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FUNGAL DISEASES

N,*N*-dimethylsphingosine, an inhibitor of sphingosine kinase, induces phytoalexin production and hypersensitive cell death of Solanaceae plants without generation of reactive oxygen species

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Abstract Plant recognition of elicitors derived from pathogens induces various resistant reactions, including production of reactive oxygen species, hypersensitive cell death and accumulation of phytoalexins. Previously, we isolated a ceramide elicitor from *Phytophthora infestans*, which activates O_2^- production of potato suspension-cultured cells. In this study, we employed nine ceramide-related chemicals to test their elicitor activity. Although, none of the tested chemicals induced O_2^- production, *N*,*N*-dimethylsphingosine (DMS) induced accumulation of phytoalexin in potato tubers. In potato, tobacco and *Nicotiana benthamiana*, DMS also induced rapid cell death. DMS-treated potato cells stained with 4′,6-diamidino-2-phenylindole (DAPI) showed chromatin condensation, and isolated DNA from DMS-treated cells had ladder pattern, confirming that DMS-induced plant cell death is a hypersensitive reaction-like programmed cell death. Involvement of ceramide signaling in induction of plant defense reactions is discussed.

Keywords Hypersensitive cell death · Phytoalexin · *Phytophthora infestans* · Potato · Reactive oxygen species · *N*,*N*-dimethylsphingosine

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Introduction

Plants have the ability to recognize molecules derived from potential pathogens to induce various disease resistances. Surface-derived structural molecules from plant pathogens, such as fungal cell wall constituents (chitin, glucan, protein and glycoprotein) and bacterial lipopolysaccharide (LPS) and flagellin, elicit defense responses in a wide range of plant species. Such nonspecific elicitors are generally conserved structural components of microorganisms termed microorganism-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs), which are recognized as a result of the innate immunity of plants (Jones and Takemoto 2004). Another group of elicitors are recognized by specific range of plant species or cultivars. Originally, specific elicitors were named avirulence (Avr) proteins because they were identified as determinants of Avr of plant pathogens, which now are commonly called "effectors" because they are very often also virulence factors for pathogens during the interactions with susceptible plants (Greenshields and Jones 2008).

Both non-specific and specific elicitors generally induce a similar set of disease resistance reactions (Mysore and Ryu 2004). Production of reactive oxygen species (ROS), a hallmark of plant resistance reactions, is involved in the induction of resistance reactions such as the expression of defense genes, accumulation of phytoalexins and induction of hypersensitive cell death (Doke et al. 1996; Perrone et al. 2003; Yoshie et al. 2005; Yoshioka et al. 2003). Because the ROS produced by animal phagocytes is microbiocidal to attacking pathogens (Klebanoff 2005), the ROS produced by plant cells after challenge by pathogens was initially considered to be a toxic agent that inhibits the growth of microorganisms. However, recently emerging

evidence indicates that ROS is a universal second messenger of plant signaling for various physiological activities including the induction of disease resistance (Torres et al. 2006).

One such disease mechanism is hypersensitive cell death, a form of plant programmed cell death that limits the attacking pathogen to the infection site. The processes that induce programmed plant cell death are largely unknown, in contrast to well-studied programmed animal cell death, but animal and plants share many of the common morphological and biochemical changes, such as chromatin condensation, transition of mitochondrial permeability and DNA fragmentation (Lam et al. 2001). Recent reports also suggest that programmed cell death in plants and animals in regulated by similar mechanisms. Caspases are a family of cysteine proteases essential for the induction of programmed cell death in animal cells (Cohen 1997). Although caspase activity was detected in plant cells, no orthologous sequence of animal caspases has been found in plant genomes (Bonneau et al. 2008). Recently, Hatsugai et al. (2004) identified vacuolar processing enzyme (VPE) as a caspase of plant that is essential for TMV-induced hypersensitive cell death. Bax is a mammalian pro-apoptotic protein that has been shown to induce programmed cell death of plant cells when overexpressed in plant cells (Kawai-Yamada et al. 2001). While there is no obvious homologue of the *Bax* gene in the plant genome, there is a homologous gene for Bax inhibitor (*BI-1*), which can inhibit Bax activity, H_2O_2 generation and salicylic acid-induced cell death (Kawai-Yamada et al. 2004). These reports strongly suggest that machineries for inducing programmed cell death are evolutionally conserved between animal and plant systems even though sequences of genes for the factors involved are not necessarily conserved.

In this study, we tested ceramide-related compounds as candidate elicitors of plant defense responses. Most of the chemicals had no elicitor activity, but *N*,*N*-dimethylsphingosine (DMS) induced a HR-like cell death and phytoalexin accumulation. Interestingly, DMS did not induce ROS production, which suggests that DMS is not recognized as an exogenous elicitor but is active later in the signaling pathway that leads to the induction of defense reactions.

Materials and methods

Plant materials

Suspension-cultured potato cells (cv. Sayaka) were grown by agitation at 130 rpm at 23°C in 95 ml of MS medium supplemented with 30 mg/ml sucrose, 1 μg/ml thiamine, 100 μg/ml myo-inositol, 200 μg/ml KH_2PO_4, and 0.2 μg/ml 2,4-dichlorophenoxyacetic acid. Cells were subcultured every week and used for experiments 3–5 days after the subculturing.

