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Phosphocholine-Containing Glycosyl Inositol-Phosphoceramides from *Trichoderma viride* Induce Defense Responses in Cultured Rice Cells

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We isolated two major zwitterionic glycosphingolipids (ZGLs) from the phytopathogenic filamentous fungus *Trichoderma viride*. Structural analyses showed that the ZGLs (designated Tv-ZGL2 and Tv-ZGL3) were the same as the glycosphingolipids ZGL2 and ZGL4 from *Acremonium* sp., which are described in our previous paper. ZGLs have the following structure: Man(α1-6)GlcN(α1-2)Ins-P-Cer (Tv-ZGL2) and phosphocholine (PC) → 6Man(α1-6)GlcN(α1-2)Ins-P-Cer (Tv-ZGL3). To determine whether these ZGLs have functional roles in plant-fungus interaction, we tested to determine whether they would induce defense responses in cultured rice cells. We found that *T. viride*'s ZGLs elicited expression of the *PAL* and *PBZ1* genes, both of which are associated with pathogen resistance. Tv-ZGL2 induced cell death at a moderate rate. Tv-ZGL3, which contains a PC moiety, induced a high level of cell death in rice cells.

Key words: glycosphingolipid; defense response; *Trichoderma viride*; rice cells; phytopathogen

Sphingolipids are major components of cell membranes in mammals, bacteria, and fungi. In mammalian cells, they act as a second messenger and are involved in intracellular responses to environmental conditions and extracellular stress, in addition to their role in cell membrane structure.[1)] In addition, recent studies have shown that glycosphingolipids have important roles in immune responses to cancer[2)] and infectious diseases.[3)] Although many studies have been carried out on sphingolipids of eukaryotes such as mammals and yeast (*Saccharomyces cerevisiae*),[4)] there have been very few studies on the structure and functional roles of sphingolipids from filamentous fungi.

Filamentous fungi are major pathogens of higher plants. Plants have developed defense responses against attack by pathogenic fungi. Such responses are initiated by the recognition of specific molecules derived from microbes.[5)] These molecules, known as elicitors, include flagella from bacteria,[6)] and chitin[7)] and glucan[8)] from fungi. Koga *et al.* found that cerebroside from *Magnasporthe grisea* acts as an elicitor and induces phytoalexin gene expression in rice plants.[9,10)] This suggests that glycosphingolipids from fungi may have important roles in plant-fungi interactions.

We have reported a novel phosphocholine (PC)-containing glycosylinositol-phosphoceramide in the filamentous fungus *Acremonium* sp.[11)] This novel glycosphingolipid was found in a zwitterionic glycolipid (ZGL) fraction eluted through ion-exchange column chromatography on two occasions. ZGL is a glycosphingolipid having both ion charges. Our preliminary findings indicated that PC-containing glycosphingolipids also exist in the phytopathogenic fungus *Trichoderma viride*. Lochnit *et al.* found that PC-containing glycosphingolipids from nematodes induced the production of inflammatory cytokines from human monocytes *in vitro*.[12)] In light of such findings, we attempted to elucidate the role of PC-containing glycosphingolipids from filamentous fungi in a plant-fungus interaction.

In this study, we isolated and purified two zwitterionic glycosphingolipids (ZGLs) from *T. viride*. Structural analysis indicated that the ZGLs contained the same components as those from *Acremonium* sp. Furthermore, we found that these ZGLs, especially the PC-containing ZGL, induced defense responses in rice cells. Our findings suggest that PC-containing glycosphingolipids have a pivotal role in plant-fungi interactions.

Materials and Methods

Cultures of fungi and rice cells. Trichoderma viride (IFO 30498) was obtained from the Institute for Fermentation, Osaka, Japan. *Acremonium* sp. no. 413[13)] and *Mucor hiemalis* no. 314[14)] were isolated from soil and identified in our laboratory. These fungi were cultured at

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Abbreviations: ZGL, zwitterionic glycosphingolipid; Man, D-mannose; GlcN, D-glucosamine; Ins, inositol; GlcNAc, *N*-acetyl-D-glucosamine; Cer, ceramide; PC, phosphocholine; PAL, phenylalanine ammonia lyase; PBZ, probenazol; ROS, reactive oxygen species; GL, glycosphingolipid; MIPC, mannose-inositol phosphorylceramide; CMS, ceramide monosaccharide; TLC, thin layer chromatography; MALDI-TOF/MS, Matrix-Assisted Laser-Desorption Ionization Time-of-Flight MS; GLC, gas-liquid chromatography

