When plants interact with certain pathogens, they protect themselves by generating various chemical and physical barriers called the hypersensitive response. These barriers are induced by molecules called elicitors that are produced by pathogens. In the present study, the most active elicitors of the hypersensitive response in rice were isolated from the rice pathogenic fungus *Magnaporthe grisea*, and their structures were identified as cerebrosides A and C, sphingolipids that were previously isolated as inducers of cell differentiation in the fungus *Schizophyllum commune*. Treatment of rice leaves with cerebroside A induced the accumulation of antimicrobial compounds (phytoalexins), cell death, and increased resistance to subsequent infection by compatible pathogens. The degradation products of cerebroside A (fatty acid methyl ester, sphingoid base, and glucosyl sphingoid base) showed no elicitor activity. Hydrogenation of the 4E-double bond in the sphingoid base moiety or the 3E-double bond in the fatty acid moiety of cerebroside A did not alter the elicitor activity, whereas hydrogenation of the 4E-double bond in the sphingoid base moiety led to a 12-fold decrease in elicitor activity. Furthermore, glucocerebrosides from Gaucher’s spleen consisting of (E)-4-sphingenine and cerebrosides from rice bran mainly consisting of (4E,SE)-4,8-sphingadienine and (4E,SE)-4,8-sphingadienine showed no elicitor activity. These results indicate that the methyl group at C-9 and the 4E-double bond in the sphingoid base moiety of cerebrosides A and C are the key elements determining the elicitor activity of these compounds. This study is the first to show that sphingolipids have elicitor activity in plants.

Sphingolipids are ubiquitous components of the membranes of all eukaryotic cells and are particularly abundant in plasma membranes. In animals, they play important roles in general membrane function, cell-to-cell contact, cell recognition, and regulation of cell growth, differentiation, and apoptosis (1–3). Sphingolipids modulate transmembrane signal transduction via their effects on protein kinases associated with growth factor receptors (2, 4) and on protein kinase C (2, 5), thereby regulating cell proliferation (1, 6) and inducing cell differentiation (7–9) and apoptosis (3). Recently, it has been shown that sphingolipid metabolic products, ceramides, function as second messengers in the signal transduction pathway involved in apoptosis (10, 11) and that the 4E-double bond in the sphingoid base moiety is important for this activity (12). In fungi, sphingolipids are known to function as inducers of cell differentiation. Fungal cerebrosides, including cerebrosides B and C, induce cell differentiation in the fungus *Schizophyllum commune*, with resultant formation of the fruiting body (13–16), and the 8E-double bond and methyl group at C-9 in the sphingoid base moiety are important for the induction of cell differentiation. In animals and fungi, sphingolipids are known to have various biological functions as mentioned above, whereas in the case of plants, no reports have appeared to date demonstrating the involvement of sphingolipids in biological processes, such as the hypersensitive response (HR), cell growth, differentiation, or apoptosis.

When plants interact with certain pathogens, they protect themselves by generating various chemical and physical barriers called the HR (17). During the HR, 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. This is accompanied by the accumulation of antimicrobial compounds (phytoalexins), the induction of pathogenesis-related proteins (chitinases, β-1,3-glucanases and proteinase inhibitors), increased expression of defense-related genes, the formation of lignin, a burst of active oxygen, and increased resistance to subsequent infection by pathogens (18–20).

The HR is induced by molecules called elicitors that are produced by pathogens (21). Elicitors have been studied extensively and, in most cases, have been shown to be oligo- or polysaccharides derived from the mycelia of pathogenic fungi. The chemical structures of some elicitors from pathogens have been determined. An oligosaccharide elicitor of phytoalexin synthesis in soybean has been isolated from the soybean pathogenic fungus *Phytophthora megasperma* and identified as hepta-β-glucopyranoside (22, 23). Unsaturated fatty acids, such as eicosapentaenoic acid and arachidonic acid from the potato pathogenic fungus *Phytophthora infestans*, have been isolated as elicitors of phytoalexin accumulation in potato (24).

Syringolides 1 and 2, glycolipid elicitors in soybean, have been isolated as products of avirulence gene D from the pathogenic
bacterium Pseudomonas syringae (25).

The HR in rice leaves in response to elicitor treatment is highly dependent on environmental conditions, and establishing the right combination of conditions had been difficult. Hence, no bioassay for measuring elicitor activity in rice plants had yet been established. In a previous study, we succeeded in developing a suitable bioassay for measuring elicitor activity in rice leaves by cultivating rice plants under conditions of low temperature, high light intensity, and high humidity (26). In the present study, the most active elicitors of the HR in rice plants were isolated from the rice pathogenic fungus Magnaporthe grisea, and their structures were identified as cerebroside A and C, sphingolipids that were previously isolated as inducers of cell differentiation in the fungus S. commune (14). Treatment of rice leaves with cerebroside A induced the accumulation of phytoalexins, cell death, and increased resistance to subsequent infection by compatible pathogens. Structure-activity relationship experiments indicated that the methyl group at C-9 and the 4E-double bond in the sphingoid base moiety are the key elements determining the elicitor activity of cerebrosides A and C.

