Cholic Acid, a Bile Acid Elicitor of Hypersensitive Cell Death, Pathogenesis-Related Protein Synthesis, and Phytoalexin Accumulation in Rice

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When plants interact with certain pathogens, they protect themselves by generating various defense responses. These defense responses are induced by molecules called elicitors. Since long ago, composts fermented by animal feces have been used as a fertilizer in plant cultivation, and recently, have been known to provide suppression of plant disease. Therefore, we hypothesized that the compounds from animal feces may function as elicitors of plant defense responses. As a result of examination of our hypothesis, an elicitor of rice defense responses was isolated from human feces, and its structure was identified as cholic acid (CA), a primary bile acid in animals. Treatment of rice (*Oryza sativa*) leaves with CA induced the accumulation of antimicrobial compounds (phytoalexins), hypersensitive cell death, pathogenesis-related (PR) protein synthesis, and increased resistance to subsequent infection by virulent pathogens. CA induced these defense responses more rapidly than did fungal cerebroside, a sphingolipid elicitor isolated from the rice pathogenic fungus *Magnaporthe grisea*. Furthermore, fungal cerebroside induced both types of rice phytoalexins, phytocassanes and momilactones, whereas CA mainly induced phytocassanes, but not momilactones. In the structure-activity relationship analysis, the hydroxyl groups at C-7 and C-12, and the carboxyl group at C-24 of CA contributed to the elicitor activity. These results indicate that CA is specifically recognized by rice and is a different type of elicitor from fungal cerebroside. This report demonstrated that bile acid induced defense responses in plants.

In animals, bile acids play important roles in the absorption of dietary lipids, regulation of cholesterol catabolism, and detoxification of secondary bile acids (Chiang, 2002). Cholic acid (CA) and chenodeoxycholic acid (CDCA) are known as representative primary bile acids, and deoxycholic acid (DCA) and lithocholic acid (LCA) metabolized from the primary bile acids by intestinal microorganisms as representative secondary bile acids. Among them, in animals, CDCA and LCA are known to act as ligands for nuclear receptors, such as farnesoid X receptor (FXR; Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999), vitamin D receptor (VDR; Makishima et al., 2002), and pregnane X receptor (PXR; Staudinger et al., 2001). By contrast, to our knowledge, no reports have yet demonstrated the specific function for CA in plants and animals.

When plants interact with certain pathogens, they protect themselves by generating various defense responses, such as the accumulation of antimicrobial compounds (phytoalexins), the induction of pathogenesis-related (PR) proteins (chitinases, β-1,3-glucanases, and proteinase inhibitors) and hypersensitive cell death, increased expression of defense-related genes, the formation of lignin, a burst of active oxygen, and increased resistance to subsequent infection by pathogens (Keen, 1992; Staskawicz et al., 1995). These defense responses (hypersensitive responses) are induced by molecules called elicitors (Keen, 1975) and the chemical structures of various elicitors have been determined. Oligosaccharide elicitors of phytoalexin synthesis in soybean (*Glycine max*) and rice (*Oryza sativa*) have been isolated and identified as reduced compounds, hexaglucosyl glucitol and tetraglucosyl glucitol, respectively (Sharp et al., 1984a, 1984b; Yamaguchi et al., 2000). Unsaturated fatty acids, such as eicosapentaenoic acid and arachidonic acid have been isolated as elicitors of phytoalexin accumulation in potato (*Solanum tuberosum*; Bostock et al., 1981). Syringolides 1 and 2, glycolipid elicitors in soybean have been isolated as products of avirulence gene D (Midland et al., 1993). Fungal cerebrosides have been isolated as sphingolipid elicitors of disease resistance in rice (Koga et al., 1998b). Various compounds are known to have elicitor

activity as mentioned above, whereas there are no reports that have appeared to date, to our knowledge, demonstrating the elicitor activity of bile acid.

Since long ago, composts fermented by animal feces have been used as a fertilizer in plant cultivation and, recently, have been known to provide suppression of plant disease (Haug, 1993; Hoitink and Grebus, 1994; Veeken et al., 2001). However, the detailed mechanism of the suppression of plant disease by composts remains unclear. Therefore, we hypothesized that the compounds from animal feces may induce plant defense responses. As a result of examination of our hypothesis, an elicitor of rice defense responses was isolated from human feces, and its structure was identified as CA, a primary bile acid in animals. Treatment of rice leaves with CA induced the accumulation of antimicrobial compounds (phytoalexins), hypersensitive cell death, PR protein synthesis, and increased resistance to subsequent infection by virulent pathogens. In this study, we describe the isolation and characterization of bile acid elicitor and the further discussion of its function in plant defense responses.

