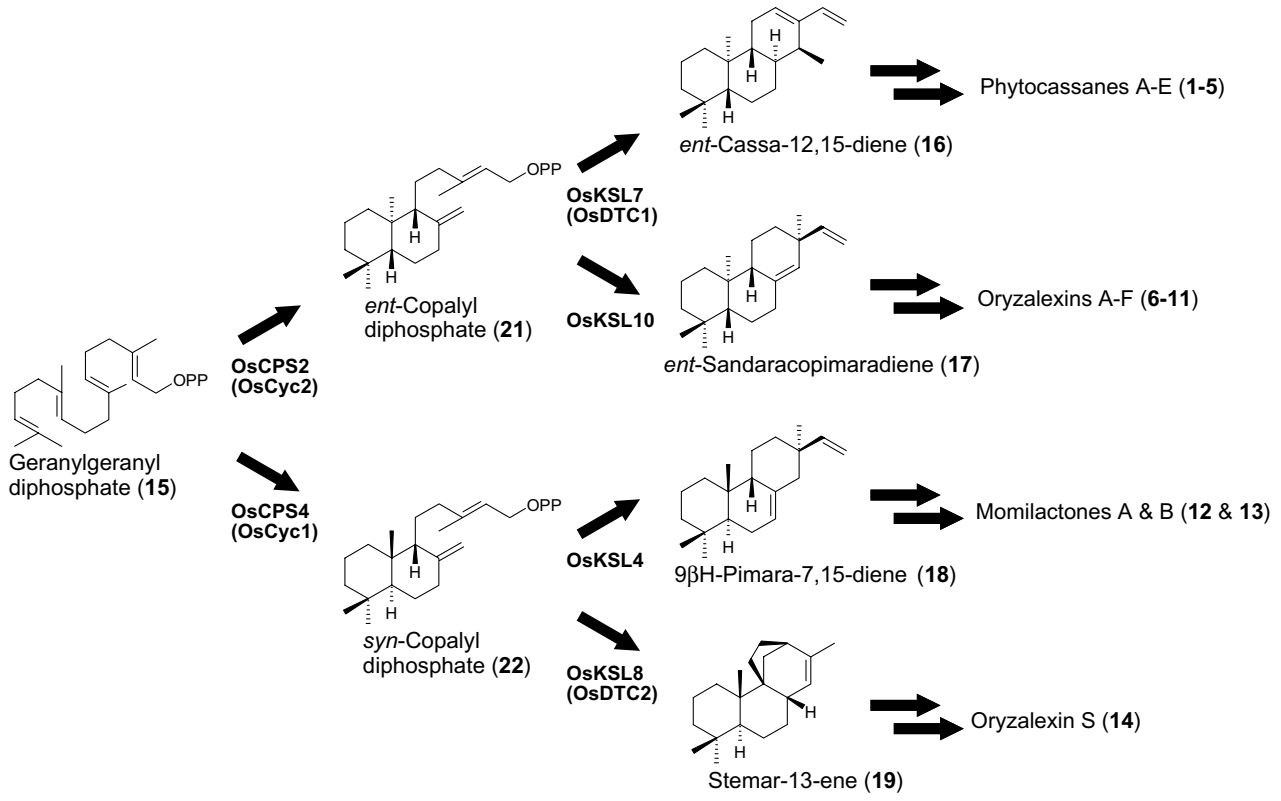

Fig. 2. Proposed pathways for the biosynthesis of diterpenoid phytoalexins in rice.

been identified (Fig. 1; Cho et al., 2004; Nemoto et al., 2004; Otomo et al., 2004a,b; Prisic et al., 2004; Wilderman et al., 2004; Xu et al., 2004; Peters, 2006).

An elicitor of rice defense responses was recently isolated from human feces (Koga et al., 2006). This elicitor was identified as cholic acid (CA, **20**; see Fig. 1), a primary bile acid in animals. Treatment of rice leaves with CA (**20**) induced phytoalexin production, hypersensitive cell death, and pathogenesis-related protein synthesis. These defense responses have the effect of increasing resistance to subsequent infections by virulent pathogens. Pathogen infection in rice leaves induces phytocassanes (**1–5**) and momilactones (**12** and **13**) (Koga et al., 1995), both of which are major diterpenoid phytoalexins in rice, whereas CA (**20**) treatment mainly induces the production of phytocassanes (**1–5**), with negligible production of momilactones (**12** and **13**) (Koga et al., 2006). The mechanisms for the preferential production of phytocassanes (**1–5**) by CA (**20**) are unknown.