 EXPERIMENTAL PROCEDURES

Analysis Procedures

IR spectra were recorded on a Nippondenshi JIR-100 FT-IR spectrophotometer. 1H NMR and 13C NMR spectra were recorded in CD3OD using a JOEL JNM-GSX 500 spectrometer (500.2 MHz for 1H, 125.8 MHz for 13C), using SiMe3 and CD3OD as internal standards. Fast atom bombardment mass spectra (FAB-MS) were recorded on a VG ZAB-HF mass spectrometer using m-nitrobenzyl alcohol as a matrix. Mamilactones (27) and phytocassanes (28, 29) were analyzed by reverse-phase high-performance liquid chromatography (HPLC) using a TSKgel ODS-120T column (4.6 mm i.d. 30 cm; TOSOH Co., Ltd., Tokyo, Japan) column at a flow rate of 1.2 ml/min at 50 °C. Cerebrosides and their derivatives were purified by reverse-phase HPLC using a TSKgel ODS-120T column (21.5 mm i.d. 37.5 cm; TOSOH) column at a flow rate of 10 ml/min at 40 °C, a TSKgel ODS-120A column (7.8 mm i.d. 30 cm; TOSOH) column at a flow rate of 2 ml/min at 50 °C, a TSKgel ODS-120S column (4.6 mm i.d. 30 cm; TOSOH) column at a flow rate of 1.2 ml/min at 55 °C, and a TSKgel CN-80TS column eluted with a mixture of acetonitrile and 20% ethanol. The fatty acid methyl esters were extracted with hexane and then purified by TSKgel ODS-120T column eluted with 76% ethanol. The structures of the two purified esters were determined by 1H NMR and FAB-MS analysis.

The residues extracted with hexane were adjusted to pH 10.7 by the addition of 1 M NaOH and the sphingoid bases were extracted with hexane. 5 ml of H2O were added to these residues, and then the glucosyl sphingoid bases were extracted with ethyl acetate. Furthermore, the sphingoid bases and glucosyl sphingoid bases were purified by HPLC on a TSKgel CN-80TS column eluted with a mixture of acetonitrile and 20% potassium phosphate buffer, pH 7.4 (29:71, v/v). The structures of the sphingoid bases and glucosyl sphingoid bases were determined by 1H NMR and FAB-MS analysis.

Purification of Fatty Acid Methyl Esters, Sphingoid Bases, and Glucosyl Sphingoid Bases from Cerebrosides A and C

5 mg of cerebrosides A and C were incubated with 5 ml of 0.75 M anhydrous methanolic HCl at 80 °C for 12 h to decomposed into n-fatty acid methyl esters and glucosyl sphingoid bases. The fatty acid methyl esters were extracted with hexane and then purified by TSKgel ODS-120T column eluted with 74% ethanol. The structures of the two purified esters were determined by 1H NMR and FAB-MS analysis.

The residues extracted with hexane were adjusted to pH 10.7 by the addition of 1 M NaOH, and the sphingoid bases were extracted with hexane. 4 ml of H2O were added to these residues, and then the glucosyl sphingoid bases were extracted with ethyl acetate. Furthermore, the sphingoid bases and glucosyl sphingoid bases were purified by HPLC on a TSKgel CN-80TS column eluted with a mixture of acetonitrile and 20% potassium phosphate buffer, pH 7.4 (29:71, v/v). The structures of the sphingoid bases and glucosyl sphingoid bases were determined by 1H NMR and FAB-MS analysis.