28 °C for 3 d in YPD medium containing 0.5% yeast extract, 0.5% peptone, 0.5% NaCl, and 1% glucose, pH 6.5. Cultured rice (*Oryza sativa*) cells (Oc line) were grown in R2S medium for 4 d.[6)]

Extraction and fractionation of fungal glycosphingolipids. Preparation of fungal glycosphingolipids was carried out by the method described in a previous paper.[11)] Mycelia of *T. viride* were collected and washed with distilled water. To extract glycosphingolipids (GLs), washed mycelia were extracted with chloroform/methanol (2:1, v/v) and then with chloroform/methanol/water (30:60:8, v/v/v). Extracts were combined and concentrated by evaporation at 40 °C. Next, the concentrated extract was subjected to mild alkaline hydrolysis with 0.5 M KOH in methanol/water (95:5, v/v) at 37 °C for 6 h. The hydrolysate was acidified to pH 1.0 with conc. HCl, dialyzed, concentrated, and then separated on a QAE-Sephadex A-25 column (Amersham Pharmacia Biotech, Piscataway, NJ) to obtain the neutral GL fraction. Polar compounds were recovered from the column by elution with 0.45 M ammonium acetate in methanol, and the eluate was dialyzed against water and then separated on a DEAE-Sephadex A-25 column (Amersham Pharmacia Biotech). The ZGL (Tv-ZGL) fraction was eluted with chloroform/methanol/water (30:60:8, v/v/v). Next, ZGLs were fractionated on a column of porous silica gel (Iatrobeads 6RS-8060, Iatron Laboratories, Tokyo) and eluted using a linear gradient (from C:M:W = 60:40:4 to 60:35:5 for Tv-ZGL2, and from C:M:W = 60:40:6 to 60:40:10 for Tv-ZGL3). Fractions were collected and analyzed by TLC (Merck Biosciences, San Diego, CA). Purification of mannose-inositol phosphorylceramide (MIPC) from *S. cerevisiae*, ZGLs from *Acremonium* sp., and ceramide monosaccharide (CMS) from *M. hiemalis* was carried out as described previously.[11,15)]

Structural analysis of fungal glycosphingolipids. To determine the fatty acid and sugar compositions of the glycosphingolipids, approximately 200 µg of glycosphingolipid was methanolyzed in thick glass test tubes (16 mm × 125 mm, with Teflon-lined screw caps; Pyrex, Iwaki Glass, Japan) with 200 µl of freshly prepared 1 M anhydrous methanolic HCl. Methanolyzation was carried out at maximum power (500 W) in a microwave oven (Sharp RE-Z3W6, 100 V, Sharp Electric, Japan) for 1 min. Samples were cooled to room temperature after methanolysis. The fatty acid methyl esters produced were extracted 3 times with 400 µl n-hexane, and then analyzed using a Shimadzu GC-18A gas chromatograph (Shimadzu, Kyoto, Japan) with a Shimadzu HiCap-CBP 5 capillary column (0.22 mm × 25 m). The temperature program was 4 °C/min from 140 °C to 230 °C. The remaining methanolic phase was evaporated to dryness for de-acidification under a nitrogen stream. The residue containing methylglycosides was trimethylsilylated with pyridine/hexamethyldisilazane/trimethylchlorosilane (9:3:1, v/v/v) at 60 °C for 30 min. The reaction mixture was analyzed by GLC on the capillary column described above using a temperature program of 4 °C/min from 170 °C to 230 °C. Sphingoids prepared from glycosphingolipids by methanolysis with 1 M aqueous methanolic HCl at 70 °C for 18 h were converted into their O-trimethylsilyl (nitrogen-free) derivatives, and then analyzed by GLC on the same capillary column using a temperature program of 4 °C/min from 210 °C to 230 °C.

