Cerebroside Elicitor Confers Resistance to Fusarium Disease in Various Plant Species

Kenji Umemura, Shigeki Tanino, Tadako Nagatsuka, Jinichiro Koga, Michiaki Iwata, Kenji Nagashima, and Yoshimiki Amemiya

First, second, and fifth authors: Agricultural & Veterinary Research Labs, Meiji Seika Kaisha Ltd., 760, Morooka, Kohoku-ku, Yokohama 222-8567, Japan; third and fourth authors: Health & Bioscience Labs, Meiji Seika Kaisha Ltd., 5-3-1, Chiyoda Sakado-shi, Saitama 350-0289, Japan; and sixth and seventh authors: Laboratory of Plant Pathology, Faculty of Horticulture, Chiba University, 648, Matsudo, Matsudo-shi, Chiba 271-8510, Japan.

Accepted for publication 23 March 2004.

ABSTRACT

Umemura, K., Tanino, S., Nagatsuka, T., Koga, J., Iwata, M., Nagashima, K., and Amemiya, Y. 2004. Cerebroside elicitor confers resistance to Fusarium disease in various plant species. *Phytopathology* 94:813-818.

In the rice blast fungus pathosystem, cerebroside, a compound categorized as a sphingolipid, was found in our previous study to be a non-race-specific elicitor, which elicits defense responses in rice. Here we describe that cerebroside C is produced in diverse strains of *Fusarium oxysporum*, a common soilborne agent of wilt disease affecting a wide range of plant species. In addition, some type of cerebroside elicitor involving cerebroside A, B, or C was detected in other soilborne phytopathogens, such as *Pythium* and *Botrytis*. Treatment of lettuce (*Lactuca sativa*), tomato (*Ly-*

opersicon esculentum), melon (*Cucumis melo*), and sweet potato (*Ipomoea batatas*) with cerebroside B resulted in resistance to infection by each pathogenic strain of *F. oxysporum*. Induction of pathogenesis-related genes and H₂O₂ production by treatment with cerebroside B were observed in tomato root tissues. The cerebroside elicitor showed no antifungal activity against *F. oxysporum* in vitro, indicating that the cerebroside elicitor activates defense mechanisms to confer resistance to Fusarium disease. These results suggest that cerebroside functions as a non-race-specific elicitor in a wide range of plant-phytopathogenic fungus interactions. Additionally, cerebroside elicitor serves as a potential biologically derived control agent.

Additional keywords: biocontrol, plant defense activator, probenazole.

A study of similarities in the innate immune systems between plants and animals was recently completed (44). The innate immune system is defined as a battery of mechanisms serving as the line of first defense in response to pathogenic organisms. These defense mechanisms are initiated based on the discrimination between self and nonself in a host. In higher plants, the signal molecules of this event are assumed to be race-specific elicitors, which are encoded by pathogen avirulence (*avr*) genes (43,47), or non-race-specific elicitors, which are various chemical derivatives of phytopathogenic origin (6,14,41,53). *Avr* gene products are recognized by receptor proteins that contain mostly nucleotide binding site/leucine-rich repeat (NBS-LRR) sequences, encoded by the corresponding plant disease resistance (*R*) genes (38). These characteristic NBS-LRR sequences are homologous to certain animal gene products involved in apoptotic cell death (31). On the other hand, numerous pathogen-derived compounds, referred to as non-race-specific elicitors, have been shown to activate plant defense responses in both host and nonhost plants (5,36). This type of resistance to most phytopathogens is assumed to be the predominant form of disease resistance in plants. In the animal innate immune system, it has been shown that pathogen surface-derived molecules, called pathogen-associated molecular patterns (PAMPs), are recognized by the host to induce the expression of defense genes and the production of antimicrobial compounds (1,46). It is clear that PAMPs include bacterial flagellin, fungal glucan, and chitin, which are regarded as non-race-specific elicitors in the plant kingdom (21,51).

In our previous reports, cerebroside A, B, and C, categorized as sphingolipids, have been isolated as novel elicitors found in a wide range of phytopathogens (27,50). Treatment with cerebroside elicitor has been demonstrated not only to induce rice phytoalexins (7,25,26,49) and pathogenesis-related (PR) proteins synthesis (32), but to confer disease resistance in rice plants to rice blast fungus in paddy fields (50). Furthermore, cerebroside elicitor has been demonstrated to activate defense signaling in rice cell suspension cultures and in rice plants (45,48). Although sphingolipid is regarded as a structural component of many eukaryotic cell membranes, recent studies suggest the involvement of sphingolipids and their metabolites in signal transduction pathways of various cellular responses in mammalian cells (10,18-20,42). Previously, we demonstrated that the elicitor activity of cerebroside and its derivatives depends on the structural specificity of sphingolipid in rice (27,50). The function of sphingolipids in fungi remained unclear, until a recent report indicated that conversion of sphingolipid to glycosphingolipid by glucosyltransferase is essential for normal development in *Aspergillus* species (30). No assessment of the biological activity of sphingolipids in plants had been reported except for our own work; however, in a recent study sphingosine-1-phosphate derived from sphingolipid was demonstrated to be involved in the signal transduction pathway controlling guard cell turgor (9,34).