Purification of Hydrogenation Products of Cerebroside A

Hydrogenation of the double bonds in cerebrosides A was performed according to the procedure developed by Schwarzmann (31). 30 mg of cerebroside A was dissolved in 3 ml of tetrahydrofuran in a screw-capped vial, and 83 μl of 1 M NaOH were added to the solution. After flushing with N2, the hydrogenation was started by the addition of 42 μmol each of KBH3 and PdCl2, and the sample was immediately mixed for 3 min at room temperature. Next, 83 μl of 1 M NaOH were added to the solution. After flushing with N2, the second hydrogenation was started by the addition of 42 μmol each of KBH3 and PdCl2, and the sample was immediately mixed for 3 min at room temperature. To obtain the cerebroside hydrogenated at the 4E-double bond in the sphingoid base moiety or at the 3E-double bond in the fatty acid moiety, this second hydrogenation procedure was repeated 10 times. To obtain the cerebroside hydrogenated at the 3E-double bond in the sphingoid base moiety, this second hydrogenation procedure was repeated 30 times. Each sample after hydrogenation was centrifuged, and the tetrahydrofuran layer was collected. The tetrahydrofuran layer was partitioned between ethyl acetate and 0.05 M Na2CO3 (pH 11). The ethyl acetate layer was evaporated, and the residue was dissolved in 76% ethanol. The solution was fractionated by HPLC on a TSKgel ODS-120S column (7.8 mm i.d. 30 cm) eluted with 76% ethanol, and the resultant fractions corresponding to two peaks at Rf 50 and 67 min were collected. The fraction corresponding to the peak at Rf 50 min was further fractionated by HPLC on a TSKgel ODS-120T column (4.6 mm i.d. 30 cm) eluted with a mixture of ethanol, acetonitrile, and water (20:65:15, v/v) to yield two purified cerebrosides. By 1H NMR analysis, the two purified cerebrosides were confirmed to be (8E)-N-α'-2'-hydroxy-(E)-3'-hexadecenoyl-1-O-β-D-glucopyranosyl-9-methyl-8-sphingen-
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Table I

| 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 | 33350 | 476000 | 0.014 | 1 | 100 |
| ODS-1207/20–100% ethanol | 2084 | 208400 | 0.1 | 7 | 44 |
| ODS-1207/86% ethanol | 121 | 81300 | 0.67 | 47 | 17 |
| Elicitor 1 | 68 | 51400 | 0.76 | 53 | 11 |
| Elicitor 2 plus 46% acetonitrile | 87 | 75000 | 0.86 | 60 | 16 |
| Elicitor 1 | 57 | 47900 | 0.84 | 59 | 10 |

^a One unit of elicitor activity is defined as the amount of the sample required to induce the half-maximal amount of momilactone A per leaf.

^b The specific elicitor activity of the sample is defined as the number of units per µg dry weight of the sample.

^c 1000 g (fresh weight) of mycelia were used as the starting material.

ene (4E)-H-cerebroside A) and (4E,8E)-N-o-α-glucopyranosyl-1-O-β-glucopyranosyl-9-methyl-4,8-sphingadienine (3E) H-cerebroside A). The fraction corresponding to the peak at Rf 67 min was further fractionated by HPLC on a TSKgel ODS-120T column (4.6 mm i.d. x 30 cm) eluted with 72% ethanol to yield a purified cerebroside. By 1H NMR analysis, the purified cerebroside was confirmed to be N-o-α-glucopyranosyl-1-O-β-glucopyranosyl-9-methylsphinganine (3E,4E,8E) H-cerebroside A. Because hydrogenation of the 9E-double bond in the sphingoid base moiety was difficult as compared with the other double bonds, the cerebroside hydrogenated only at the 8E-double bond could scarcely be obtained.

Cerebroside A

IR \( \nu_{\text{max}} \) 3363, 2921, 2851, 1647, 1155, 1077, 1038, 966 cm\(^{-1}\); 1H NMR (CD3OD, 35 °C) \( \delta \) 8.00 (6H, t, J = 6.8 Hz, Me-18, 16'), 1.29 (30H, m, H-12'–17, H-7'–15'), 1.57–1.42 (45H, m, H-11, 16'), 1.60 (3H, s, Me-19), 1.98 (2H, m, H-10), 2.02 (2H, m, H-5'), 2.02 (2H, m, H-6), 2.08 (2H, m, H-7), 3.20 (1H, dd, J = 9.3, 7.8 Hz, H-2'), 3.27 (1H, m, H-4' or 5'), 3.29 (1H, m, H-4' or 5'), 3.36 (1H, dd, J = 9.3 Hz, H-3'), 3.67 (1H, dd, J = 11.7 Hz, H-6'), 3.72 (1H, dd, J = 10.3, 3.4 Hz, H-1), 3.88 (1H, d, J = 11.7 Hz, H-6), 3.97 (1H, dt, J = 5.4, 3.4 Hz, H-2), 4.11 (1H, dd, J = 10.3, 3.4 Hz, H-1), 4.14 (1H, dd, J = 7.3, 5.4 Hz, H-3), 4.27 (1H, d, J = 7.8 Hz, H-1'), 4.33 (1H, d, J = 6.4 Hz, H-2'), 5.14 (1H, t, J = 6.8 Hz, H-8), 5.47 (1H, dd, J = 15.1, 7.3 Hz, H-4), 5.50 (1H, dd, J = 15.1, 6.4 Hz, H-3'), 5.73 (1H, dt, J = 15.1, 6.8 Hz, H-5), 5.83 (1H, dt, J = 15.1, 6.8 Hz, H-4'); negative FAB-MS m/z = 724 [M-III].