To determine sugar linkages, 300 µg of purified glycolipid was partially methylated with NaOH and methyl iodide in DMSO by the method of Ciucanu and Kerek,[16)] with a slight modification. The permethylated glycolipid was acetolyzed and hydrolyzed with 300 µl of HCl/water/acetic acid (0.5:1.5:8, v/v/v) at maximum power in a microwave oven for 1 min, and then reduced with $NaBH_4$ and acetylated with acetic anhydride/pyridine (1:1, v/v) at 100 °C for 15 min. The partially methylated alditol acetates obtained were analyzed by GLC equipped with the same HiCap-CBP 5 capillary column, as described above.

MALDI-TOF/MS (Matrix-Assisted Laser-Desorption Ionization Time-of-Flight MS) analysis of the purified glycosphingolipids was performed as described previously.[11)] We used a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer equipped with a SPARC Workstation (Shimadzu), operating in the negative-ion linear mode. Ions were formed with a pulsed UV laser beam (N2 laser, 337 nm; 3 ns-wide pulses/s). The matrix used was 7-amino-4-methylcoumarin (Sigma Chemical). External mass calibration was provided by the $[M - H]^-$ ions of angiotensin II (1,046.2 mass units; Sigma Chemical) and neurotensin-(1–11) (1,446.6 mass units; Sigma Chemical).

Cell death assay of cultured rice cells. The assay for cell death of cultured rice cells was performed as described previously.[6)] Rice cells in a total volume of 10 ml were incubated for various times with and without glycolipids dissolved in DMSO. Cells were shaken at 90 rpm at 30 °C, and then 1 ml cultured cells was removed and transferred into a tissue culture plate. The supernatant was removed and cells were stained with 0.5 ml 0.05% Evans Blue in 50 mM Hepes/KOH buffer (pH 7.2) for 15 min. After washing with distilled water, the dye incorporated into the dead cells was extracted with 50% (v/v) methanol containing 1% SDS for 12 h. The concentration of the dye in the extract solution was determined by measuring the absorbance at 595 nm.

RNA isolation and Northern blot analysis. Cultured rice cells were ground in liquid nitrogen using a mortar and pestle, then Sepasol-RNA 1 Super (Nacalai Tesque, Kyoto, Japan) was added with stirring. Total RNA was isolated from the above mixture by successive extractions with chloroform, isopropanol, and 75% ethanol. In Northern blotting, 2 µg of total RNA was separated by electrophoresis on a 1% agarose gel containing 16% formaldehyde and 50% formamide in 1 × MOPS buffer (20 mM MOPS-KOH, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA), and blotted onto a Hybond-N+ (Amersham Pharmacia Biotech) membrane according to the standard procedure. The membrane was hybridized with a *PAL* probe, which was constructed from a 0.4-kbp fragment of *PAL* cDNA (introduced into the *Eco*RI site of pUC19) and labeled with [^{32}P]-dATP using Strip-EZ DNA (Ambion, Austin, TX). The hybridized membrane was washed with 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, and 0.1% SDS) and 0.1 × SSC for 15 min at 42 °C. Total RNA loadings were checked by electrophoresis on a 1% agarose gel and staining with ethidium bromide.

Reverse transcription-PCR (RT-PCR). To transcribe cDNA, total RNA (1 µg) was mixed with 4 µl of oligo $(dT)_{20}$ (Toyobo, Osaka, Japan) and distilled water to give a final volume of 12 µl. The mixture was incubated at 70 °C for 15 min, and then chilled on ice. The sample was mixed with 1 µl of SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA), 4 µl of 5 × First-Strand Buffer (Invitrogen), 2 µl of 0.1 M DTT (Toyobo), and 1 µl of RNase inhibitor (Toyobo). The reverse transcription reaction was carried out at 42 °C for 50 min, then stopped by incubation at 70 °C for 15 min. The PCR mixture consisted of 1 µl of sample cDNA, 0.5 µl of rTaq DNA polymerase (5 U/µl, Toyobo), 2.5 µl of 10 × PCR Buffer (Toyobo), 0.5 µl of 10 mM dNTP mixture (Toyobo), 0.6 µl of 50 mM $MgCl_2$ (Toyobo), 0.5 µl of primers (Toyobo), and 19.4 µl of distilled water. The sequences of the first primer set were as follows: 5′-tgtggaaggcgttcatgga-3′ and 5′-tgagcgaggtagtcctc-3′ for probezanol 1 (*PBZ1*, product is 402 bp.), and 5′-ctctctgtatgccagtggtcgta-3′ and 5′-tcgtcgtactcagccttggcaat-3′ for actin (*Act*; product, 672 bp.).[10)] Amplification was carried out in a thermal cycler as follows: 1 min denaturing at 94 °C, and 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 1 min. The reaction was terminated by heating to 68 °C for 7 min. PCR products (5 µl) were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The bands of the PCR product were visualized on a UV transilluminator.