We report herein that all tested strains of *Fusarium oxysporum* contained cerebroside C. In addition to *F. oxysporum*, cerebroside elicitor was found in other soilborne pathogens. Treatment with cerebroside B resulted in enhanced disease resistance against the pathogenic *F. oxysporum* in lettuce, tomato, melon, and sweet potato. Furthermore, cerebroside elicitor induced the synthesis of PR genes and H₂O₂ production in tomato root tissues. These results suggest that cerebroside functions as a general elicitor in several plant species, and as a potential biologically derived control agent.

Corresponding author: K. Umemura; E-mail address: kenji_umemura@meiji.co.jp

Publication no. P-2004-0607-01R

© 2004 The American Phytopathological Society

MATERIALS AND METHODS

Microorganisms and qualitative analysis of cerebroside elicitor. *Pythium graminicola* IFO32996, *Botrytis allii* IFO9430, *Glomerella cingulata* IFO5257, and *Sclerotium cepivorum* IFO32367 were purchased from the Institute for Fermentation, Osaka (IFO), Japan. Each microorganism was cultured in a potato/sucrose (PS) liquid medium containing potato extract (200 g of potato) and 20 g of sucrose per liter for 6 days at 25°C. Following collection of mycelia, the cerebroside elicitor was analyzed in a qualitative manner as previously described (48).

Plant culture. Plants such as lettuce (*Lactuca sativa* L. cv. Patriot, Nitounosan Seed Co. Ltd., Japan), tomato (*Lycopersicon esculentum* L. cv. Ponterozza, Takii Seed Co. Ltd., Japan), and melon (*Cucumis melo* L. cv. Amus, Nihon Engei Seisan, Japan) were sown in a plug-tray (2.5 × 2.5 cm cells). Plants were grown in a greenhouse at 13 to 28°C and used about 4 weeks after seeding, when they had the fourth leaf expanded. Seedlings of sweet potato (*Ipomoea batatas* L. cv. Benikomachi) were used as commercially available.

Inducing chemicals and their application. The cerebroside elicitor used in this study was purified as previously described (50). To enable comparison between cerebroside and another chemical inducer, lettuce plants were treated with probenazole (3-allyloxy-1, 2-benzisothiazole-1, 1-dioxide; Oryzemat, Meiji Seika Kaisha Ltd., Japan), as previously reported (50). As a positive control, benomyl (Benlate) purchased from Dupont Kabushiki Kaisha, was applied to sweet potato plants in the field

experiment. Glucosylceramide (GlcCer) was purchased from Funakoshi Pharmaceutical Ltd., Japan.

Cerebroside protection experiments. Cerebroside was applied to plants by a dipping treatment with solutions of various concentrations. To evaluate the efficacy of cerebroside against Fusarium disease, each plant was scored for disease symptoms according to the following scale: 0, healthy leaves; 1, lower leaves stunted and yellowing; 2, slight wilt (less than 20% of leaves wilting); 3, moderate wilt (20 to 50% of leaves wilting); 4, serious wilt (more than 50% of leaves wilting); and 5, dead. Disease scores were converted to disease severity by the following formula: disease severity = [(B × 1) + (C × 2) + (D × 3) + (E × 4) + (F × 5)]/(total number of plants), where B, C, D, E, F, and G are the number of plants corresponding to scores 1, 2, 3, 4, and 5, respectively.

Strains of *F. oxysporum* were cultured in PS liquid medium for 7 days at 25°C and then added to a soil and bran (1:1) mixed medium in flasks. After incubation for 3 weeks at 28°C, conidia was scored as a plating method, using Komada's Fusarium selection medium (28), and diluted to make an infested soil at a given density of the conidia. After cerebroside treatment, each plant was transplanted in a plastic pot (350 cm³) packed with each causal *F. oxysporum*-infested soil and kept in a greenhouse.

Field trial. Sweet potato seedlings (*I. batatas* cv. Benikomachi, weakly resistant to Fusarium stem rot in the field) were purchased from Zen-Noh, Japan. Randomized split plots were used. After water or cerebroside B or benomyl treatment for 24 h, each seedling was transplanted to subplots consisting of three 10 m rows, spaced 80 cm apart on 18 May 2000 in the Meiji Seika Kaisha test fields in Kayama, Odawara, Kanagawa Prefecture. Around 15 seedlings were grown within the rows, about 70 cm apart. Initial disease symptoms of natural infection were observed on 25 May. To evaluate the disease rate, diseased plants were scored on 3 June.

Northern blot analysis. cDNA probes were synthesized by polymerase chain reaction (PCR) amplification of insert DNA in pET28b (Novagen, Madison, WI) using forward primer (5'-CTTCTCGACGTCTCATATGAG-3') and reverse primer (5'-GCTACCAACCAATGTGTTGGT-3') for *PR-1* (accession no.