Cerebroside C

IR \( \nu_{\text{max}} \) 3378, 2921, 2851, 1648, 1538, 1467, 1165, 1080, 1039, 966 cm\(^{-1}\); 1H NMR (CD3OD, 35 °C) \( \delta \) 8.00 (6H, t, J = 6.8 Hz, Me-18, 18'), 1.29 (34H, m, H-12'–17, H-7'–15'), 1.37–1.42 (44H, m, H-11, 16'), 1.60 (3H, s, Me-19), 1.98 (2H, m, H-10), 2.02 (2H, m, H-5'), 2.02 (2H, m, H-6), 2.08 (2H, m, H-7), 3.20 (1H, dd, J = 9.3, 7.8 Hz, H-2'), 3.27 (1H, m, H-4' or 5'), 3.29 (1H, m, H-4' or 5'), 3.36 (1H, dd, J = 9.3 Hz, H-3'), 3.67 (1H, dd, J = 12.2 Hz, H-6'), 3.72 (1H, dd, J = 10.3, 3.4 Hz, H-1), 3.88 (1H, d, J = 10.3, 3.4 Hz, H-1), 4.11 (1H, dd, J = 7.3, 5.4 Hz, H-3), 4.27 (1H, d, J = 7.8 Hz, H-1'), 4.43 (1H, d, J = 5.9 Hz, H-2'), 5.14 (1H, t, J = 6.8 Hz, H-8), 5.47 (1H, dd, J = 15.1, 7.3 Hz, H-4), 5.50 (1H, dd, J = 15.6, 5.9 Hz, H-3'), 5.73 (1H, dt, J = 15.1, 6.8 Hz, H-5), 5.83 (1H, dt, J = 15.6, 6.8 Hz, H-4'); negative FAB-MS m/z = 752 [M-II].

Bioassay for Measuring Elicitor Activity in Rice Plants

A bioassay for measuring elicitor activity was performed as described previously (26). Rice seeds (Oryza sativa L. cv. Akitakomachi) were planted in soils that contained 0.6 mg/g nitrogen, 0.9 mg/g phosphorus, and 0.6 mg/g potassium and grown in a glass case in a phytotron to prevent exposure to air currents. Until the third 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 (3000 lux) and 12 h of darkness daily. Humidity was set at 80%. Once the third 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 (30,000 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 suspended in 20 mM potassium phosphate buffer, pH 6.5, that contained 0.1% Tween 20 by sonication and rapid agitation on a Vortex mixer. Ten drops (about 20 µl, total) of the sample solution were placed on ten wounded sites per leaf (2 µl per site). After this treatment, plants were grown at 25 °C during the day and 18 °C at night, with cycles of 12 h of high intense light (30,000 lux) and 12 h of darkness daily. Humidity was set at 80%. The two leaves were collected 48 h after treatment with the sample solution, and the amounts of phytoalexins produced were quantitated by HPLC as described above. Because momilactone A was predominantly induced among the various phytoalexins (26), induction of larger amounts of phytoalexins produced was taken to be indicative of higher elicitor activity. Concentration curves were prepared for cerebroside A to be assayed, and the amounts of momilactone A induced were plotted. One unit of elicitor activity was defined as the number of units per µg dry weight of the sample.

Measurement of Ion Leakage from Leaf Discs

Cell death was assayed by measuring ion leakage from leaf discs as described (32). Rice plants (O. sativa L. cv. Akitakomachi) were cultivated in a phytotron as described above. For each measurement, 20 leaf discs (3 cm in length) 48 h after treatment with 0.2 mM cerebroside A or after control treatment (20 mM potassium phosphate plus 0–400 µg/ml cerebroside A (C) or the same solution plus 0.1% Tween 20 (D) as described under “Experimental Procedures.” The amount of momilactone A induced in the leaves was measured 48 h after treatment. Each value represents the mean ± S.E. of results from 20 experiments.
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Table II
Carbon chemical shifts of cerebrosides A and C in CD$_2$OD at 35°C