RESULTS

Purification and Structural Determination of an Elicitor from Human Feces

When plants interact with pathogens, they produce antimicrobial compounds called phytoalexins as a defense response. To examine whether the compounds in animal feces function as elicitors of plant defense response, we tested the elicitor activity of an extract from human feces by measuring phytoalexin induction in rice bioassay systems. As a result, an ethyl acetate extract from an acidic water suspension of human feces effectively induced the accumulation of phytoalexins in rice leaves. The active material in the feces apparently had acidic and hydrophobic properties. On the basis of this observation, we purified elicitor from the ethyl acetate extract by monitoring the ability to induce phytoalexin production. After HPLC fractionation in three steps, one purified elicitor was isolated and designated compound 1 (Table I). Compound 1 was purified about 255-fold and 10.9% of the initial activity was recovered. The specific activity of compound 1 was 1.07 units/μg.
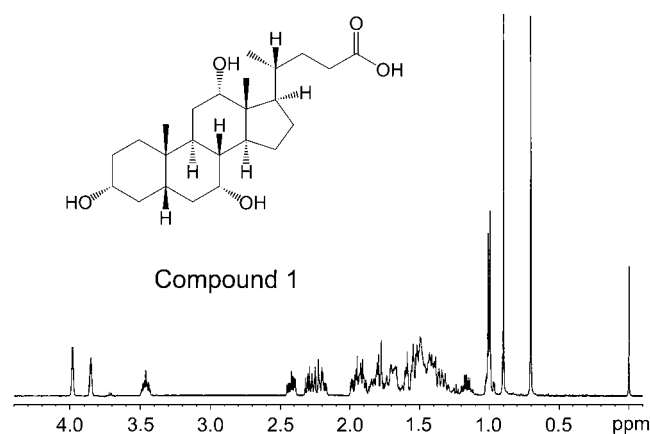

Figure 1. ^{1}H-NMR spectrum of compound 1 isolated from human feces.

The ^{1}H-NMR spectrum of compound 1 (Fig. 1) was identical to that of authentic CA. In the negative electrospray ionization mass spectrometry spectrum of compound 1, the molecular ion [M-H]$^-$ at mass-to-charge ratio (m/z) 407.3 was identical to that of CA. Furthermore, elicitor activity of compound 1 was 1.07 ± 0.13 units/mg, which was the same activity with CA (1.11 ± 0.11 units/mg). From these results, the structure of compound 1 was identified as CA, a primary bile acid in animals. To the best of our knowledge, this report is the first to demonstrate that bile acids have elicitor activity in plants.

In our previous study, fungal cerebrosides have been isolated as sphingolipid elicitors from the membrane of the rice pathogenic fungus *Magnaporthe grisea* (Koga et al., 1998b). Fungal cerebrosides induced the production of phytoalexins, PR proteins, cell death, active oxygen, and complete resistance to subsequent infection by virulent pathogens (Koga et al., 1998b; Umemura et al., 2000). Furthermore, the methyl group at C-9 in the sphingoid-base moiety, a structure unique to fungal sphingolipids, was specifically recognized by rice plants (Koga et al., 1998b). Therefore, to examine the differences between elicitors from pathogens and animal feces, we examined differences in the induction of phytoalexins by CA and fungal cerebroside. So far, five cassane-type diterpenes, phytocassanes A, B, C, D, and E (Koga et al., 1995; Koga et al., 1997; Fig. 2A), eight pimarane-type diterpenes, momilactones

Table I. *Purification of elicitor from the ethyl acetate extract of human feces*

Step	Total Dry Weight	Total Elicitor Activity	Specific Elicitor Activity	Purification Factor	Elicitor Activity Recovered
	mg	*units*[a]	*units/μg*[b]	*-fold*	*%*
Ethyl acetate extract[c]	6,278.0	26,590	0.0042	1	100.0
ODS-120T/20%–100% ethanol	187.0	9,517	0.051	12	35.8
ODS-120T/45% ethanol	8.9	3,658	0.411	98	13.8
ODS-120T/40% acetonitrile	2.7	2,890	1.070	255	10.9

[a]One unit of elicitor activity is defined as the amount of the sample required to induce 0.3 μg of phytocassanes per leaf.   [b]The specific elicitor activity of the sample is defined as the number of units per microgram dry weight of the sample.   [c]A total of 1,031 g (fresh weight) of human feces was used as the starting material.

A and B (Cartwright et al., 1977; Fig. 2A), oryzalexins A, B, C, D, E, and F (Akatsuka et al., 1985; Sekido et al., 1986; Kato et al., 1993; Kato et al., 1994), one stemarane-type diterpene, oryzalexin S (Tamogami et al., 1993), and one flavonoid, sakuranetin (Kodama et al., 1992), have been isolated as rice phytoalexins. Dose-response curves for the phytocassanes and momilactones induced by CA or fungal cerebroside are shown in Figure 2, B and C. Fungal cerebroside induced the production of significant amounts of phytocassanes and momilactones. On the other hand, CA induced the production of significant amounts of phytocassanes, but induced the production of negligible amounts of momilactones in rice leaves.

Induction of Various Defense Responses by Treatment with CA

Plant defense response to pathogen invasion (hypersensitive response) is accompanied by induction of a variety of chemical and physiological barriers as well as phytoalexins (Keen, 1992; Staskawicz et al., 1995). Therefore, to demonstrate that CA did, indeed, induce the hypersensitive response in rice plants, we examined whether three markers associated with hypersensitive response could be detected in rice leaves after treatment with CA. During the hypersensitive response, recognition of a pathogen triggers the activation of a cell death pathway that results in the formation of a zone of dead cells (necrosis) around the site of infection (Levine, et al., 1994; Mittler et al., 1995; Ryerson and Heath, 1996). Therefore, first, we examined a biochemical marker, the induction of irreversible mem-

brane damage (cell death), by measuring ion leakage from leaf discs. As shown in Figure 3A, significant amounts of ion leakage were induced 24 h or more after treatment with CA as compared with the control treatment, suggesting that the CA-induced response was accompanied by cell death.