TABLE 1. Cerebroside C in various strains of *Fusarium oxysporum*

Strains of <i>F. oxysporum</i>	Host plant
<i>F. oxysporum</i> f. sp. <i>lactucae</i> isolate SB1-1 (race 1)	Lettuce
<i>F. oxysporum</i> f. sp. <i>lactucae</i> isolate F9501 (race 2)	Lettuce
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race J-1	Tomato
<i>F. oxysporum</i> f. sp. <i>lycopersici</i> race J-2	Tomato
<i>F. oxysporum</i> f. sp. <i>melonis</i> race 0	Melon
<i>F. oxysporum</i> f. sp. <i>melonis</i> race 1	Melon
<i>F. oxysporum</i> f. sp. <i>cucumerinum</i>	Cucumber

Fig. 1. Structures of cerebroside elicitor and animal-derived glucosylceramide (GlcCer).

AI490182), and forward primer (5'-ACGTATATGGGGTCGT-ACTGGTT-3') and reverse primer (5'-TACATCCTCCAGGTA-CCTTAAGG-3') for *PR-5* (accession no. AW034472). Total RNA was isolated from tomato roots by the Phenol/sodium dodecyl sulfate (SDS) method, as described elsewhere (2). Ten micrograms of total RNA was separated to formaldehyde gels, blotted onto Hybond-N membranes (Amersham Biosciences, Tokyo), hybridized to CDP-star (Amersham Biosciences) labeled probe, and fed to a CDP-star detection module according to the manufacturer's instructions. Equal loading was monitored by ethidium bromide staining of rRNA.

Measurement of H₂O₂ production. H₂O₂ production was followed by monitoring of the luminescent increase of MCLA (2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2- α]pyrazine-3-one; Tokyo Kasei Kogyo, Tokyo). Five segments (1 cm length) of tomato roots were equilibrated in 0.1 M phosphate buffer (pH 6.8), maintained in a spectrophotometer cuvette by mild agitation, and then mixed with a solution of MCLA at a final concentration of 0.3 mM. After addition of cerebroside at a given final concentration, MCLA luminescence was continuously measured with a luminescence reader (BLR-201; Aloka Co. Ltd., Japan) for 15 min, with 5-s intervals. Authentic H₂O₂ solution was used as a standard.

RESULTS

Cerebroside elicitor was found in diverse strains of *F. oxysporum* and in other soilborne pathogens, such as *Pythium graminicola*, *Botrytis allii*, *Glomerella cingulata*, and *Sclerotium cepivorum*. We sought to confirm the presence or absence of the cerebroside in mycelia of *F. oxysporum*, listed in Table 1, by thin-layer chromatography and reversed-phase high-performance liquid chromatography, as previously described (27). Cerebroside C (Fig. 1) was detected in the mycelia of all tested strains of *F. oxysporum*. Furthermore, some type of cerebroside elicitor was found in the mycelia of all soilborne pathogens examined, including *P. graminicola*, *B. allii*, *G. cingulata*, and *S. cepivorum* (Table 2). Cerebroside elicitors A, B, and C differ only in the lipid moiety (Fig. 1). Cerebroside elicitors have been shown to specifically induce the synthesis of phytoalexins and PR protein at the same concentrations in rice (50). The elicitor activity of cerebroside in the case of rice plants has been shown to depend on the

TABLE 2. Cerebroside elicitors in diverse soilborne pathogens

Soilborne pathogens	Cerebroside species
<i>Pythium graminicola</i>	C
<i>Botrytis allii</i>	A, B
<i>Glomerella cingulata</i>	B
<i>Sclerotium cepivorum</i>	B

Fig. 2. Cerebroside-enhanced resistance to Fusarium disease of lettuce, control (left) and cerebroside treatment (right). Lettuce plants cultivated in plug-trays were treated with cerebroside B at 10 μ g/ml for 24 h by dipping treatment or solvent (0.5% EtOH). The disease symptoms were surveyed 13 days after transplanting into infected soil.

structure of the sphingoid moiety characteristic of fungi, one example being the C-9 methyl group (27). Dickson and Lester (12, 13) reported that as in other sphingolipids, cerebroside is the essential component of the fungal cell membrane.

Cerebroside elicitor enhances resistance to Fusarium disease in various plant species. To determine whether cerebroside functions as an elicitor beyond the rice-blast interaction, cerebroside was tested for its potential to enhance resistance to Fusarium disease in various plant species. As shown in Figure 2, cerebroside B induced disease resistance to lettuce (*Lactuca sativa* cv. Patriot) by dipping treatment against the *F. oxysporum* f. sp. *lactucae* isolate SB1-1 (race 1) (16). Cerebroside C also enhanced disease resistance to root rot in lettuce (data not shown), indicating that its activity is equal to that of rice. The efficacy of cerebroside B was dose-dependent, and the application of cerebroside at concentration below 5 μ g/ml resulted in reduced efficacy (Table 3). On the other hand, lettuce treated with pro-benazole (Oryzmate, Meiji Seika Kaisha) at 100 μ g/ml, considered to be a plant defense activator, showed slightly reduced disease development. The same phenomenon applied to inoculation with *F. oxysporum* f. sp. *lactucae* F9501 (race 2) as another isolate (data not shown). These results indicate that cerebroside elicitor is effective in controlling root rot caused by *F. oxysporum* f. sp. *lactucae* in lettuce.