| Carbon no. | Sugar | Fatty acid | Sphingoid base | Sugar | Fatty acid | Sphingoid base |
|------------|-------|------------|----------------|-------|------------|----------------|
| C-1        | 104.9 | 175.6      | 69.8           | 104.9 | 175.6      | 69.8           |
| C-2        | 75.2  | 74.3       | 54.9           | 75.2  | 74.3       | 54.9           |
| C-3        | 78.1  | 129.2      | 73.1           | 78.1  | 129.2      | 73.1           |
| C-4        | 71.8  | 134.9      | 131.1          | 71.8  | 134.9      | 131.1          |
| C-5        | 78.1  | 33.5       | 134.7          | 78.1  | 33.5       | 134.7          |
| C-6        | 62.9  | 30.4–30.9  | 33.9           | 62.9  | 30.4–30.9  | 33.9           |
| C-7        | 30.4–30.9 | 125.0        | 30.4–30.9 | 30.4–30.9 | 125.0        |
| C-8        | 30.4–30.9 | 125.0        | 30.4–30.9 | 30.4–30.9 | 125.0        |
| C-9        | 30.4–30.9 | 136.9        | 30.4–30.9 | 30.4–30.9 | 136.9        |
| C-10       | 30.4–30.9 | 40.9         | 30.4–30.9 | 30.4–30.9 | 40.9         |
| C-11       | 30.4–30.9 | 28.9         | 30.4–30.9 | 30.4–30.9 | 28.9         |
| C-12       | 30.4–30.9 | 30.4–30.9    | 30.4–30.9 | 30.4–30.9 | 30.4–30.9    |
| C-13       | 30.4–30.9 | 30.4–30.9    | 30.4–30.9 | 30.4–30.9 | 30.4–30.9    |
| C-14       | 33.2  | 30.4–30.9  | 30.4–30.9      | 30.4–30.9 | 30.4–30.9 | 30.4–30.9      |
| C-15       | 23.8  | 30.4–30.9  | 30.4–30.9      | 30.4–30.9 | 30.4–30.9 | 30.4–30.9      |
| C-16       | 14.5  | 33.2       | 33.2           | 33.2  | 33.2       | 33.2           |
| C-17       | 23.8  | 23.9       | 23.9           | 23.9  | 23.9       | 23.9           |
| C-18       | 14.5  | 14.6       | 14.6           | 14.6  | 14.6       | 14.6           |
| C-19       | 16.3  |            | 16.3           |        |            | 16.3           |

Kinetics of the Development of Resistance after Treatment with Cerebroside A

Rice plants (O. sativa L. cv. Akitakomachi) were cultivated in a phytotron as described above. Rice leaves (two leaves) were treated with 0.53 mg/ml of elicitor 1 (control treatment) or with the same solution plus 0.2 mg cerebrosides A (cerebrosides treatment) at different times before inoculation with M. grisea (compatible race 007) on day 0. After the treatment with the sample solutions, the leaves were sprayed with a spore suspension of a compatible race (007) of M. grisea prepared as described (33). After the rice plants had been kept 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 (3000 lux) and 12 h of darkness. Development of disease was scored by determination of the number of lesions 7 days after inoculation. The number of lesions on control plants was taken as 100%. The data presented are the means ± S.E. of results from 20 measurements for each time point.

RESULTS

Purification and Structural Determination of Elicitors from M. grisea—Inoculation of rice plants with an incompatible race of M. grisea induces the accumulation of momilactones (27) and phytocassanes (28, 29), which are the main phytoalexins found in rice. In our newly established bioassay, an ethyl acetate extract of a sonicated suspension of mycelia of M. grisea—inoculated rice plants was taken as 100%. The data presented are the means ± S.E. of results from 20 measurements for each time point.

Dose-response curves for levels of momilactones by elicitor 1 in the presence and absence of Tween 20 are shown in Fig. 1. In this bioassay, Tween 20 was added to the elicitor solution, because the surface of the rice leaf is extremely hydrophobic and the elicitor solution without Tween 20 was difficult to place on the leaf. The amount of momilactone A induced by elicitor 1 in the presence of Tween 20 was slightly greater than that induced by elicitor 1 without Tween 20. However, elicitor 1 alone induced a readily detectable amount of momilactone A, indicating that the elicitor activity of elicitor 1 is not due to the effect of the detergent. 58 μg/ml of elicitor 1 induced the half-maximal amount of momilactone A per leaf (0.53 μg). Therefore, 1 unit of elicitor activity was defined as the amount required to induce 0.53 μg of momilactone A per leaf.

13C NMR (Table II), 1H NMR, FAB-MS, and IR spectra (see under "Experimental Procedures") indicated that the two purified elicitors were sphingolipids composed of D-glucose, fatty acid, and sphingoid base. The FAB-MS spectrum, the coupling constants between the protons, the carbon chemical shifts, 1H-13C COSY spectrum, and the heteronuclear multiple bond correlation experiment revealed that the sugar moiety of the two elicitors should be D-glucose. The carbon and proton chemical shifts, 1H-13C COSY spectrum and the heteronuclear multiple bond correlation experiments of elicitors 1 and 2 revealed that a hydroxyl group and an olefinic carbon of each fatty acid moiety should be at C-3' and C-4', respectively, and that a hydroxyl group, two olefinic carbons, and a branching methyl of each sphingoid base moiety should be at C-3, C-4, C-5, C-8, and C-9, respectively. However, the length of methylene of the fatty acid and sphingoid base moieties were not able to be determined by these analyses. Therefore, to determine the length of methylene, the two elicitors were decomposed into D-glucose, fatty acid methyl esters, and sphingoid bases by hydrolysis.