Phytocassanes (**1–5**) and momilactones (**12** and **13**) have also been identified as major phytoalexins in suspension-cultured rice cells by treatment with a chitin oligosaccharide elicitor (Yamada et al., 1993; Umemura et al., 2002). In the present study, we established a high-performance liquid chromatography–electrospray ionization-tandem mass spectrometry (HPLC–ESI-MS/MS) protocol for the rapid and accurate quantification of the major phytoalexins, phytocassanes (**1–5**) and momilactones (**12** and **13**). Using this method, we examined the effects of CA (**20**) and the chitin oligosaccharide elicitor on the production of phytocassanes (**1–5**) and momilactones (**12** and **13**) in suspension-cultured rice cells to investigate whether phytocassanes are produced preferentially by CA (**20**) in cultured rice cells as well as in rice leaves. We also examined the effects of CA (**20**) and the chitin oligosaccharide elicitor on the expression of the diterpene cyclase genes involved in the biosynthesis of phytocassanes (**1–5**) and momilactones (**12** and **13**). In suspension-cultured rice cells, CA (**20**) preferentially induced phytocassanes (**1–5**) and the expression of diterpene cyclase genes involved in phytocassane biosynthesis.

2. Results and discussion

2.1. HPLC–ESI-MS/MS for the quantification of phytocassanes (1–5) and momilactones (12 and 13)

The levels of the major rice diterpenoid phytoalexins, phytocassanes (**1–5**) and momilactones (**12** and **13**), were quantified using gas chromatography–MS or HPLC (Nojiri et al., 1996; Umemura et al., 2002). An HPLC system interfaced with an ESI-tandem mass spectrometer provides a method of quantification that does not require complicated purification steps, derivatization, or elevated temperatures. This technique was expected to be a powerful tool for the rapid and accurate estimation of minute amounts of phytoalexins. In the present study, we were able to establish an HPLC–ESI-MS/MS analytical system for the quantification of phytocassanes (**1–5**) and momilactones (**12** and **13**).

The multiple reaction monitoring (MRM) mode was used in ESI-MS/MS to monitor the precursor and product ions. Momilactones A (**12**) and B (**13**) (Fig. 1) yielded the precursor ions as protonated molecular ions at m/z 315 and m/z 331, respectively, and their characteristic product ions at m/z 271 ($[M+H-CO_2]^+$) and m/z 269 ($[M+H-H_2O-CO_2]^+$), respectively, in the positive ion mode. Phytocassanes A–E (**1–5**) (Fig. 1) also gave the precursor ions as protonated molecular ions (m/z 317 for phytocassanes A (**1**), D (**4**), and E (**5**); m/z 335 for phytocassane B (**2**); m/z 319 for phytocassane C (**3**)) and their characteristic product ions as $[M+H-H_2O]^+$ ions (m/z 299 for phytocassanes A, D, and E; m/z 317 for phytocassane B; m/z 301 for phytocassane C) in the positive ion mode. Thus, diterpenoid phytoalexin levels were determined with combinations of m/z 315/271 for momilactone A (**12**); m/z 331/269 for momilactone B (**13**); m/z 317/299 for phytocassanes A (**1**), D (**4**), and E (**5**); m/z 335/317 for phytocassanes B (**2**); and m/z 319/301 for phytocassane C (**3**) in the MRM mode (Fig. 3). Although the same combination of precursor ion at m/z 317 and product ion at m/z 299 was used to measure phytocassanes A (**1**), D (**4**), and E (**5**), these were discriminated on the basis of their retention times in HPLC. Using the HPLC–ESI-MS/MS conditions that we established, all of the tested diterpenoid phytoalexins (**1–5**, **12** and **13**) could be quantified within a range of 0.1–50 ng.

Currently, standard samples of *ent*-sandaracopimaradiene type and stemar-13-ene type phytoalexins (oryzalexins A–F (**6–11**) and S (**14**)) are not available, except for oryzalexin A (**6**), and the HPLC–MS/MS analytical methods for quantification of these phytoalexins remains to be investigated.

2.2. Phytoalexin production in response to treatment with a chitin oligosaccharide elicitor or CA (20)

CA (**20**) preferentially induces phytocassane synthesis in rice leaves (Koga et al., 2006), whereas a chitin oligosaccharide induces production of both phytocassanes (**1–5**) and momilactones (**12** and **13**) in suspension-cultured rice cells (Umemura et al., 2002). By using the HPLC–ESI-MS/MS quantification system established in the present study, we compared the effects of a chitin oligosaccharide and CA (**20**) on the production of phytocassanes (**1–5**) and momilactones (**12** and **13**) in suspension-cultured rice cells, in order to examine whether phytocassane-specific mechanisms operate in CA (**20**)-treated rice cells.