Statistical analysis. Student's *t*-tests were used to determine statistical significance, and $P < 0.05$ was considered significant.

Materials. Unless specifically described, all reagents were of guaranteed grade and are available commercially.

Results

Isolation and analysis of ZGLs from Trichoderma viride

To determine whether ZGLs of *T. viride* would induce defense responses in rice cells, we attempted partially to purify ZGLs from the mycelia by chloro-

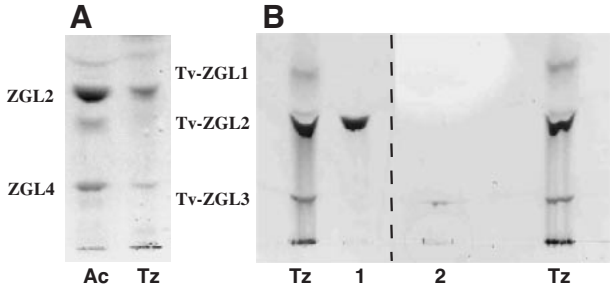

Fig. 1. Thin-Layer Chromatographs of ZGLs Extracted from *Trichoderma viride* and *Acremonium* sp.

Thin-layer plates were developed with chloroform/methanol/water (60:40:10, v/v/v), and spots were visualized with orcinol/H_2SO_4 reagent. A, Lane Ac, ZGLs from *Acremonium* sp.; lane Tz, ZGLs from *T. viride*. B, lane 1, Tv-ZGL2; lane 2, Tv-ZGL3. Broken line indicates cropped border where irrelevant lanes were removed.

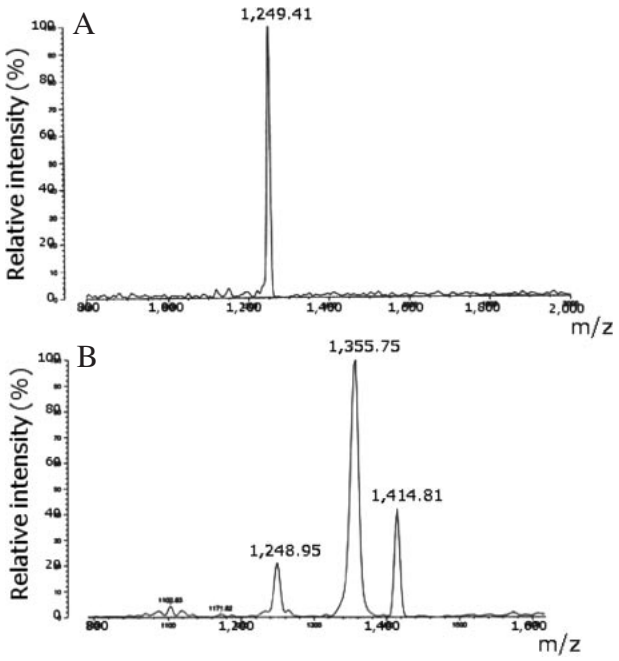

Fig. 2. MALDI-TOF/MS Spectra of Tv-ZGL2 and Tv-ZGL3.

Analyses were carried out in negative-ion linear mode. A, Tv-ZGL2; B, Tv-ZGL3.