β-1,3-Glucanase induced in the hypersensitive response is considered to degrade β-1,3-glucan, a component of pathogenic fungal cell wall. Therefore, second, we measured the induction of β-1,3-glucanase, a PR protein belonging to the PR-2 family (Cote et al., 1991). As shown in Figure 3B, significant amounts of β-1,3-glucanase activity were induced 24 h or more after treatment with CA as compared with the control treatment.

Finally, we examined whether treatment with CA could induce significant resistance to a virulent pathogen. The data presented in Figure 3C show that treatment of rice plants with CA 16 h or more before inoculation of the pathogen resulted in resistance to infection by a virulent race of *M. grisea*. However, treatment with CA 0 to 8 h before inoculation hardly enhanced resistance to infection. These results indicate that CA did not inhibit proliferation of the pathogen but, rather, it induced resistance against the pathogen. The three sets of results described above clearly show that CA induced hypersensitive response in rice plants.

Determination of Minimal Amounts of CA to Induce Significant Disease Resistance

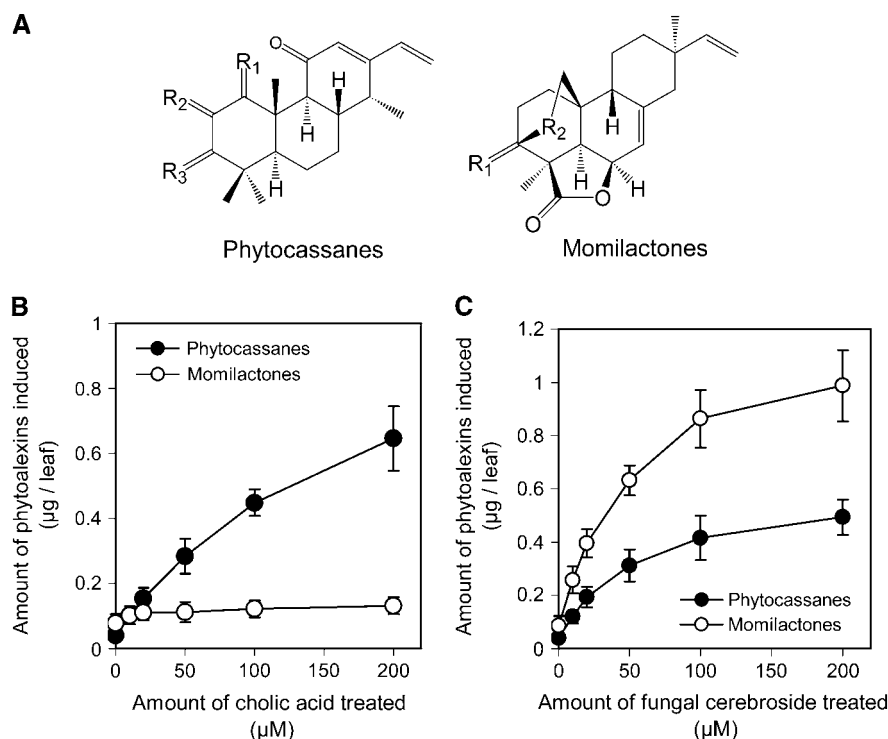Treatment of rice leaves with 20 μM or more of CA induced significant amounts of phytocassanes (Fig.

Figure 2. Induction of phytoalexins in rice leaves by CA (cholic acid) or fungal cerebroside. A, Chemical structures of phytocassanes and momilactones. R1, R2, and R3 = H, OH, or O. B, Dose-response curves for total production of phytocassanes A to E and of momilactones A and B induced by CA. C, Dose-response curves for total production of phytocassanes A to E and of momilactones A and B induced by fungal cerebroside. The total production of phytocassanes A to E and of momilactones A and B induced in the leaves 48 h after treatment with 20 mM potassium phosphate, pH 6, 0.1% Tween 20, plus CA or fungal cerebroside was measured. The results are expressed as the mean $\pm$ SE of five experiments.

2B). Therefore, to examine whether such a low concentration of CA (20 μM) has physiological significance in rice defense responses, we measured minimal amounts of CA to induce resistance to a virulent pathogen. The data presented in Figure 4A show that treatment of rice leaves with 20 μM or more of CA before inoculation of the pathogen resulted in significant resistance to infection by a virulent race of *M. grisea*. Although high concentrations of CA (about 10 mM) are used as a surfactant for membrane solubilization, our results indicate that such a low concentration of CA (20 μM) acts as a specific elicitor in rice plants rather than as a nonspecific surfactant.