Phytoalexins were extracted from the culture medium 48 h after CA (**20**) treatment and were quantified using HPLC–ESI-MS/MS. CA (**20**) treatment induced the dose-dependent accumulation of phytocassanes (**1–5**), increasing at least until 2 mM CA (**20**) (Fig. 4). CA (**20**)

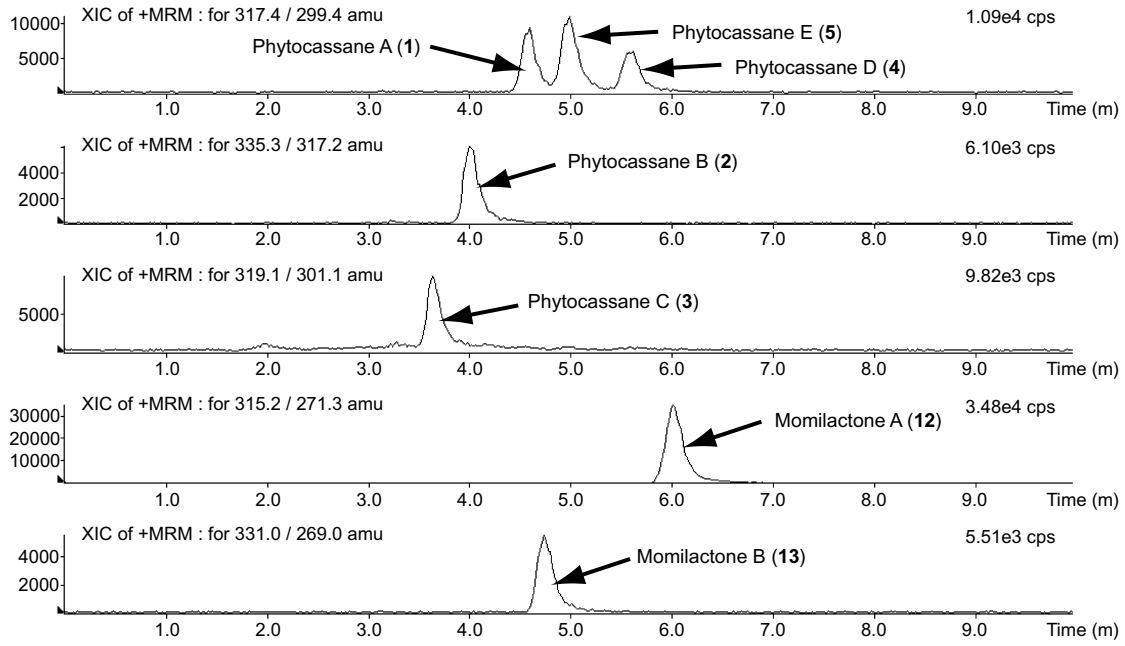

Fig. 3. Multiple reaction monitoring chromatograms for phytocassanes (**1**–**5**) and momilactones (**12** and **13**). An aliquot (5 μL) of a solution mixture of purified phytocassanes A–E (**1**–**5**) (0.1 ng each) and momilactones A (**12**) and B (**13**) (0.1 ng each) was injected onto a RP-HPLC column, with the eluate monitored using ESI-MS/MS.

only slightly induced accumulation of momilactones (**12** and **13**), even at 2 mM. Thus, in the following experiments, rice cells were treated with 2 mM CA (**20**).

A time-course study was conducted to measure the induction of accumulation of phytocassanes (**1**–**5**) and momilactones (**12** and **13**) in suspension-cultured rice cells treated with either the chitin oligosaccharide *N*-acetylchitooctaose (0.6 μM) or CA (**20**) (2 mM). Phytoalexins that accumulated in the culture medium of the suspension-cultured rice cells were sampled at 0, 4, 8, 12, 24, 48, 72, 96, and 120 h after the addition of elicitors. The chitin oligosaccharide led to an increase, beginning at 8 h, in the level of phytocassanes (**1**–**5**), which peaked at 167 μg/g fresh weight at 48 h and gradually decreased thereafter (Fig. 5a). The level of momilactones (**12** and **13**) increased beginning at 8 h and reached a maximum of 76 μg/g fresh weight at 48 h; this maximum was still maintained at the final measurement at 120 h. CA (**20**) treatment led to an increase in phytocassanes (**1**–**5**) beginning at 48 h, and the level continued to increase through to the final measurement of 667 μg/g fresh weight at 120 h (Fig. 5b), at which time the level was approximately four-times the maximum level induced by the chitin oligosaccharide. Momilactones (**12** and **13**) also increased from 48 h through 120 h in response to CA (**20**). However, the level of momilactones (**12** and **13**) was less than half the maximum in the chitin oligosaccharide treatment, at 33 μg/g fresh weight, even at 120 h. These results indicate that, similar to its effect in rice leaves, CA (**20**) preferentially induces accumulation of phytocassanes (**1**–**5**) over momilactones (**12** and **13**) in suspension-cultured rice cells and that the effect of CA (**20**) is different from that of the chitin oligosaccharide in terms of both the timing and the magnitude of the change in phytoalexin accumulation. The differences in the time-course of induction between CA (**20**) and the chitin oligosaccharide suggest that different regulatory mechanisms control the responses to the two elicitors in rice cells.