Pretreatment with nonpathogenic *F. oxysporum* has been reported to reduce Fusarium disease in several plant species (15,24, 37). However, there seems to be no established theory to explain whether these microorganisms compete for nutrients with pathogenic microbes or enhance disease resistance in host plants. To determine whether cerebroside from the cell membrane of *F. oxysporum* is involved in one broad mechanism of action or the other, we tried to determine whether cerebroside enhanced resistance to Fusarium diseases in other plant species, as in lettuce. We observed the results of treating tomato (*Lycopersicon esculentum* cv. Ponterozza) roots with cerebroside B at 10 μ g/ml 2 days before challenge inoculation with *F. oxysporum* f. sp. *lycopersici* race J-1. As shown in Table 4, cerebroside B enhanced disease resis-

TABLE 3. Dose-dependency of cerebroside-enhanced resistance to Fusarium disease in lettuce (*Lactuca sativa* L. cv. Patriot)

Concentration (μ g/ml)	14 days postinoculation		28 days postinoculation	
	Disease severity ^a	% Diseased plants	Disease severity ^a	% Diseased plants
Control	1.2 $\pm$ 1.2 ₄	60	4.6 $\pm$ 0.7 ₃	100
1	0.4 $\pm$ 0.7 ₂	30	1.9 $\pm$ 1.5 ₂	60
5	0.3 $\pm$ 0.4 ₂	25	1.8 $\pm$ 1.5 ₂	60
10	0 ₁	0	0.6 $\pm$ 1.0 ₁	30
20	0 ₁	0	0.5 $\pm$ 0.8 ₁	30
Probenazole (100 μ g/ml)	0.6 $\pm$ 0.7 ₃	25	4.3 $\pm$ 0.9 ₃	90

^a Mean value $\pm$ standard deviation is based on two independent experiments encompassing 10 pots (one plant per pot). Inoculum density of *Fusarium oxysporum* f. sp. *lactucae* isolate SB1-1 (race 1) was measured at 2×10^5 conidia per gram of soil. The statistical significance of the values followed by various numbers in each column was determined using Duncan's multiple range test ($P = 0.05$).

TABLE 4. Cerebroside-enhanced resistance to Fusarium disease of tomato (*Lycopersicon esculentum* L. cv. Ponterozza) surveyed 21 days postinoculation

Concentration	Disease severity ^a	% Diseased plants
Control	3.1 $\pm$ 1.3 ₂	93
5	2.1 $\pm$ 1.7 ₁	68
10	1.9 $\pm$ 1.7 ₁	70

^a Mean value $\pm$ standard deviation is based on three independent experiments encompassing 20 pots (one plant per pot). Inoculum density of *Fusarium oxysporum* f. sp. *lycopersici* race J-1 was measured at 2×10^4 conidia per gram of soil. The statistical significance of the values followed by various numbers was determined using Duncan's multiple range test ($P = 0.05$).

tance in aerial parts of tomato compared with that of the untreated control. In the melon (*C. melo* cv. Amus)–*F. oxysporum* f. sp. *melonis* race 0 interaction, it was found that cerebroside B also enhanced disease resistance (Table 5). Therefore, it seems reasonable to conclude that cerebroside enhances Fusarium disease resistance in several plant species, and that cerebroside acts as a non-race-specific elicitor in several plant–*F. oxysporum* interactions.

Cerebroside B enhances resistance to Fusarium disease in sweet potato in the field. To examine the effects of cerebroside elicitor on resistance to Fusarium disease under practical conditions, cerebroside treatment was extended to the field. After seedlings of sweet potato (*I. batatas* cv. Benikomachi) were treated with cerebroside B for 24 h, they were transplanted in *F. oxysporum* f. sp. *batatas*-infested soil (virulence factor not determined) for challenge-inoculation. As shown in Table 6, cerebroside effectively protected sweet potato from stem rot caused by *F. oxysporum* f. sp. *batatas*, as well as the fungicide benomyl. This indicates that cerebroside elicitor may have potential applications as a biologically produced control agent.

Cerebroside elicitor induces PR gene expression and H₂O₂ production in tomato plants. Earlier, we demonstrated that cerebroside treatment induces the expression of several PR proteins in rice plants (50). In the present study, we monitored the expression of genes coding for proteins of the PR-1 and PR-5 family in tomato root tissues by northern blot analysis. Accumulation of PR-1 mRNA was induced 48 h after cerebroside B dipping treatment (Fig. 3). As expected, no PR-1 mRNA induction was observed in control plants treated with water. GlcCer (Fig. 1), an animal-derived glycosphingolipid and an analogue of cerebroside, showed very weak PR-1 inducing activity, consistent with the lack of elicitor activity in rice, as described previously (27,48). Expression of PR-5 mRNA was also induced by cerebroside treatment, although there was relatively low expression of this gene in both the water control and in GlcCer-treated roots. A few studies of the expression of PR proteins in tomato roots were examined (8,35). The biological functions of these PR proteins in tomato roots remain uncertain, but cerebroside was demonstrated to induce their gene expression.