Since the composts fermented by animal feces should contact with plant roots, we examined whether treatment of rice roots with CA could induce significant resistance to a virulent pathogen. The data presented in Figure 4B show that the treatment of rice roots with 20 μM or more of CA before inoculation
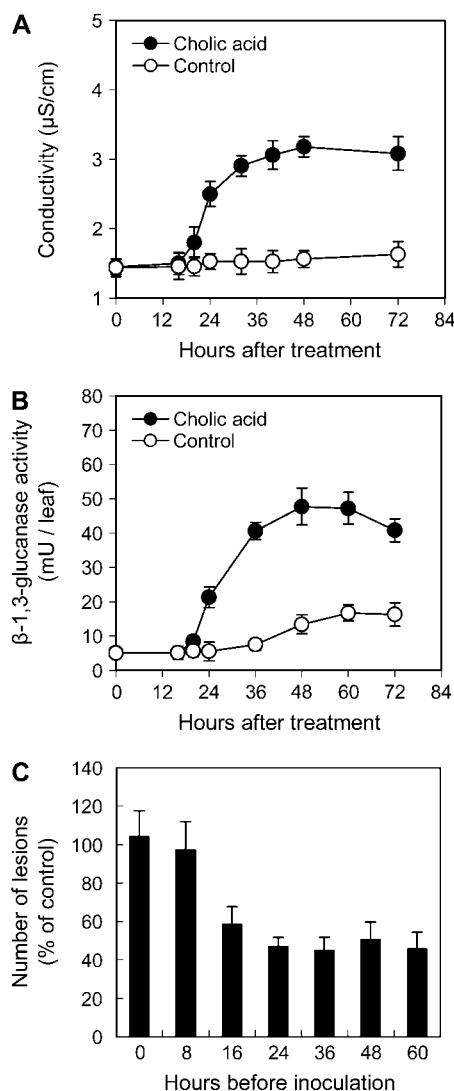
Figure 3. Induction of various defense responses induced in rice leaves by CA (cholic acid). A, Induction of cell death by CA. Ion leakage from leaf discs as an indicator of cell death was measured. Twenty leaf discs 0 to 72 h after treatment with 20 mM potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or with the same solution plus 200 μM of CA were floated on 80 mL of 0.01% Tween 20 and the increase in conductivity (μS/cm) of the solution was measured with a conductivity meter. B, Induction of β-1,3-glucanase by CA. β-1,3-Glucanase activities induced in the leaves 0 to 72 h after treatment with 20 mM potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or with the same solution plus 200 μM of CA was measured using curdlan as a substrate. C, Induction of disease resistance by CA. Forty rice leaves were sprayed with 4 mM potassium phosphate, pH 6, plus 0.01% Tween 20 (control) or with the same solution plus 20 μM of CA at different times before inoculation with *M. grisea*. The leaves 0 to 60 h after treatment were sprayed with a spore suspension of a virulent race (007) of *M. grisea*. Development of disease was scored by counting the number of lesions 7 d after inoculation. When the number of lesions on control plants was taken as 100%, the relative percentage at each time was measured to estimate the disease resistance. The results are expressed as the mean ± SE of five experiments.
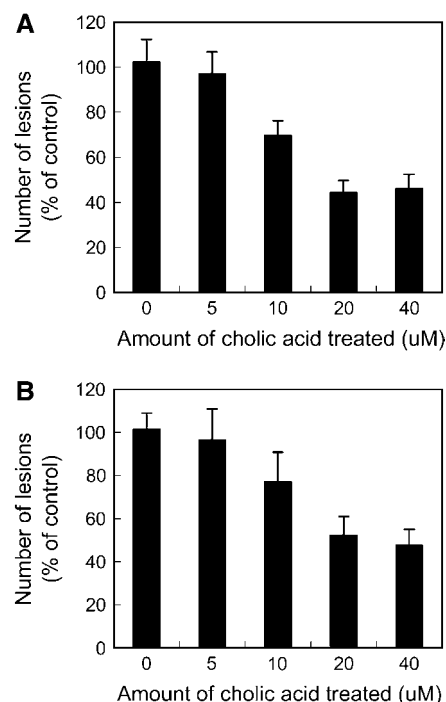
Figure 4. Determination of minimal amounts of CA (cholic acid) to induce significant disease resistance. A, Induction of disease resistance by treatment of rice leaves with various amounts of CA. Forty rice leaves were sprayed with 4 mM potassium phosphate, pH 6, plus 0.01% Tween 20 (control) or with the same solution plus 0 to 40 μM of CA. The leaves 48 h after treatment were sprayed with a spore suspension of a virulent race (007) of *M. grisea*. Development of disease was scored by counting the number of lesions 7 d after inoculation. When the number of lesions on control plants was taken as 100%, the relative percentage at each amount of CA treated was measured to estimate the disease resistance. The results are expressed as the mean ± SE of five experiments. B, Induction of disease resistance by treatment of rice roots with various amounts of CA. Forty rice roots were soaked in tap water (tap water was adjusted to pH 6 by acetic acid) or with the same solution plus 0 to 40 μM of CA. The leaves 48 h after treatment were sprayed with a spore suspension of a virulent race (007) of *M. grisea* and development of disease was scored as described.

of the pathogen resulted in significant resistance to infection by a virulent race of *M. grisea*.

Timing of Induction of Various Defense Responses by CA and Fungal Cerebroside

To examine the differences between elicitors from pathogens and animal feces, we tested the timing of induction of rice defense responses. First, significant amounts of phytocassanes were induced 24 h or more after treatment with CA, but they were not induced until 36 h or more after treatment with fungal cerebroside (Fig. 5A). Thus, induction by CA was clearly more rapid relative to the induction by fungal cerebroside. Second, significant cell death was induced 24 h or more after treatment with CA, whereas fungal cerebroside did not induce cell death until 48 h or more after treatment (Fig. 5B). Thus, CA clearly induced cell death more rapidly than did fungal cerebroside. Finally, CA induced β-1,3-glucanase 24 h earlier than did fungal cerebroside (Fig. 5C). Based on these three sets of results, CA induced various defense responses more rapidly than did fungal cerebroside.