FIG. 2. Structures of cerebrosides A and C.
The amounts of phytoalexins induced by treatment of rice leaves with cerebroside A

| Time after treatment (h) | Control treatment | Cerebroside treatment |
|-------------------------|-------------------|-----------------------|
|                         | Phytocassane      | Phytoalexin production |
|                         | A                 | B                     |
|                         |                   | µg/g fresh weight of leaves |
| 0                       | ND                | ND                    |
| 12                      | ND                | ND                    |
| 24                      | 0.5 ± 0.2         | ND                    |
| 36                      | 1.3 ± 0.4         | ND                    |
| 60                      | ND                | 6.9 ± 1.9             |
| 72                      | ND                | 0.4 ± 0.2             |
| 96                      | ND                | 0.2 ± 0.1             |

*ND, not detectable.

The ³H NMR spectrum of the fatty acid methyl ester of elicitor 1 showed signals for a terminal methyl group (δ 0.88), a methyl group of carbomethoxy ester (δ 3.80), a proton geminal to a hydroxyl group (δ 4.60), and two olefinic protons (δ 5.50, 5.89). The negative FAB-MS spectrum showed a molecular ion [M-H]⁻ at m/z 283. Thus, the structure of the fatty acid methyl ester of elicitor 1 was confirmed to be 2-hydroxy-(E)-3-hexadecenoic acid methyl ester. The ³H NMR spectrum of the fatty acid methyl ester of elicitor 2 was almost identical to that for elicitor 1, whereas the negative FAB-MS spectrum showed a molecular ion [M-H]⁻ at m/z 311. Therefore, the structure of the fatty acid methyl ester of elicitor 2 was confirmed to be 2-hydroxy-(E)-3-octadecenoic acid methyl ester.

The positive FAB-MS spectrum of the sphingoid base of each elicitor showed a molecular ion [M]⁺ at m/z 312.445, suggesting that the structure of the sphingoid base of elicitors 1 and 2 was (4E,8E)-9-methyl-4,8-sphingadienine. Consequently, the two elicitors were identified as cerebrosides A and C (Fig. 2), sphingolipids that were previously isolated from Penicillium funiculosum (14) and Pachybasium species (34).

Demonstration of the HR Induction by Treatment with the Cerebroside—The HR to pathogen invasion is accompanied by the expression of a variety of biochemical and phenomenological markers (18–20). Therefore, to demonstrate that the cerebroside elicitor did, indeed, induce the HR in rice plants, we examined whether three markers associated with the HR could be detected in rice leaves after treatment with the cerebroside. Because the total amounts of cerebrosides A plus C isolated from mycelial extracts of M. grisea were 144 µg/g fresh weight of mycelia, subsequent experiments were performed with the similar concentration (0.2 mM, 145.6 µg/g) of cerebroside A.

First, we examined whether treatment with the cerebroside induced the production of sufficient phytoalexin to limit proliferation of the pathogen. The amounts of phytoalexins induced after treatment with the cerebroside are shown in Table III. Momilactones and phytocassanes have high antifungal activity against M. grisea and the ED₅₀ values of momilactone A and phytocassanes A and B in the prevention of growth of germ tubes of M. grisea were 5, 5, and 1.5 µg/ml, respectively (27, 28). Thus, the amounts of phytoalexins induced 24 h or more after treatment with the cerebroside were sufficient to inhibit growth of germ tubes of M. grisea. Second, we examined a biochemical marker, the induction of irreversible membrane damage (cell death), by measuring ion leakage from leaf discs. As shown in Fig. 3, the amount of ion leakage from leaves upon treatment with the cerebroside was significantly greater than that upon control treatment, suggesting that the cerebroside-induced response was accompanied by cell death. Finally, we examined whether treatment with the cerebroside could induce significant resistance to a compatible pathogen. The data presented in Fig. 4 show that treatment of rice plants with the cerebroside 24 h or more before inoculation of the pathogen resulted in complete resistance to infection by a compatible race of M. grisea. However, treatment with the cerebroside 0–6 h before inoculation hardly enhanced resistance to infection. These results indicate that the cerebroside elicitor did not inhibit proliferation of the pathogen but, rather, induced resistance against the pathogen. The three sets of results described above clearly show that the cerebroside elicitor induced HR in rice plants. To the best of our knowledge, this is the first report to demonstrate that sphingolipids induce the HR in plants.