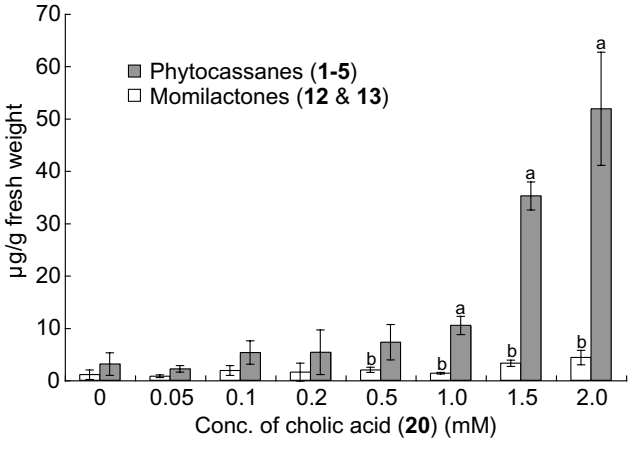

Fig. 4. Cholic acid (**20**) dose responses for total amounts of phytocassanes A to E (**1**–**5**) and momilactones A (**12**) and B (**13**) accumulated in the medium of suspension-cultured rice cells 48 h after treatment. The phytoalexin contents were quantified using HPLC–ESI-MS/MS. The results are expressed as the mean ± standard error of three replicates. Means designated by letters are significantly different at $^{a}P < 0.01$ and $^{b}P < 0.05$ compared to the value at 0 mM (*t*-test).

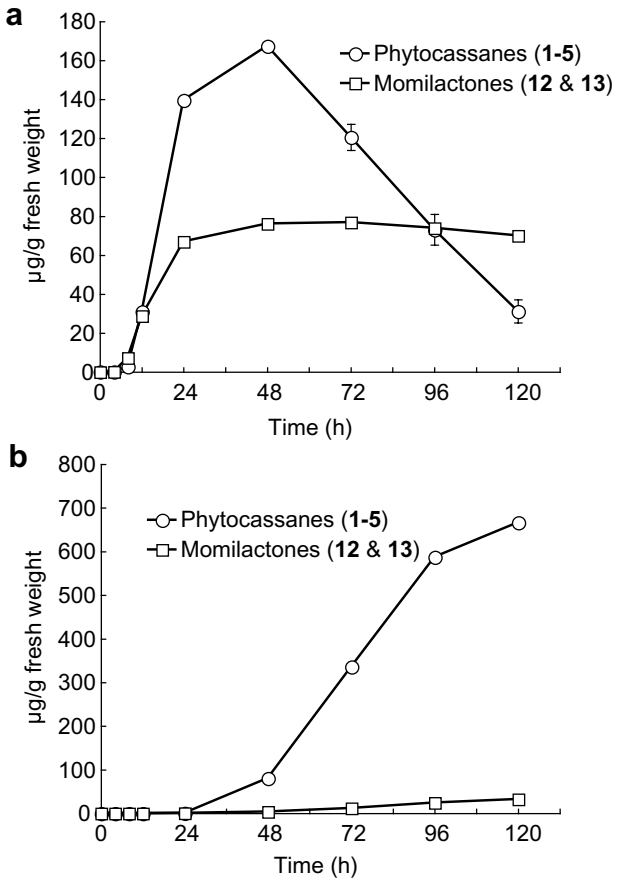

Fig. 5. Induction of phytocassanes (**1–5**) and momilactones (**12** and **13**) by (a) a chitin oligosaccharide, *N*-acetylchitooctaose, or (b) cholic acid (**20**) in suspension-cultured rice cells. The total amounts of phytocassanes A–E (**1–5**) and momilactones A (**12**) and B (**13**) accumulated in the culture medium of the rice cells from 0 to 120 h after treatment with 0.6 μM chitin oligosaccharide or 2 mM cholic acid (**20**) were quantified using HPLC–ESI-MS/MS. The results are expressed as the mean ± standard error of three experiments. In the control medium, the levels of phytocassanes (**1–5**) and momilactones (**12** and **13**) were less than 1.5 ng/g fresh weight of rice cells throughout the experiment.