Cerebroside from fungal membrane components may participate in the initial step of plant–pathogen interactions. Rapid generation of active oxygen species involving H₂O₂ production is one of the earliest detectable defense reactions in plants. Thus, we

tested whether cerebroside induced H₂O₂ production in tomato roots. As shown in Figure 4, H₂O₂ production correlated with treatment with cerebroside in a dose-dependent manner. In agreement with the results of efforts to enhance disease resistance in lettuce, cerebroside A, B, and C showed the same specific activity in producing H₂O₂ in tomato roots (data not shown). Induction of H₂O₂ production in rice by cerebroside elicitor has already been reported by Suharsono et al. (45). These results suggest that cerebroside elicitor may function as a general determinant of nonself recognition by host plants in the early stages of several plant–pathogen interactions.

DISCUSSION

This report indicates that cerebroside elicitor confers resistance to Fusarium diseases in various plant species. Cerebroside induced the expression of PR proteins in tomato plants and had no direct antifungal activity against *F. oxysporum* in vitro (data not shown), indicating that cerebroside activates plant defense mechanisms to confer resistance to Fusarium diseases. We also confirmed that cerebroside is produced by various species of *F. oxysporum*. Cerebroside functions as an elicitor in a wide range of plant–*F. oxysporum* interactions, in addition to the rice–blast fungus pathosystem. Moreover, cerebroside elicitor was found in a wide variety of phytopathogens, it appears that cerebroside from the cell membrane of phytopathogen is recognized by plants as a signal molecule of pathogen attack. These results suggest that cerebroside functions as an elicitor in many plant–pathogen interactions.

F. oxysporum is a soilborne parasite and an economically devastating agent of wilt disease in a wide range of plant species. Our field test demonstrated that cerebroside reduces Fusarium disease incidence on sweet potato even in an infested field. It has been proposed that application of elicitors can help control plant disease (4). This is based on the idea that pretreatment of a plant with elicitor molecules may sensitize the plant, allowing it to respond more rapidly to an actual pathogen infection. In our

TABLE 5. Cerebroside-enhanced resistance to Fusarium disease of melon (*Cucumis melo* L. cv. Amus) surveyed 21 days postinoculation

Concentration	Number of plants	Disease severity ^a	% Diseased plants
Control	30	1.6 ± 1.5 ₂	57
10	23	0.4 ± 1.0 ₁	17
20	25	0.5 ± 1.1 ₁	20

^a Mean value ± standard deviation is based on two independent experiments encompassing 10 pots (one plant per pot). Inoculum density of *Fusarium oxysporum* f. sp. *melonis* race 0 was measured at 1 × 10⁵ conidia per gram of soil. The statistical significance of the values followed by various numbers was determined using Duncan's multiple range test (*P* = 0.05).

TABLE 6. Cerebroside-enhanced resistance to Fusarium disease of sweet potato (*Ipomoea batatas* L. cv. Benikomachi) surveyed 16 days postinoculation

Concentration	Number of diseased plants/healthy plants	% Disease rate ^a
Control	34/47	72 ₃
20	10/44	23 ₂
50	10/46	22 ₂
Benomyl (500 µg/ml)	7/47	15 ₁

^a The statistical significance of the values followed by various numbers was determined using Duncan's multiple range test (*P* = 0.05).

Fig. 3. Northern blot analysis of pathogenesis-related (PR) proteins induction in tomato roots. Tomato roots were treated by dipping with solvent (0.5% EtOH; control) or glucosylceramide (GlcCer) solution (10 µg/ml) or cerebroside B solution (10 µg/ml). Total RNA was extracted from roots 48 h after sample treatment. Ten micrograms of total RNA was loaded per slot. The blot was developed with CDP-star module (Amersham Biosciences) using the probes indicated on the right. The experiment was carried out in triplicate with similar results.

future work, we plan to develop cerebroside as a biologically produced control agent for agricultural use.

In this study, probenazole, considered a plant defense activator (23,40), resulted in a slight reduction of disease development of *F. oxysporum* in lettuce. Probenazole is a chemical compound that has been used successfully for 30 years in Japan to protect rice plants against the rice blast fungus by inducing disease resistance in the field (54). Although cerebroside and probenazole have been demonstrated to share several common features, they appear to have different modes of action (50). Probenazole is a useful compound for analyzing signal pathways in plant defense mechanisms (22,40,56). Cerebroside may also prove useful as a tool for improving our understanding of defense systems in plants (45).