Elicitor Activity of a Number of Bile Acids and Their Derivatives

To examine the structure-activity relationship, we tested a number of bile acids and their derivatives for elicitor activity by measuring phytoalexin induction in rice leaves (Fig. 6B). CA had higher elicitor activity than similar compounds that lack either the hydroxyl group at C-12 (CDCA) or at C-7 (DCA; Fig. 6A). Furthermore, LCA and hyodeoxycholic acid (HDCA), both of which lack the hydroxyl groups at C-12 and C-7 (Fig. 6A), had lower activity than CDCA and DCA, suggesting that the hydroxyl groups at C-12 and C-7 are important for the activity. Methylation of the carboxyl group at C-24 of CA (cholic acid methyl ester [methyl-CA]) led to a 7-fold decrease in activity, and conjugate bile acids, such as taurocholic acid (tauro-CA), glycocholic acid (glyco-CA), and tauroglycocholic acid (tauroglyco-CA) had lower activity than CA, suggesting that the carboxyl group at C-24 also contribute to the activity. Thus, the hydroxyl groups at C-7 and C-12, and the carboxyl group at C-24 of CA seem to be key elements determining the activity, and CA is specifically recognized by rice.

DISCUSSION

Comparisons of the elicitor activity of bile acid derivatives provide some important insights into the structural features required for elicitor activity in rice plants. The hydroxyl groups at C-7 and C-12, and the carboxyl group at C-24 of CA were key elements determining the elicitor activity. Such specificity for CA indicates that rice plants can specifically recognize CA in feces and thereby induce various defense
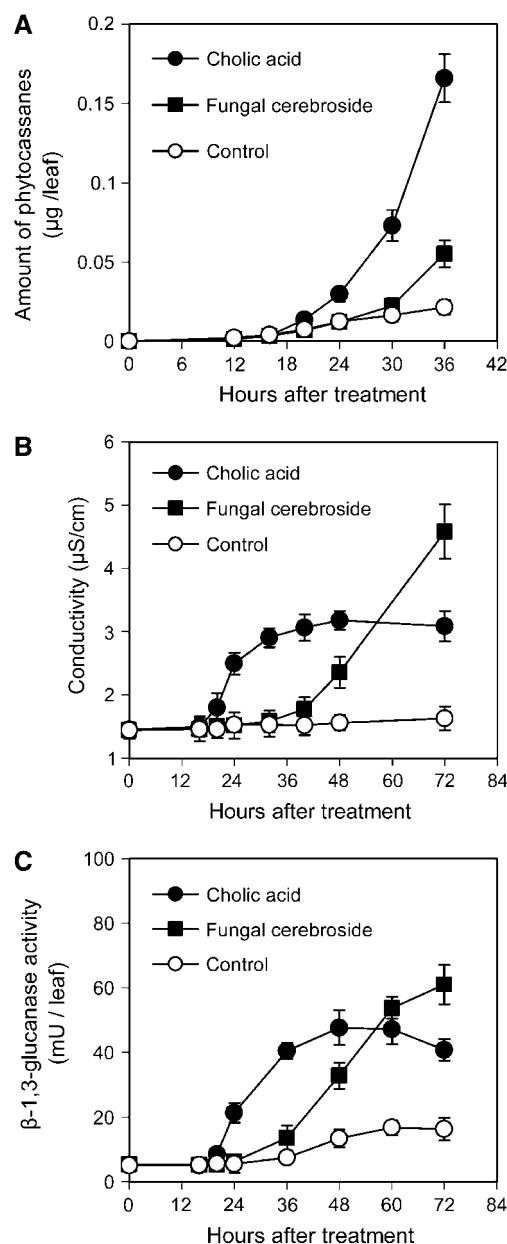
Figure 5. Timing of induction of various defense responses by CA (cholic acid) and fungal cerebroside. A, Timing of induction of phytocassanes by CA or fungal cerebroside. The total production of phytocassanes A to E induced in the leaves 0 to 36 h after treatment with 20 mM potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or with the same solution plus 200 μM of CA or 200 μM of fungal cerebroside was measured. B, Timing of induction of cell death by CA or fungal cerebroside. Twenty leaf discs 0 to 72 h after treatment with 20 mM potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or with the same solution plus 200 μM of CA or 200 μM of fungal cerebroside were floated on 80 mL of 0.01% Tween 20 and the increase in conductivity (μS/cm) of the solution was measured with a conductivity meter. C, timing of induction of β-1,3-glucanase by CA or fungal cerebroside. β-1,3-Glucanase activities induced in the leaves 0 to 72 h after treatment with 20 mM potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or with the same solution plus 200 μM of CA or 200 μM of fungal cerebroside was measured using curdlan as a substrate. The results are expressed as the mean $\pm$ SE of five experiments.