form-methanol extraction, alkaline hydrolysis, and column chromatography using QAE-Sephadex A-25 and DEAE-Sephadex A-25. Figure 1A shows TLCs of the partially purified ZGLs. Two major bands were visualized after spraying with orcinol/H_2SO_4. These have almost the same Rf values as the two major ZGLs from *Acremonium* sp.[11] We further purified these two ZGLs by porous silica gel chromatography (Fig. 1B), and designated these compounds Tv-ZGL2 and Tv-ZGL3. Both purified ZGLs reacted positively with Dittmer-Lester and Hanes-Isherwood reagents, in addition to the orcinol/H_2SO_4 reagent, confirming the presence of phosphorus. Using MALDI-TOF/MS analyses, we found that the mass values of Tv-ZGL2 (m/z 1,248.85) and Tv-ZGL3 (1,414.80) (Fig. 2) were almost the same as those of ZGL2 (m/z 1,249.0) and ZGL 4 (1,414.7) from *Acremonium* sp.[11] As reported previously, the structure of ZGL2 is Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer, and the structure of ZGL4 is PC $\rightarrow$ 6Man(α1-6)GlcN(α1-2)Ins1-*P*-1Cer. We also identified a lower peak of m/z 1,355.80 in the laser desorption spectrum of Tv-ZGL3, which probably represents a loss of 58 mass units from the molecular ion. This may reflect the elimination of $CH_2 = N(CH_3)_2$ from the PC group, as discussed previously.[11]

Table 1. Sugar and Ceramide Components of ZGLs from *T. viride*

	Tv-ZGL2	Tv-ZGL3
Sugar component	*myo*-inositol	*myo*-inositol
	1,6-GlcN	1,6-GlcN
	1-Man	1-Man
Fatty acid	2-hydroxy tetracosanoic acid	2-hydroxy tetracosanoic acid
Long chain base	4-hydroxy-sphinganine	4-hydroxy-sphinganine
Phosphocholine	−	+
Mass value	1,248.85	1,414.80

Confirmation of the structures of ZGLs from T. viride

We attempted to confirm the structures of *T. viride* ZGLs. The aliphatic components of ZGLs were determined by gas-liquid chromatography (GLC), and were identified by comparison with chromatograms of authentic standards. It was confirmed that the fatty acid of ceramide in the ZGLs was 2-hydroxytetracosanoic acid and that the sphingoid was 4-hydroxyoctadecasphinganine (phytosphingosine). The ZGLs were methylated, *N*-acetylated, and completely hydrolyzed to determine their sugar components. The alditol acetate derivatives of the hydrolysates were analyzed by GLC. The sugar components were identified as *N*-acetylglucosamine, *myo*-inositol, and mannose, by comparison with the retention times of the authentic carbohydrates. From these observations, it was confirmed that Tv-ZGL2 is composed of inositol 1-monophosphate, glucosamine, and mannose. Furthermore, mass spectra analysis confirmed the presence of choline phosphate in Tv-ZGL3, in addition to the above sugar components.

To determine sugar linkages, the partially methylated alditol acetate derivatives of ZGLs were analyzed by GLC. Methylation analysis demonstrated the presence of 1-substituted mannose (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol; 1Man) and 1,6-substituted *N*-acetylglucosamine (1,5,6-tri-*O*-acetyl-3,4-di-*O*-methyl-*N*-acetylglucosaminitol; 1,6GlcNAc) for *N*-acetylated ZGL2. A 1,6-substituted *N*-acetylglucosamine was detected in the *N*-acetylated ZGL3, but no terminal sugar residue was detected. This suggests that PC is linked to the non-reducing end of the sugar chain.

We confirmed that the structure of Tv-ZGL2 is Man(α1-6)GlcN(α1-2)Ins-*P*-Cer, and that the structure of Tv-ZGL3 is PC $\rightarrow$ 6Man(α1-6)GlcN(α1-2)Ins-*P*-Cer. Table 1 shows the sugar and ceramide components of the Tv-ZGLs. We also detected minute quantities of ZGL such as Tv-ZGL1 (Fig. 1B), which appears to be the same as ZGL1 (GlcN(α1-2)Ins1-*P*-1Cer) from *Acremonium* sp.[11]

Defense responses of rice cells induced by ZGLs from T. viride

We have found that mannose-binding glycosphingolipids (ZGL2 and 4 from *Acremonium* sp.) induced defense responses in rice cells, and that ZGL1 from *Acremonium* sp. did not induce such responses, because it lacks a mannose residue. Since Tv-ZGL 2 and 3 appear to be the same as the mannose-containing ZGL2 and ZGL4 from *Acremonium* sp., we investigated their ability to induce plant defense responses. We used the

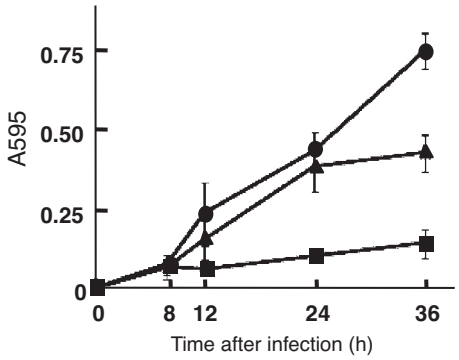

Fig. 3. Cell Death of Cultured Rice Cells after Treatment with Various Glycolipids.