*2.3. Effects of treatment with the chitin oligosaccharide or CA (**20**) on the expression of diterpene cyclase genes involved in the biosynthesis of diterpenoid phytoalexins*

As described in Section 2.2, treatment with the chitin oligosaccharide induced the accumulation of both phytocassanes (**1–5**) and momilactones (**12** and **13**) in suspension-cultured rice cells, whereas treatment with CA (**20**) mainly induced accumulation of phytocassanes (**1–5**). To investigate the mechanisms regulating the preferential accumulation of phytocassanes (**1–5**) by CA (**20**) in suspension-cultured rice cells, time-course analysis of diterpene cyclase gene transcripts involved in the biosynthesis of phytocassanes (**1–5**) and momilactones (**12** and **13**) was examined by real-time reverse transcription polymerase chain reaction (RT-PCR). The chitin oligosaccharide led to increases in the mRNA levels of *OsCPS4, OsCPS2, OsKSL4,* and *OsKSL7*, which peaked at 4–8 h after treatment and thereafter decreased rapidly (Figs. 6a–d). In contrast, CA induced increasing levels of *OsCPS2* and *OsKSL7* mRNA from 48 h through 120 h (Figs. 6b and d). These two genes are involved in the biosynthesis of phytocassanes (**1–5**). The levels of *OsCPS2* and *OsKSL7* mRNA at 120 h after treatment were about 330% and 56%, respectively, of the maximum levels detected following chitin oligosaccharide induction. Although the mRNA levels of *OsCPS4* and *OsKSL4*, which are involved in the biosynthesis of momilactones (**12** and **13**), also increased from 48 h through 120 h, these mRNA levels, even at 120 h after CA (**20**) treatment, were about 27% and 20%, respectively, of the maximum levels induced by the chitin oligosaccharide (Figs. 6a and c). These results indicate that, as with the accumulation of phytoalexins, differences exist in both the timing and levels of mRNA accumulation between the responses to CA (**20**) and the chitin oligosaccharide. Compared with the chitin oligosaccharide, CA (**20**) induced a slower accumulation of diterpene cyclase mRNA; however, the induction continued until the final measurement at 120 h. The mRNA increases correspond to those of CA (**20**)-induced phytoalexin accumulation (Fig. 5b and 6). It is also noteworthy that CA (**20**) very strongly induced the accumulation of the phytocassane biosynthetic gene *OsCPS2* (Fig. 6b), suggesting that it is one of the main mechanisms by which CA (**20**) induces high levels of phytocassanes (**1–5**).

The observed stronger induction of the *ent*-CDP (**21**) synthase gene *OsCPS2* relative to the *syn*-CDP (**22**) synthase gene *OsCPS4* suggests that the production of the *ent*-CDP (**21**)-derived oryzalexins A-F (**6-11**) may be strongly induced by CA (**20**), as observed with the *ent*-CDP (**21**)-derived phytocassanes. We therefore quantified oryzalexin A (**6**), which is a major component of oryzalexins A-F (**6-11**), by gas chromatography-selected ion monitoring (GC-SIM); we could not determine the levels of oryzalexins B-F (**7-11**) because of the unavailability of standard samples of these phytoalexins. With CA (**20**) treatment, the level of oryzalexin A (**6**) was about one-hundredth the level of total phytocassanes (**1–5**) even at 120 h, indicating that the production of oryzalexins A–F (**6–11**) is negligible in CA (**20**)-treated suspension-cultured rice cells. In fact, it was indicated by RT-PCR that, in CA (**20**)-treated suspension-cultured rice cells, the mRNA of *OsKSL10* involved in biosynthesis of oryzalexins A–F (**6–11**) was at undetectable levels at 0–96 h and at trace levels at 120 h after treatment (data not shown). These results suggest that the strong induction of *OsCPS2* in the CA (**20**)-treated rice cells contributes primarily to the abundant accumulation of phytocassanes (**1–5**). On the other hand, the observed weaker induction of the *syn*-CDP (**22**) synthase gene *OsCPS4* relative to *OsCPS2* suggests that the production of the *syn*-CDP (**22**)-derived oryzalexin S (**14**) is not strongly induced by CA (**20**) treatment, although we did not determine the levels of oryzalexin S (**14**) because of the unavailability of a standard sample. In fact, real-time RT-PCR analysis indicated that, although the mRNA level