Biological control of Fusarium disease with nonpathogenic strains of *F. oxysporum* has been studied in several plant species. Likewise, it has been reported that certain microorganisms also protect plants by inducing resistance. This biological-induced resistance was demonstrated in cases involving fungal root pathogens and cases involving fungal leaf pathogens (39,52). Nevertheless, little is known about the molecular mechanisms involved in this bioprotection. In the current study, cerebroside elicitor was demonstrated to enhance resistance to soilborne Fusarium diseases. In earlier work with rice, we showed that cerebroside elicitor enhanced fungal foliar disease resistance (50). Therefore, it seems plausible that cerebroside elicitor is an important factor in this type of bioprotection.

In this report, cerebroside elicitor was demonstrated to induce the expression of PR proteins in tomato roots. PR-1 protein in tomato leaves has been reported to be involved in induced resistance (8,35). However, it was demonstrated that accumulation of PR proteins in tomato roots is not always associated with bioprotection. It is unclear whether PR proteins belonging to the PR-1 and PR-5 protein family are involved in the bioprotection of tomato roots (11), but cerebroside elicitor was demonstrated to induce the expression of PR proteins, as did infection by pathogens. On the other hand, GlcCer derived from an animal source

did not induce the expression of PR proteins in tomato roots, consistent with the lack of elicitor activity in rice. These results suggest that cerebroside represents a host-non-specific determinant of fungal recognition and several plant species possess a recognition system for cerebroside (55).

Rapid H₂O₂ production was reported to be an early response during interactions between plants and pathogens (29). There are two phases (phases I and II) in the kinetics of H₂O₂ production. A rapid and weak production of H₂O₂ (phase I) occurs after inoculation with either a virulent or an avirulent race of *Pseudomonas syringae* pv. *glycinea* in soybean cells (3). Phase II is a second and strong production of H₂O₂ in inoculation with an avirulent of *Pseudomonas fluorescens* but not a virulent strain (17). Therefore, it is proposed that phase I is a non-race-specific reaction, whereas phase II is responsible for race-specific disease resistance in plants. The second production phase of H₂O₂ in tomato roots after treatment with cerebroside was not observed until 10 h posttreatment (data not shown). Further experiments need to be performed to determine the function of cerebroside-induced H₂O₂ production in defense signaling.

In conclusion, our findings suggest that cerebroside elicitor may function as a nonspecific elicitor in many plant–fungus interactions as well as serving as a useful chemical for practical agro-nomic use.

ACKNOWLEDGMENTS

We thank M. Fujinaga and H. Ogiso (Nagano Vegetable and Ornamental Crops Experiment Station) for providing *F. oxysporum* f. sp. *lactucae* isolate SB1-1 (race 1) and isolate F9501 (race 2); Y. Amemiya (Faculty of Horticulture, Chiba University) for providing *F. oxysporum* f. sp. *lycopersici* race J-1 and *F. oxysporum* f. sp. *cucumerinum*; F. Namiki (National Agricultural Research Center for Kyushu-Okinawa Region) for providing *F. oxysporum* f. sp. *melonis* race 0 and race 1 (33); and our colleague, M. Matsumura (Meiji Seika Kaisha Ltd.), for technical support.

LITERATURE CITED

1. Aderem, A., and Ulevitch, R. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406:782-787.
2. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1987. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
3. Baker, C. J., and Orlandi, E. W. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:299-321.
4. Benhamou, N. 1996. Elicitor-induced plant defence pathways. *Trends Plant Sci.* 1:233-240.
5. Boller, T. 1995. Chemoperception of microbial signals in plant cells. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46:189-214.
6. Bostock, R. M., Kuc, J. A., and Laine, R. A. 1981. Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato. *Science* 212:67-69.
7. Cartwright, D., Langcake, P., Pryce, R. J., and Leworthy, D. P. 1977. Chemical activation of host defence mechanisms as a basis for crop protection. *Nature* 267:511-513.
8. Cohen, Y., Niderman, T., Mosinger, E., and Fluhr, R. 1994. β -Aminobutyric acid induces the accumulation of pathogenesis-related proteins in tomato (*Lycopersicon esculentum* L.) plants and resistance to late blight infection caused by *Phytophthora infestans*. *Plant Physiol.* 104:59-66.
9. Coursol, O., Fan, L.-M., Stunff, H. L., Sphiegel, S., Gilroy, S., and Assmann, S. M. 2003. Sphingolipid signaling in *Arabidopsis* guard cells involves heterotrimeric G proteins. *Nature* 423:651-654.
10. Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, J. S., and Spiegel, S. 1996. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* 381:800-803.
11. Dassi, B., Dumas-Gaudot, E., and Gianinazzi, S. 1998. Do pathogenesis-related (PR) proteins play a role in bioprotection of mycorrhizal tomato roots towards *Phytophthora parasitica*? *Physiol. Mol. Plant Pathol.* 52:167-183.
12. Dickson, R. C., and Lester, R. L. 1999. Metabolism and selected functions of sphingolipids in the yeast *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 10:305-321.
13. Dickson, R. C., and Lester, R. L. 2002. Sphingolipid functions in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* 1583:13-25.