■, MIPC from *S. cerevisiae* (50 μM); ▲, ZGLs from *T. viride* (50 μM); ●, CMS from *M. hiemalis* (50 μM). The fraction eluted with DEAE-Sephadex A-25 column chromatography was used as ZGLs from *T. viride*. Each data point represents the mean value for three independent experiments; bars show S.D. ($^*P < 0.05$)

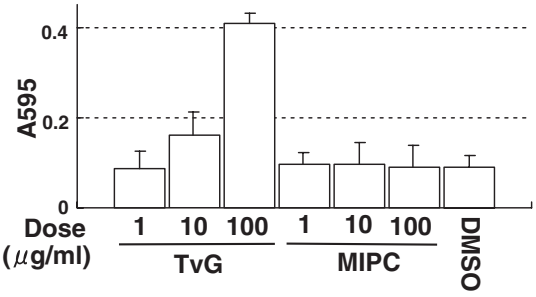

Fig. 4. Effects of Tv-ZGL Concentration on Rice Cell Death.

Cell death assay was carried out after treatment with Tv-ZGLs and MIPC for 30 h. The fraction eluted with DEAE-Sephadex A-25 column chromatography was used as Tv-ZGLs. Each data point is the mean value for three independent experiments; bars show S.D. ($^*P < 0.05$)

Evans Blue dye exclusion assay to examine their effects, and found that Tv-ZGLs induced a high level of cell death (Fig. 3). In contrast, MIPC from *S. cerevisiae*, a major component of microbes' cell membranes, only weakly induced rice cell death (Fig. 3). The cell death response increased as the concentration of Tv-ZGLs increased (Fig. 4). Northern blot analyses showed that the two ZGLs from *T. viride* increased expression of the *PAL* (phenylalanine ammonia lyase) (Fig. 5A) gene and the *PBZ1* (protein induced by probenazol) (Fig. 5B) gene, both of which are related to the defense responses of rice. Ceramide monosaccharide (CMS) also induced expression of the *PAL* and *PBZ1* genes (Fig. 5A, B). These results indicate that ZGLs from *T. viride* induced defense responses in rice cells.

Importance of the PC portion in the induction of rice cell death

To determine the relationship between *T. viride* ZGL structure and the induction of rice cell death, we compared the effects of purified Tv-ZGL2 and Tv-ZGL3 on the induction of rice cell death. Figure 6 shows rice cell death stimulated by mannose-inositol phosphorylceramide (MIPC), Tv-ZGL2, and Tv-ZGL3. Tv-ZGL2 induced a higher level of cell death than MIPC. Tv-ZGL3, which contains PC, induced the highest rate of cell death. These results suggest that the PC moiety of *T. viride* ZGL has a pivotal role in the induction of the defense response in rice cells.

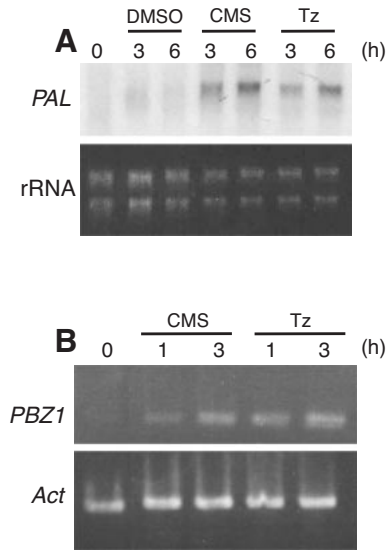

Fig. 5. Accumulation of *PAL* and *PBZ1* mRNA in Cultured Rice Cells Stimulated with Various Glycolipids.