Elicitor Activity of Various Sphingolipids and of the Derivatives of Cerebroside A—To examine the structure-activity relationship, we tested whether various cerebrosides and derivatives of cerebroside A have elicitor activity by measuring phytoalexin induction in rice leaves (Table IV). Cerebrosides A and C had almost the same elicitor activity, but their degradation products, 2-hydroxy-(E)-3-hexadecenoic acid methyl ester, (4E,8E)-9-methyl-4,8-sphingadienine and (4E,8E)-9-glucopyranosyl-9-methyl-4,8-sphingadienine, showed no elicitor activity. In potato, unsaturated fatty acids, such as eicosapentaenoic acid and arachidonic acid, have been shown to have elicitor activity (24), whereas, in rice, the entire structure of the cerebroside or ceramide, but not the fatty acid moiety, appears to be important for the activity.

Furthermore, to examine which double bonds in the ceramide moiety are involved in the elicitor activity, the double bonds of cerebroside A were hydrogenated. Hydrogenation of the 3E-double bond in the fatty acid moiety of cerebroside A did not
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alter the elicitor activity, whereas hydrogenation of the 4E-double bond in the sphingoid base moiety led to a 12-fold decrease in elicitor activity. (3E,4E,8E) H₂-cerebroside A, in which all of the double bonds are hydrogenated, had almost the same activity as (4E) H₂-cerebroside A, in which only the 4E-double bond in the sphingoid base is hydrogenated, suggesting that the 8E-double bond in the sphingoid base is not involved in the elicitor activity. The glucocerebrosides from Gaucher's spleen and the galactocerebrosides from bovine brain showed hardly any elicitor activity. The methyl group at C-9 and the 8E-double bond in the sphingoid base, which are present in cerebroside A and C but absent in these cerebroside from mammals, are thought to be related to the observed difference in elicitor activity. However, the 8E-double bond in the sphingoid base is not involved in the activity, suggesting that the methyl group at C-9 is important for the elicitor activity. Among the cerebroside from rice bran, about 14% contain the cerebroside consisting of (4E,8E)-4,8-sphingadienine (30), which is structurally similar to cerebroside A and C but lacking the methyl group at C-9. The view that the methyl group at C-9 is important for the elicitor activity is also supported by the finding that the cerebroside from rice bran showed hardly any activity. The results presented above indicate that the methyl group at C-9 and the 4E-double bond in the sphingoid base moiety are the key elements determining the elicitor activity of cerebroside A and C.

DISCUSSION

To the best of our knowledge, our findings are the first to show that sphingolipids have elicitor activity in plants.

Comparisons of the elicitor activity of cerebroside derivatives provide some important insights into the structural features required for the elicitor activity in rice plants. The 4E-double bond and methyl group at C-9 in the sphingoid base moiety of cerebroside A and C were the key elements determining the elicitor activity in rice. Such a specificity for fungal cerebroside indicates that rice plants can specifically recognize the pathogenic fungi and thereby protect themselves. In the fungus S. commune, cerebroside C induces cell differentiation, with resultant formation of the fungus body (14), and the 8E-double bond and methyl group at C-9 in the sphingoid base moiety are important for inducing cell differentiation (13–15). In mammalian cells, ceramides function as second messengers in the

![Figure 4. Kinetics of the development of resistance after treatment with cerebroside A.](image)

**Elicitor activity of various sphingolipids and of the derivatives of cerebroside A**

(3E) H₂-Cerebroside A (cerebroside B), (4E) H₂-cerebroside A, and (3E,4E,8E) H₂-cerebroside A are the hydrogenation products of cerebroside A. Hexadecenoic acid methyl ester (2-hydroxy-(E)-3-hexadecenoic acid methyl ester), methyl sphingadienine (4E,8E)-9-methyl-4,8-sphingadienine), and glucosyl methyl sphingadienine (4E,8E)-O-glucopyranosyl-9-methyl-4,8-sphingadienine) are the hydrolysis products of cerebroside A. The elicitor activity of each compound was measured as described under “Experimental Procedures.”