Figure 6. Elicitor activity of various bile acids. A, Chemical structures of bile acids. B, Elicitor activity of various bile acids. CA, Cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HDCA, hyodeoxycholic acid; Methyl-CA, cholic acid methyl ester; Glyco-CA, glycocholic acid; Tauro-CA, taurocholic acid; Tauroglyco-CA, tauroglycocholic acid; Tauro-LCA, taurolithocholic acid.
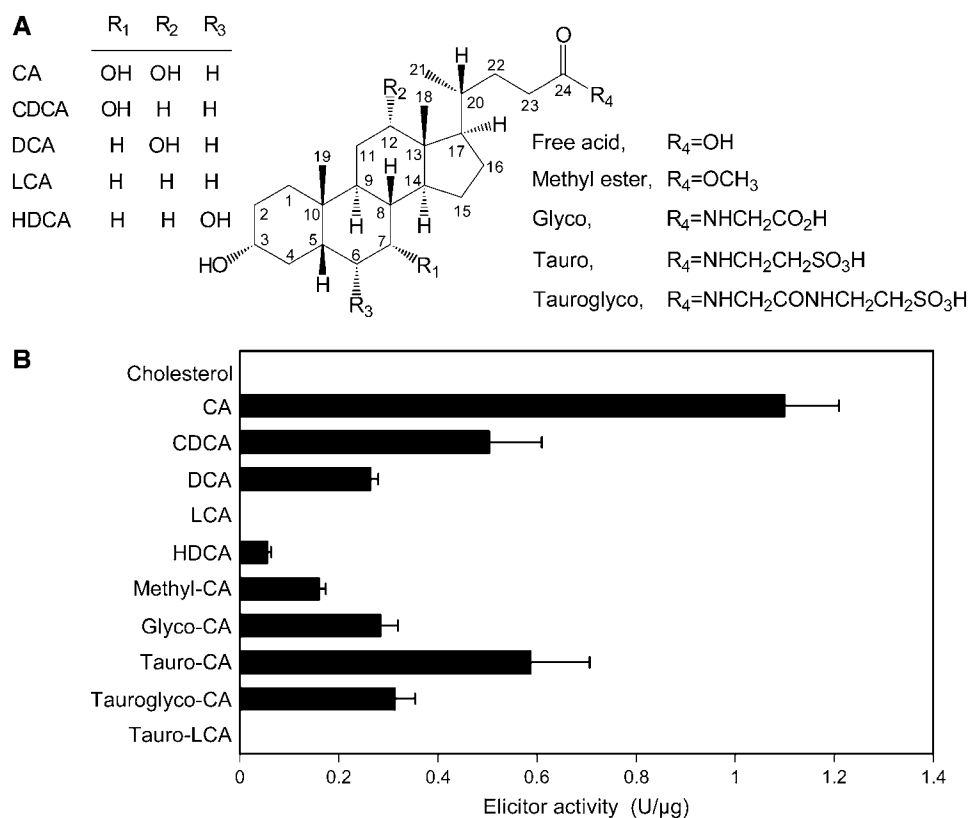
responses. Furthermore, the low concentration of CA required for elicitor activity provides further evidence that CA is specifically recognized by rice. In animals, bile acids play important roles in the absorption of dietary lipids, regulation of cholesterol catabolism, and detoxification of secondary bile acids (Chiang, 2002). In particular, bile acids have attracted attention as natural ligands for nuclear receptors such as FXR (Makishima et al., 1999; Parks et al., 1999; Wang et al.,1999), VDR (Makishima et al., 2002), and PXR (Staudinger et al., 2001). When FXR receptor, an orphan nuclear receptor bound to CDCA, repressed transcription of the gene encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis, and activated the gene encoding intestinal bile acid-binding protein, a candidate bile acid transporter (Makishima et al., 1999; Parks et al., 1999; Wang et al.,1999). Activation of VDX or PXR by LCA induced the expression of CYP3A, a cytochrome P450 enzyme that detoxifies LCA in the liver and intestine (Staudinger et al., 2001; Makishima et al., 2002). CDCA and LCA have been extensively studied as natural ligands for nuclear receptors as mentioned above, whereas no reports have yet demonstrated the presence of any specific receptor for CA in animals. Hence, the molecular mechanisms in the absorption of dietary lipids and regulation of cholesterol catabolism by CA have not been fully elucidated yet. From this point of view, the isolation of rice receptor for CA in defense responses might assist bile acid studies in animals.

Until now, both types of phytoalexins, phytocassanes and momilactones, had always been induced together under various conditions (e.g. infection with pathogens such as *M. grisea* [Koga et al., 1995, 1998a; Umemura et al., 2003] and *Rhizoctonia solani* [Koga et al., 1995], and treatment with elicitors, fungal cerebroside [Koga et al., 1998b] and *N*-acetyl-chitooligosaccharides [Umemura et al., 2002], and with a signal transducer, jasmonic acid [Umemura et al., 2002]), suggesting that the signaling pathway for the phytocassane synthesis shares the same pathway with that responsible for the momilactone synthesis and this pathway branches in the downstream. Surprisingly, CA induced production of significant amounts of phytocassanes but not momilactones and induced defense responses more rapidly than did fungal cerebroside, indicating the possibility that CA skips this common signaling pathway and directly acts in the downstream signaling pathway specific to phytocassane synthesis. Fungal cerebrosides have been known to activate defense signaling pathway via membrane-associated heterotrimeric G-protein α-subunit, small GTPase Rac, and mitogen-activated protein kinase (Suharsono et al., 2002; Lieberherr et al., 2005). Furthermore, Lieberherr et al. (2005) have shown that the two G proteins regulate a fungal cerebroside-inducible mitogen-activated protein kinase in rice at the protein level. It seems probable that CA activates a different signaling pathway from that of fungal cerebroside.