Fig. 4. Dose dependency of H₂O₂ production in tomato roots by cerebroside elicitor. Five segments (1 cm length) of tomato roots were equilibrated in 0.1 M phosphate buffer (pH 6.8) and then mixed with a solution of MCLA at a final concentration of 0.3 mM. Following addition of cerebroside B at given final concentration, MCLA luminescence was continuously measured using a luminescence reader for 15 min, at 5-s intervals. The values shown are the mean and standard deviation of the results from four measurements.

14. Ebel, J., and Cosio, E. G. 1994. Elicitors of plant defense responses. *Int. Rev. Cytol.* 148:1-35.
15. Fuchs, J. G., Moenne-Loccoz, Y., and Defago, G. 1997. Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to Fusarium wilt in tomato. *Plant Dis.* 81:492-496.
16. Fujinaga, M., Ogiso, H., Tuchiya, N., Saito, H., Yamanaka, S., Nozue, M., and Kojima, M. 2003. Race 3, a new race of *Fusarium oxysporum* f. sp. *lactucae* determined by a differential system with commercial cultivars. *J. Gen. Plant Pathol.* 69:23-28.
17. Glazener, J. A., Orlandi, E. W., and Baker, J. C. 1996. The active oxygen response of cell suspensions to incompatible bacteria is not sufficient to cause hypersensitive cell death. *Plant Physiol.* 110:759-763.
18. Hakomori, S. 1990. Bifunctional role of glycosphingolipids. *J. Biol. Chem.* 265:18713-18716.
19. Hannun, Y. A. 1994. The sphingomyelin cycle and the second messenger function of ceramide. *J. Biol. Chem.* 269:3125-3128.
20. Hannun, Y. A. 1996. Functions of ceramide in coordinating cellular responses to stress. *Science* 274:1855-1859.
21. Imler, J.-L., and Hoffman J. A. 2001. Toll-like receptors in innate immunity. *Trends Cell Biol.* 11:304-311.
22. Iwata, M. 2001. Probenazole: A plant defense activator. *Pesticide Outlook* 12:28-31.
23. Iwata, M., Umemura, K., Teraoka, T., Usami, H., Fujisawa, Y., and Iwasaki, Y. 2003. Role of the α subunit of heterotrimeric G-protein in probenazole-inducing defense signaling in rice. *J. Gen. Plant Pathol.* 68:83-86.
24. Katsube, K., and Akasaka, Y. 1997. Control of Fusarium wilt of spinach by transplanting seedling pretreated with nonpathogenic *F. oxysporum*. (In Japanese with English summary) *Ann. Phytopathol. Soc. Jpn.* 63:389-394.
25. Koga, J., Ogawa, N., Yamauchi, T., Kikuchi, M., Ogasawara, N., and Shimura, M. 1997. Functional moiety for the antifungal activity of phytocassane E, a diterpene phytoalexin from rice. *Phytochemistry* 44:249-253.
26. Koga, J., Shimura, M., Oshima, K., Ogawa, N., Yamauchi, T., and Ogasawara, N. 1995. Phytocassanes A, B, C, D, novel diterpene phytoalexins from rice, *Oryza sativa* L. *Tetrahedron* 51:7907-7918.
27. Koga, J., Yamauchi, T., Shimura, M., Ogawa, N., Oshima, K., Umemura, K., Kikuchi, M., and Ogasawara, N. 1998. Cerebrosides A and C, sphingolipid elicitors of hypersensitive cell death and phytoalexin accumulation in rice plants. *J. Biol. Chem.* 273:31985-31991.
28. Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. *Rev. Plant Prot. Res.* 8:115-125.
29. Lamb, C., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:251-275.
30. Levery, S. B., Momany, M., Lindsey, R., Tiledo, M. S., Shayman, J. A., Fuller, M., Brooks, K., Doong, R. L., Straus, A. H., and Takahashi, H. K. 2002. Disruption of the glucosylceramide biosynthetic pathway in *Aspergillus nidulans* and *Aspergillus fumigatus* by inhibitors of UDP-Glc:ceramide glucosyltransferase strongly affects spore germination, cell cycle, and hyphal growth. *FEBS Letts.* 525:59-64.
31. Li, P., Nijhawan, D., Budihardjyo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/Caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489.
32. Midoh, N., and Iwata, M. 1996. Cloning and characterization of a probenazole-inducible gene for an intracellular pathogenesis-related protein in rice. *Plant Cell Physiol.* 37:9-18.
33. Namiki, F., Shiomi, T., Nishi, K., Kayamura, T., and Tsuge, T. 1998. Pathogenic and genetic variation in the Japanese strains of *Fusarium oxysporum* f. sp. *melonis*. *Phytopathology* 88:804-810.
34. Ng, C. K., Carr, K., McAlinsh, M. R., Powell, B., and Hetherington, A. M. 2001. Drought-induced guard cell signal transduction involves sphingosine-1-phosphate. *Nature* 410:596-599.