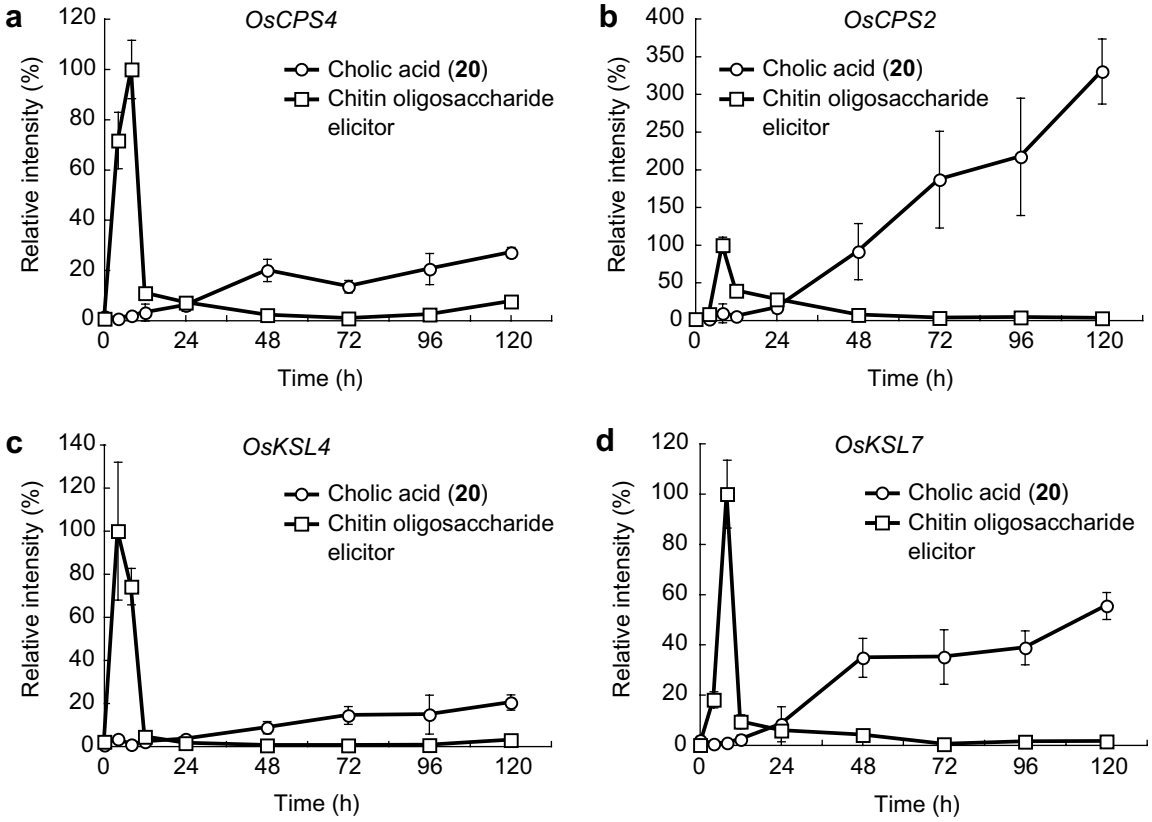

Fig. 6. Time course of relative mRNA levels of the diterpene cyclase genes (a) *OsCPS4*, (b) *OsCPS2*, (c) *OsKSL4*, and (d) *OsKSL7* in suspension-cultured rice cells from 0 to 120 h after treatment with 0.6 μM chitin oligosaccharide or 2 mM cholic acid (**20**). The mRNA levels were determined using quantitative reverse-transcription PCR. The percentage mRNA level at each time was determined relative to the maximum induction level of each gene obtained by treatment with the chitin oligosaccharide (100%). Values of 100% in a, b, c, and d correspond to the following relative mRNA levels normalized by *OsUBQ* mRNA levels: 0.54 (*OsCPS4*), 0.10 (*OsCPS2*), 0.55 (*OsKSL4*), and 0.57 (*OsKSL7*) mRNA level. The results are expressed as the mean ± standard error of three experiments.

of *OsKSL8* involved in biosynthesis of oryzalexin S (**14**) in suspension-cultured rice cells increased from 48 h through 120 h after CA (**20**) treatment, the mRNA level at 120 h after treatment were about 39% of the maximum level induced by the chitin oligosaccharide (data not shown).