**Table IV**

| Compound | Sugar | Fatty acid | Sphingoid base | Specific elicitor activity* |
|----------|-------|-----------|----------------|-----------------------------|
| Cerbroside A | Glu | 16:1 | d19:2(4E,8E,9Me) | 0.86 |
| Cerbroside C | Glu | 18:1 | d19:2(4E,8E,9Me) | 0.84 |
| (3E) H₂-Cerbroside A (Cerebroside B) | Glu | 16:0 | d19:2(4E,8E,9Me) | 0.87 |
| (4E) H₂-Cerbroside A | Glu | 16:1 | d19:1(4E,8E,9Me) | 0.072 |
| (3E,4E,8E) H₂-Cerbroside A | Glu | 16:0 | d19:0(9Me) | 0.071 |
| Hexadecenoic acid methyl ester | | | | <0.020* |
| Methyl sphingadienine | | | | <0.020* |
| Glucosyl methyl sphingadienine | Glu | | | <0.020* |
| Sphingolipids from animals | | | | |
| Glucosylceramides | Glu | 14–24:0 | d18:1(4E) | <0.020* |
| from Gaucher’s spleen | | 18–24:1 | | |
| Galactosylceramides | Gal | 18–26:0 | d18:1(4E) | <0.020* |
| from bovine brain | | | | |
| Sphingolipids from plants | | | | |
| Cerbroside from rice bran | Glu | 14–26:0 | d18:0–2(4E,8E or Z) | <0.020* |
| | | 14–26:0 | t18:0–1(8E or Z) | |

*One unit of elicitor activity is defined as the amount of the sample required to induce the half-maximal amount of momilactone A per leaf. The specific elicitor activity of the sample is defined as the number of units per µg dry weight of the sample.

In this table, minor components are omitted. The abbreviations used are: 14–26:0, 2-hydroxy fatty acids having carbon chain length 14–26; 16:0, hexadecanoic acid; 16:1, (E)-3-hexadecenoic acid (hexadecanoic acid with 1 double bond); 16:1, 2-hydroxy-(E)-3-hexadecenoic acid; d18:0–2, sphinganine with 0–2 double bonds; d18:0, sphinganine; t18:0, 4-hydroxysphinganine; d18:1(4E), (E)-4-sphingine; d19:0(9Me), 9-methylysphinganine; d19:1(8E,9Me), (8E)-9-methyl-8-sphingine, d19:2(4E,8E,9Me), (4E,8E)-9-methyl-4,8-sphingadienine.

The amount of each compound required to induce maximal momilactone production was about 50 µg per leaf. The amount of momilactone A induced by treatment with the cerebroside from rice bran (50 µg per leaf) was 2-fold higher than that induced by control treatment (20 µg potassium phosphate plus 0.1% Tween 20). The amount of momilactone A induced by treatment with the glucocerebroside from Gaucher’s spleen (50 µg per leaf) was about 50% higher than that induced by control treatment. The amounts of momilactone A induced by treatment with the other compounds (50 µg per leaf) were about 20–30% higher than that induced by control treatment. However, these amounts were very much lower than the amount (about 21-fold the amount induced by control treatment) required for 1 unit of elicitor activity.
signal transduction pathway involved in apoptosis (10, 11), and the 4E-double bond in the sphingoid base moiety is important for this activity (12). The structural features required for the elicitor activity in rice and for the induction of cell differentiation in *S. commune* are similar in that the methyl group at C-9 is important for both types of activity. However, the structural features required for the elicitor activity in rice and for the induction of apoptosis in mammalian cells are similar in that the 4E-double bond is important for both types of activity.

It is well established that programmed cell death plays an important role in the development of plant organs, as it does in the development of animal organs. Recent studies suggest that the HR in plants is a type of programmed cell death (35, 36), because the appearance of the HR is genetically controlled (37, 38) and the HR-associated cell death is activated by certain elicitors (39, 40). Furthermore, the induction of the HR by some pathogens and elicitors has been shown to be mechanistically similar to apoptosis in animals, because apoptotic features in animals are present in some plant cells undergoing the HR (41–44). In consideration of our finding that the HR is induced by sphingolipids in rice plants, it seems plausible that the HR in plants may be induced by the same mechanism as that responsible for the sphingolipid or ceramide-mediated apoptosis in animals.

Because, in animal cells, sphingolipids modulate various protein kinases, our findings suggest the possibility that a signal transduction pathway involved in the HR might play a role in plant disease resistance. Gene-for-gene interactions, in which a single disease resistance gene in the plant responds specifically to a single avirulence gene in the pathogen, have been described. Recently, numerous resistance genes have been cloned from several plant species (19, 45–49). The rice *Xa21* gene, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6 (49), and the tomato *Pto* gene, which confers resistance to races of *P. syringae* pv. *tomato* (45), encode serine-threonine protein kinases, suggesting that the protein kinases play an important role in plant disease resistance. If sphingolipids produced by pathogens can modulate these plant protein kinases in a manner similar to that observed in animal systems, the sphingolipid-mediated signaling pathway may function in plant disease resistance. If we are to understand the relationship between sphingolipid elicitors and plant disease resistance, further studies on the effects of these molecules on the signal transduction pathway are now essential.

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