In light of the two facts that bile acids regulate gene transcription by acting as natural ligands for nuclear receptors such as FXR, PXR, or VDR in animals and that CA might directly act downstream in the defense signaling pathway in rice, it seems possible that CA operates as a ligand for a nuclear receptor that regulates phytocassane and PR protein synthesis and cell death in a manner similar to that observed in animal systems. Recently, a gene encoding *ent*-cassa-12,15-diene synthase, an enzyme that specifically catalyzes phytocassane synthesis, has been cloned (Cho et al., 2004). If our hypothesis is correct, this enzyme will be regulated by a nuclear receptor for CA. Regardless, bile acids will be a powerful tool for the elucidation of defense signaling pathways in rice.

Recently, because of environmental pollution by chemically synthesized fungicides, natural substances that induce plant disease resistance have attracted attention as alternatives (Koga, 2003). For example, harpin (Wei et al., 1992; Dong et al., 1999) and fungal cerebroside (Koga et al., 1998b; Umemura et al., 2000, 2004; Deepak et al., 2003) prevented infection by inducing defense systems in various plants. However, the high cost involved in the practical use of such natural substances has been prohibitive. On the other hand, because bile acid can be produced from domestic animals at a very low cost and is effective at very low concentrations to induce plant disease resistance, it may prove to be an ideal alternative to chemically synthesized fungicides in the future.

MATERIALS AND METHODS

Analysis Procedures

^{1}H-NMR spectrum was recorded in $CDCl_3$ solutions on a Varian UNITY plus 500 spectrometer (500.2 MHz), using $SiMe_4$ as an internal standard. Electrospray ionization mass spectrometry was recorded on a LCT mass spectrometer. Electron ionization mass spectra (EI-MS) were recorded on a Nippondenshi DX-303 mass spectrometer. Momilactones and phytocassanes were analyzed by reverse-phase HPLC using a TSKgel ODS-120T (4.6 mm i.d. × 30 cm; TOSOH) column at a flow rate of 1.2 mL/min at 50°C as described (Koga et al., 1995). Compound 1 was purified by reverse-phase HPLC using a TSKgel ODS-120T (7.8 mm i.d. × 30 cm; TOSOH) column at a flow rate of 2 mL/min at 50°C and a TSKgel ODS-120T (4.6 mm i.d. × 30 cm; TOSOH) column at a flow rate of 1 mL/min at 50°C. The eluate was monitored with an UV detector at 215 nm for compound 1.

Microorganisms and Materials

Magnaporthe grisea (virulent race 007) was kindly provided by Dr. Michiaki Iwata (Meiji Seika Kaisha, Ltd.). Fungal cerebroside, (4*E*,8*E*)-*N*-D-2′-hydroxy-(*E*)-3′-hexadecenoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (cerebroside C) was isolated from *M. grisea* as described (Koga et al., 1998b).

Quantitative Analysis of Momilactones and Phytocassanes from Rice Leaves

Six rice (*Oryza sativa*) leaves treated with the elicitor solution were collected and cut into pieces. The sample was shaken with 5 mL of ethyl acetate and 5 mL of 0.1 N Na_2CO_3 (pH 10.5) for 16 h to yield ethyl acetate and aqueous layers. The ethyl acetate layer was evaporated to yield a crude extract, which was dissolved in 1.6 mL of ethanol before adding 2.4 mL of 0.02 N HCl. The solution was mixed and centrifuged at 15,000g for 30 min. The supernatant

(0.2 mL) was subjected to HPLC on a TSKgel ODS-120T column (4.6 mm i.d. × 30 cm; TOSOH) eluted with 45% acetonitrile. The peaks of phytoalexins were monitored with an UV detector at 280 nm for phytocassanes and at 215 nm for momilactones as described. The structures of the compounds were confirmed by EI-MS as described (Koga et al., 1995). In the EI-MS spectra of phytocassane A, B, C, D, and E, the molecular ions $[M]^+$ at m/z 316, $[M-18]^+$ at m/z 316, $[M]^+$ at m/z 318, $[M]^+$ at m/z 316, and $[M]^+$ at m/z 316 were confirmed. In the EI-MS spectra of momilactone A and B, the molecular ions $[M]^+$ at m/z 314 and $[M]^+$ at m/z 330 were confirmed.

Bioassay for Measuring Elicitor Activity in Rice Plants

A bioassay for measuring elicitor activity was performed according to the modified method as described (Koga et al., 1998a, 1998b). Rice seeds (*Oryza sativa* L. cv Akitakomachi) were planted in soils that contained 0.6 mg/g of nitrogen, 0.9 mg/g of phosphorus, and 0.6 mg/g of kalium, then grown in a glass case in a phytotron to prevent exposure to air currents. Until the second leaf was fully expanded, the phytotron was operated at 22°C during the day and 18°C at night with cycles of 12 h of light (3,000 lux) and 12 h of darkness daily. Humidity was set at 80%. Once the second leaf was fully expanded, the phytotron was operated at 22°C during the day and 18°C at night with cycles of 12 h of more intense light (25,000 lux) and 12 h of darkness daily. When the fifth leaf was fully expanded, the surface of the fourth leaf was wounded slightly by pressure from the tapered end of a Pipetman tip (Gilson). The sample to be assayed was dissolved in 20 mm potassium phosphate buffer, pH 6.0, plus 0.1% Tween 20. One drop (2 μL per site) of the sample solution was placed on each of 20 wounded sites per leaf. Plants were then grown at 24°C during the day and 18°C at night with cycles of 12 h of high intense light (25,000 lux) and 12 h of darkness daily. Humidity was set at 80%. The six leaves were collected 48 h after treatment with the sample solution, and the amounts of phytoalexins produced were quantitated by reverse-phase HPLC. A dose-response curve for total production of phytocassanes A, B, C, D, and E induced by bile acid was plotted. One unit of activity was defined as the amount of bile acid required to induce 0.3 μg of phytocassanes per leaf. The elicitor activity of bile acid was calculated as the number of units per microgram dry weight of bile acid. The results are expressed as the mean ± SE of results from three experiments.