A, Total RNA was isolated from rice cells incubated with CMS from *M. hiemalis* and with ZGLs from *T. viride* (Tz). The fraction eluted by DEAE-Sephadex A-25 column chromatography was used as ZGLs from *T. viride*. RNA (2 μg) was analyzed by probing with *PAL* cDNA (upper panel). The ethidium bromide-stained gel showed equal RNA loading before blotting (lower panel). B, Total RNA was isolated from rice cells and subjected to RT-PCR for detection of *PBZ1* and *actin* mRNA.

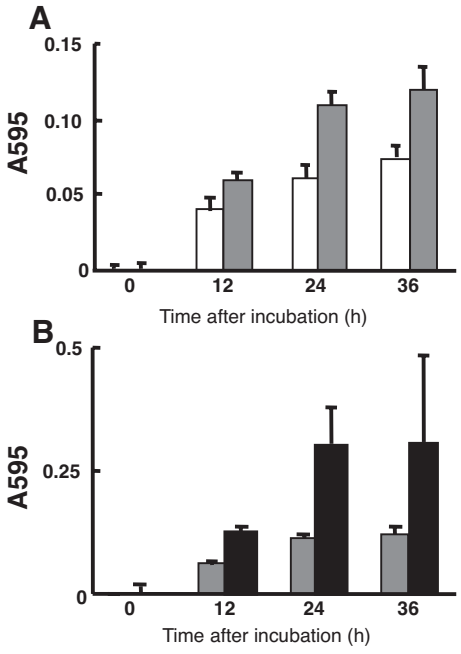

Fig. 6. Time Course of Cell Death of Cultured Rice Cells Incubated with MIPC, Tv-ZGL2, and Tv-ZGL3.

A, cell death of cultured rice cells was examined in the presence of MIPC (50 μM, open bars) and purified Tv-ZGL2 (50 μM, hatched bars). B, cell death of cultured rice cells was examined in the presence of purified Tv-ZGL2 (50 μM, hatched bars) and Tv-ZGL3 (50 μM, closed bar). Each data point is the mean value for three independent experiments; bars show S.D. ($^*P < 0.05$)

Discussion

Our previous paper was the first report of ZGLs from filamentous fungi.[11] The two ZGL-containing fungi *Acremonium* sp. and *Trichoderma viride* are phytopathogenic. In the present study, we found that

ZGLs from *T. viride* contained two main glycosphingolipids that are the same as the major glycosphingolipids from *Acremonium* sp. reported previously.[11] In addition, *T. viride* ZGLs induced expression of defense response-related genes and cell death in rice cells.

We found that fungal ZGLs increased gene expression of *PAL* and *PBZ*, both of which are related to defense responses in rice cells. Tanaka *et al.* reported that expression of these genes was induced by the accumulation of intracellular Ca^{2+} in rice cells.[17] This might support that ZGLs cause an influx of Ca^{2+} into the cytosolic space as a result of increased membrane permeability, and this might be attributable to the hydrophilic portion of the PC.

Cell death in higher plants has a pivotal role in protection against parasite microbes. Kawasaki *et al.* found that cell death in rice cells was regulated by cytosolic reactive oxygen species (ROS) through the small GTP-binding protein Rac.[18] Iwano *et al.* also found that ROS production occurred in rice cells infected with an incompatible strain of *Pseudomonas avenae*.[19] These results indicate that ROS production is related to defense responses against infectious microbes. However, even though *T. viride* is a representative pathogenic fungus for rice cells, we did not find any such phenomenon when we stimulated rice cells with *T. viride* ZGLs (data not shown). In addition, we did not detect ROS production in the supernatant of rice cells after treatment with CMS, a strong inducer of cell death (data not shown). These results indicate that bacteria and glycolipid elicitors from fungi induce cell death *via* different mechanisms. PC-containing Tv-ZGL3 induced a high level of cell death as compared with Tv-ZGL2, though its content was not very high (see Fig. 1B). This fact indicates that PC has an important role in the induction of defense responses in rice cells.

In conclusion, our results indicate that the PC-containing glycolipid Tv-ZGL3 has a novel role in rice-fungus interaction. Further analysis is necessary to determine the mechanisms by which the PC-containing Tv-ZGL3 induces defense responses in rice.

Acknowledgments

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