35. Niderman, T., Genetet, I., Bruyere, T., Gees, R., Stintzi, A., Legrand, M., Fritig, B., and Mosinger, E. 1995. Pathogenesis-related PR-1 proteins are antifungal. *Plant Physiol.* 108:17-27.
36. Nurnberger, T. 1999. Signal perception in plant pathogen defense. *Cell Mol. Life Sci.* 55:167-182.
37. Ogawa, K., and Kodama, H. 1986. Fusarium wilt of sweet potato by non-pathogenic *Fusarium oxysporum*. (In Japanese with English summary) *Ann. Phytopathol. Soc. Jpn.* 52:15-21.
38. Pan, O., Wendel, J., and Fluhr, R. 2000. Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *J. Mol. Evol.* 50:203-213.
39. Pieterse, C. M., van Wees, S. C., Hoffland, E., van Pelt, J. A., and van Loon, L. C. 1996. Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* 8:1225-1237.
40. Sekizawa, Y., Aoyama, H., Kimura, M., and Yamaguchi, I. 1995. GTPase activity in rice plasma membrane preparation enhanced by a priming effect for plant defense reactions. *J. Pesticide Sci.* 20:165-168.
41. Sharp, J. K., Valent, B., and Albersheim, P. 1984. The primary structures of one elicitor-active and seven elicitor-inactive hexa (β -D-glucopyranosyl)-D-glucitols isolated from the mycelial walls of *Phytophthora megasperma* f. sp. *glycinea*. *J. Biol. Chem.* 259:11321-11336.
42. Simons, K., and Ikonen, E. 1997. Functional rafts in cell membranes. *Nature* 387:569-572.
43. Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. *Science* 268:661-667.
44. Staskawicz, B. J., Mudgett, M. B., Dangi, J. L., and Galan, J. E. 2001. Common and contrasting themes of plant and animal diseases. *Science* 292:2285-2289.
45. Suharsono, U., Fujisawa, Y., Kawasaki, T., Iwasaki, Y., Satoh, H., and Shimamoto, K. 2002. The heterotrimeric G protein α subunit acts upstream of the small GTPase Rac in disease resistance of rice. *Proc. Natl. Acad. Sci. USA* 99:13307-13312.
46. Thoma-Uzynski, S., Stenger, S., Takeuchi, O., Ochoa, M. T., Engele, M., Sieling, P. A., Barnes, P. F., Rollinghoff, M., Bolcskei, P. L., Wagner, M., Akira, S., Norgard, M. V., Belisle, J. T., Godowski, P. J., Bloom, B. R., and Modlin, R. L. 2001. Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science* 291:1544-1547.
47. Tompson, J. N., and Burdon, J. J. 1992. Gene-for-gene coevolution between plants and parasites. *Nature* 360:121-125.
48. Umemura, K., Ogawa, N., Koga, J., Iwata, M., and Usami, H. 2002. Elicitor activity of cerebroside, a sphingolipid elicitor, in cell suspension cultures of rice. *Plant Cell Physiol.* 41:676-683.
49. Umemura, K., Ogawa, N., Shimura, M., Koga, J., Usami, H., and Kono, T. 2003. Possible role of phytocassane, rice phytoalexin, in disease resistance of rice against the rice blast fungus *Magnaporthe grisea*. *Biosci. Biotechnol. Biochem.* 67:899-902.
50. Umemura, K., Ogawa, N., Yamauchi, T., Iwata, M., Shimura, M., and Koga, J. 2000. Cerebroside elicitors found in diverse phytopathogens activate defense responses in rice plants. *Plant Cell Physiol.* 41:676-683.
51. Underhill, D. M., and Ozinsky, A. 2002. Toll-like receptors: Key mediator of microbe interaction. *Curr. Opin. Immunol.* 14:103-110.
52. van Wees, S. C., Pieterse, C. M., Trijssenaar, A., van't Westende, Y. A., Hartog, F., and van Loon, L. C. 1997. Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol. Plant-Microbe Interact.* 6:716-724.
53. Waspi, U., Blanc, D., Winkler, T., Ruedi, P., and Dudler, R. 1998. Syringolin, A novel peptide elicitor from *Pseudomonas syringae* pv. *syringae* that induces resistance to *Pyricularia oryzae* in rice. *Mol. Plant-Microbe Interact.* 11:727-733.
54. Watanabe, T., Igarashi, H., Matsumoto, K., Seki, S., Mase, S., and Sekizawa, Y. 1977. The characteristics of probenazole (Oryzmate) for the control of rice blast. *J. Pesticide Sci.* 2:291-296.
55. Worrall, D., Ng, C. K., and Hetherington, A. M. 2003. Sphingolipids, new players in plant signaling. *Trends Plant Sci.* 8:317-320.
56. Yoshioka, K., Nakashita, H., Klessig, D. F., and Yamaguchi, I. 2001. Probenazole induces systemic acquired resistance in *Arabidopsis* with a novel type of action. *Plant J.* 25:149-157.