Purification Procedure of Elicitor from Human Feces

A total of 1,031 g of human fecal samples was suspended in 5 L of water and homogenized five times for 1 min at intervals of 20 min at 4°C. The homogenized sample was centrifuged 18,000g for 30 min at 4°C and the supernatant was adjusted to pH 3 with HCl and extracted with ethyl acetate. The ethyl acetate extract was evaporated, then fractionated by HPLC on a TSKgel ODS-120T column (7.8 mm i.d. × 30 cm; TOSOH) that was eluted with a linear gradient of 20% to 100% ethanol containing 0.3% acetate. This chromatography was repeated and fractionated. A resultant fraction with the highest elicitor activity was then fractionated by HPLC on an ODS-120T column (4.6 mm i.d. × 30 cm; TOSOH) that was eluted with a mixture of ethanol, acetate, and water (45:0.3:54.7, v/v). This chromatography was also repeated and fractionated. A resultant fraction with the highest elicitor activity was then fractionated by HPLC on an ODS-120T column (4.6 mm i.d. × 30 cm; TOSOH) that was eluted with a mixture of acetonitrile, acetate, and water (40:0.2:59.8, v/v) yielded one purified elicitor.

Measurement of Ion Leakage from Leaf Discs

Cell death was assayed by measuring ion leakage from leaf discs as described (Mittler et al., 1996; Koga et al., 1998b). Rice plants (*Oryza sativa* L. cv Akitakomachi) were cultivated in a phytotron as described before. When the fifth leaf was fully expanded, the fourth leaf was treated with 20 mm potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or the same solution plus 200 μM of CA or 200 μM of fungal cerebroside as described before. Plants were then grown at 24°C during the day and 18°C at night with cycles of 12 h of high intense light (25,000 lux) and 12 h of darkness daily. Humidity was set at 80%. For each measurement, 20 leaf discs (3 cm in length) 0 to 72 h after treatment were collected. The collected sample was floated on 80 mL of 0.01% Tween 20 and gently mixed. The solution was incubated for 3 h at room temperature and conductivity (μS/cm) of the solution was measured with a conductivity meter. Each value presented represents the mean ± SE of results from five experiments.

β-1,3-Glucanase Assay

β-1,3-Glucanase activities were measured using curdlan as a substrate as described (Inui et al., 1997). Rice plants (*Oryza sativa* L. cv Akitakomachi) were cultivated in a phytotron as described earlier. When the fifth leaf was fully expanded, the fourth leaf was treated with 20 mM potassium phosphate, pH 6, plus 0.1% Tween 20 (control) or the same solution plus 200 μM of CA or 200 μM of fungal cerebroside as described earlier. Plants were then grown at 24°C during the day and 18°C at night with cycles of 12 h of high intense light (25,000 lux) and 12 h of darkness daily. Humidity was set at 80%. For each measurement, six leaves 0 to 72 h after treatment were collected and suspended in 10 mL of 50 mM sodium acetate buffer, pH 5.0, containing 0.3% Triton X-100 plus 2 mM 2-mercaptoethanol. The leaves were then homogenized for 1 min at 4°C and centrifuged 15,000g for 15 min at 4°C. The supernatant was used as a crude enzyme solution that was combined with 2 mL of reaction mixture that contained 20 mg curdlan, 50 mM Na_2HPO_4-citric buffer, pH 5.0. The reaction mixture was shaken (60 strokes/min) at 37°C for 60 min, and the reducing sugars in the supernatant were measured by the dinitrosalicylic acid assay as described (Miller, 1959). One unit of activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min. Each value presented represents the mean ± SE of results from five experiments.

Kinetics of the Development of Resistance after Treatment with CA

Rice plants (*Oryza sativa* L. cv Akitakomachi) were cultivated in a phytotron as described earlier. When the fifth leaf was half expanded, 40 rice leaves were sprayed with 4 mM potassium phosphate, pH 6, plus 0.01% Tween 20 (control) or with the same solution plus 20 μM of CA at different times (0–60 h) before inoculation with *M. grisea* as described earlier. Plants were then grown at 24°C during the day and 18°C at night with cycles of 12 h of high intense light (25,000 lux) and 12 h of darkness daily. Humidity was set at 80%. For each measurement, the leaves 0 to 60 h after treatment were sprayed with a spore suspension of a virulent race (007) of *M. grisea* that was prepared as described (Matsumoto et al., 1980). After the rice plants had been grown in a moist chamber (100% humidity) at 25°C for 48 h, they were removed and cultivated in the phytotron that was operated at 23°C during the day and 18°C at night with cycles of 12 h of light (3,000 lux) and 12 h of darkness. Development of disease was scored by counting the number of lesions 7 d after inoculation. When the number of lesions on control plants was taken as 100%, the relative percentage at each time was measured to estimate the disease resistance. The data presented are the means ± SE of results from five measurements for each time.

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