Most of the chitin oligosaccharide elicitor-inducible genes have been reported to show transient expression in rice (Sakamoto et al., 2004; Chujo et al., 2007; Okada et al., 2007). The chitin oligosaccharide elicitor caused transient increases in the levels of diterpene cyclase gene transcripts, whereas the animal elicitor CA caused continuous increases in the transcript levels of diterpene cyclase genes for at least 120 h after treatment. In the present study, using HPLC as previously published (Koga et al., 2006), we confirmed that the CA (**20**) content in the culture medium of the rice cells treated with 2 mM CA (**20**) did not decrease significantly until 120 h after treatment (data not shown). Therefore, the continuous increase in the expression levels of diterpene cyclase genes might be, at least in part, because CA (**20**) is not turned over, although it is also possible that CA (**20**) does not induce down-regulation mechanisms of gene expression. We have no information on the turnover rate of chitin oligosaccharides in suspension-cultured rice cells. It remains to be clarified whether the transient increases in the expression of diterpene cyclase genes caused by a chitin oligosaccharide elicitor results from the down-regulation of gene expression or a rapid turnover of the elicitor in the suspension-cultured cells.

2.4. Conclusions

In the present study, we established an HPLC–ESI-MS/MS system for the rapid and accurate quantification of the major rice diterpenoid phytoalexins, phytocassanes (**1–5**) and momilactones (**12** and **13**). Using this method, we showed that CA (**20**) preferentially induces the biosynthesis of phytocassanes (**1–5**) in suspension-cultured rice cells, whereas a fungal chitin oligosaccharide elicitor induces formation of both phytocassanes (**1–5**) and momilactones (**12** and **13**). We further showed that CA (**20**) more strongly induces the transcription of the genes *OsCPS2* and *OsKSL7*, which are involved in phytocassane biosynthesis, than the genes *OsCPS4* and *OsKSL4*, which are involved in momilactone biosynthesis, *OsCPS2* being especially strongly induced. The elucidation of the regulatory mechanisms of CA (**20**)-induced *OsCPS2* gene expression may

provide important information on the preferential induction of phytocassanes (**1–5**) by CA (**20**). Clearly, different mechanisms regulate the chitin oligosaccharide and CA (**20**) responses, and the reasons for these differences require clarification.

3. Experimental

3.1. Chemicals

Purified chitooctaose was kindly supplied by Yaizu Suisankagaku Industry Co. Ltd., Tokyo, Japan, and was re-*N*-acetylated to give *N*-acetylchitooctaose as described (Ito et al., 1997). This substance was used as a chitin oligosaccharide elicitor of rice defense responses. Cholic acid (CA, **20**) was purchased from Kanto Kagaku Co., Ltd., Tokyo, Japan.

3.2. Plant material and cell culture

Calli of *O. sativa* L. cv. Nipponbare were maintained on gerangum (0.3%) plates using modified N6 medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) as described previously (Cho et al., 2004). For suspension cultures, approximately 2.5-mL aliquots of the calli were transferred to 500-mL Erlenmeyer flasks containing 150 mL of N6 medium with 1 mg/L 2,4-D. The cultures were maintained on a rotary shaker at 120 rpm and 25 °C in the dark. The suspension-cultured cells were collected every week and filtered through a 20-mesh screen to create fine aggregates. At the time of each collection, 2.5 mL of the resultant cells were transferred to a new 500-mL Erlenmeyer flask containing 150 mL of the above medium and cultured further, as described above.

3.3. Treatment of rice cells with the chitin oligosaccharide or CA (*20*)

Separate stock solutions of the chitin oligosaccharide and CA (**20**) were sterilized by filtration through 0.20-μm filters. The stock solutions were added to rice cells 6 days after transfer to fresh medium, and the reaction mixture was incubated on a rotary shaker at 120 rpm and 25 °C in the dark. For the analysis of diterpenoid phytoalexins, 0.5 mL of the culture medium from the cell suspension was collected at 0, 4, 8, 12, 24, 48, 72, 96, and 120 h after the start of treatment.

3.4. Sample preparation for HPLC–ESI-MS/MS

An 0.5-mL aliquot of the cell culture medium at pH ca. 5.8 of rice cells treated with either the chitin oligosaccharide or CA (**20**) was extracted with EtOAc (3×0.5 mL). The combined EtOAc extracts were concentrated *in vacuo* with the resulting residue dissolved in 5 mL of EtOH:H_2O:MeCN:AcOH (79:13.99:7:0.01, v/v/v/v), and 5 μL of the resultant solution were subjected to HPLC–ESI-MS/MS.

3.5. HPLC

An Agilent1100 separation module (Agilent Technologies, Palo Alto, CA, USA) equipped with a Pegasil C_{18} column (150 mm long, 2.1 mm in diameter; Senshu Scientific, Tokyo, Japan) was used for HPLC analyse. For diterpenoid phytoalexins, elution was conducted in MeCN:H_2O:AcOH (70:29.9:0.1, v/v/v) at a flow rate of 0.2 mL/min, and the eluate monitored by ESI-MS/MS.

3.6. ESI-MS/MS

Standard solutions (1 mg/L) of the respective samples of diterpenoid phytoalexins were prepared in MeOH:H_2O:AcOH (55:44.9:0.1, v/v/v). For the selection of diagnostic precursor-to-product ion transitions, the standard solutions were directly infused at a flow rate of 5 mL/min into a quadropole tandem mass spectrometer (API-3000, Applied Biosystems Instruments, Foster City, CA, USA) fitted with an electrospray ion source. All diterpenoid phytoalexins were analyzed in positive ion mode. N_2 was used as the collision gas. The ES capillary was 3.0 kV, and the source temperature was 400 °C. Other parameters were optimized using spectrometer software (Applied Biosystems Instruments). The MRM mode was used to monitor the precursor and product ions.

3.7. Effects of treatment with CA (*20*) on phytoalexin production and expression of diterpene cyclase genes involved in phytoalexin biosynthesis

Aliquots (ca. 2.5 mL) of the calli were transferred to 500-mL Erlenmeyer flasks containing 150 mL of N6 medium with 1 mg/L 2,4-D and were cultured on a rotary shaker at 120 rpm and 25 °C in the dark. Aliquots (1 mL) of cell suspensions were collected at 0, 4, 8, 12, 24, 48, 72, 96, and 120 h after the start of treatment. Each sample was centrifuged at 14,000*g*, with precipitated cells used

Table 1
Primers used in the present study

Gene name	Primer name	Primer sequence
OsCPS4	OsCPS4 F	5′-TGACGAGGCTGGGCATATC-3′
	OsCPS4 R	5′-TCTGGAGTCCAGTTCCTGAAA-3′
OsCPS2	OsCPS2 F	5′-TTAGGAAAATGGTTGACTAC-3′
	OsCPS2 R	5′-ATCGACTAAATTCATCTCAC-3′
OsKSL4	OsKSL4 F	5′-CGCTTTGTAACTCTAAGGTA-3′
	OsKSL4 R	5′-ACGTAAAAGGCTTGTATATC-3′
OsKSL7	OsKSL7 F	5′-TTCATCTCTGTCACTTTTTCTTTTT-3′
	OsKSL7 R	5′-ATCCCAACGAAGTCATCCAC-3′
UBQ	OsUBQ F	5′-GGACTGGTTAAATCAATCGTCA-3′
	OsUBQ R	5′-CCATATACCACGACCGTCAAAA-3′

for the extraction of total RNA. Each supernatant was used for quantification of diterpenoid phytoalexins by HPLC–ESI-MS/MS. Total RNA was isolated from rice cells using Sepasol®-RNA I (Nakalai tesque, Kyoto, Japan) according to the manufacturer's protocol and was quantified using a spectrometer. First-strand cDNA was synthesized from total RNA (1 μg) using a QuantiTect® reverse transcription kit (Qiagen) according to the manufacturer's protocol. The cDNA solution was diluted 10-fold in deionized water before PCR amplification.

Real-time RT-PCR analysis was performed in a 20-μL reaction volume using an ABI Prism® 7700 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). The optimized reaction contained 10 μL of *Power* SYBR® Green PCR Master Mix (Applied Biosystems), 0.4 μL each of oligonucleotide primer sets (Table 1), 1 μL of cDNA, and 8.2 μL of H_2O. Real-time PCR was performed using the following program: 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. The levels of *OsCPS4*, *OsCPS2*, *OsKSL4*, *OsKSL7,* and *OsUBQ* mRNA were determined by generating standard curves using a series of known concentrations of the target sequences and were analyzed by the difference in threshold cycles (ΔCT) method. The specificity of the real-time PCR was verified by dissociation curve analysis and agarose gel electrophoresis of the PCR products. The mRNA levels of the target genes *OsCPS4*, *OsCPS2*, *OsKSL4*, and *OsKSL7* were normalized to that of the rice housekeeping gene *OsUBQ* to correct for among-sample variation.

3.8. GC-SIM analysis

A JEOL JMS-GCmate II GC–MS system (JEOL, Akishima, Japan) fitted with a fused-silica capillary column HP-5 (30 m long, 0.32 mm in diameter, 0.25-mm film thickness, Hewlett-Packard) was used for GC-SIM analysis. The sample was dissolved in acetone, and the resulting sample solution was injected onto the column at 70 °C in a splitless mode. One minute after injection, the column temperature was programmed to increase to 300 °C at a rate of 10 °C/min. The flow rate of the He carrier gas was 1 mL/min. The injection temperature was 280 °C The retention time of oryzalexin A (**6**) was 20.3 min, and the level of oryzalexin A (**6**) was determined from the peak area for *m*/*z* 302, by reference to a calibration curve.

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