Tubers of potato cultivar Rishiri carrying the *R1* gene were stored at 4°C until use. Tuber discs, 2 cm in diameter and 2 mm thick, were prepared in the dark. Because aging of the tubers, after slicing the potato tubers, is thought to be essential for the rapid expression of host resistance (Furuichi et al. 1979), tuber discs were aged for 24 h before treatment with elicitors and chemicals. The volume of all solutions applied to the tuber surface was 100 μl and all the treated discs were incubated at 20°C in the dark.

Tobacco BY-2 cells were grown by agitating at 130 rpm at 25°C in 95 ml of MS medium supplemented with 30 mg/ml sucrose, 1 μg/ml thiamine, 100 μg/ml myo-inositol, 200 μg/ml KH_2PO_4, and 0.2 μg/ml 2,4-dichlorophenoxyacetic acid (Sasabe et al. 2000). Cells were subcultured every week and used for experiments 3–5 days after the subculturing.

N. benthamiana plants were grown at 23°C with 16 h of light and 8 h dark in a growth room.

Treatment with elicitors and ceramide-related chemicals

Hyphal wall components (HWC) elicitor was prepared from cultured mycelia of *P. infestans*, race 1.2.3.4 according to methods previously described (Doke and Tomiyama 1980). The lyophilized elicitor was dissolved in 10 mM Tris–HCl buffer (pH 7.4) for use. The INF1 elicitor was prepared from *Escherichia coli* cells carrying a chimeric plasmid (pFB53) with the *P. infestans infl* gene (Kamoun et al. 1997) according to the methods of Yamamoto et al. (2004). Ceramide-related chemicals, C2-ceramide (BIOMOL, Plymouth Meeting, PA, USA), C2-dihydroceramide (BIOMOL), C6-ceramide (Cayman Chemical, Ann Arbor, MI, USA), C8-ceramide (Cayman Chemical), C18-ceramide (Avanti Polar Lipids, Alabaster, AL, USA), DMS (Cayman Chemical) and sphingosine-1-phosphate (BIOMOL), were solubilized in DMSO for 1 mM stock solutions, except for DMS which was dissolved in 3% ethanol to make the 1 mM stock.

Detection of ROS production

The relative intensity of ROS generation was determined by counting photons from L-012-mediated chemiluminescence. L-012 (Wako, Osaka, Japan) is a luminol derivate that has a high sensitivity for superoxide radicals. For the detection of ROS produced by suspension-cultured cells, the cells were suspended in buffer [175 mM mannitol, 50 mM MES-KOH (pH 5.7), 0.5 mM $CaCl_2$ and 0.5 mM

K_2SO_4], and shaken at 100 rpm at 23°C for 1 h. Cells were then treated with elicitors and, incubated at same condition for 3 h, and chemiluminescence was then measured after the addition of 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) with a plate reader Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany).

For the detection of ROS production in plant tissues, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) was placed on the surface of potato tubers or used to infiltrate *N. benthamiana* leaves via a syringe without needle. Chemiluminescence was monitored continuously using a photon image processor equipped with a sensitive CCD camera in the dark chamber at 20°C (Aquacosmos 2.5; Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan) and quantified using the U7501 program (Hamamatsu Photonics).

Detection of phytoalexins

Potato phytoalexins, exuded into the well from the potato tissue, was extracted with ethyl acetate as described previously (Noritake et al. 1996; Sakai et al. 1981). The extract were separated on TLC plates (TLC aluminum sheet of silica gel 60, Merck, Whitehouse Station, NJ, USA), which were developed with cyclohexane:ethyl acetate (1:1, v/v) and visualized by spraying with sulfuric acid containing 0.5% vanillin followed by heating at 120°C.

Detection of cell death

For quantifying dead cells, 0.05% Evans blue (Merck) was added to cell suspensions. After 15 min, a 500 µl sample of the cell suspension was washed five times with 1.5 ml of distilled water each time to remove excess stain. Dye bound to dead cells was solubilized in 500 µl of 50% methanol that contained 1% SDS for 30 min at 50°C. The dye in solution (50 µl) was then diluted with 450 µl of distilled water and quantified by monitoring the absorbance at 600 nm (Yano et al. 1998).

Preparation of silencing constructs

Partial cDNA of *NbICS1*, *NbEIN2*, *NbNPR1* and *NbCOI1* was amplified from *N. benthamiana* cDNA using gene-specific primers with restriction sites at the 5′ ends. The gene-specific primer (Supplemental Table 1) combinations used to construct the silencing constructs were as follows: NbICS1-F and NbICS1-R1 (*NbICS1*), NbEIN2-F1 and NbEIN2-R1 (*NbEIN2*), NbNPR1-F and NbNPR1-R1 (*NbNPR1*) and NbCOI1-F and NbCOI1-R1 (*NbCOI1*). Amplified fragments were digested with restriction enzymes and ligated into the pTV00 vector (Ratcliff et al. 2001) digested with the corresponding restriction enzymes to generate pTV00:ICS1, pTV00:NPR1, pTV00:EIN2 and pTV00:COI1. For all constructs, amplified inserts were sequenced to confirm cloning of the correct gene fragment. The pTV00 vectors were transformed into *A. tumefaciens* cells (strain GV3101) by electroporation with MicroPulser electroporator (Bio Rad, Hercules, CA, USA).

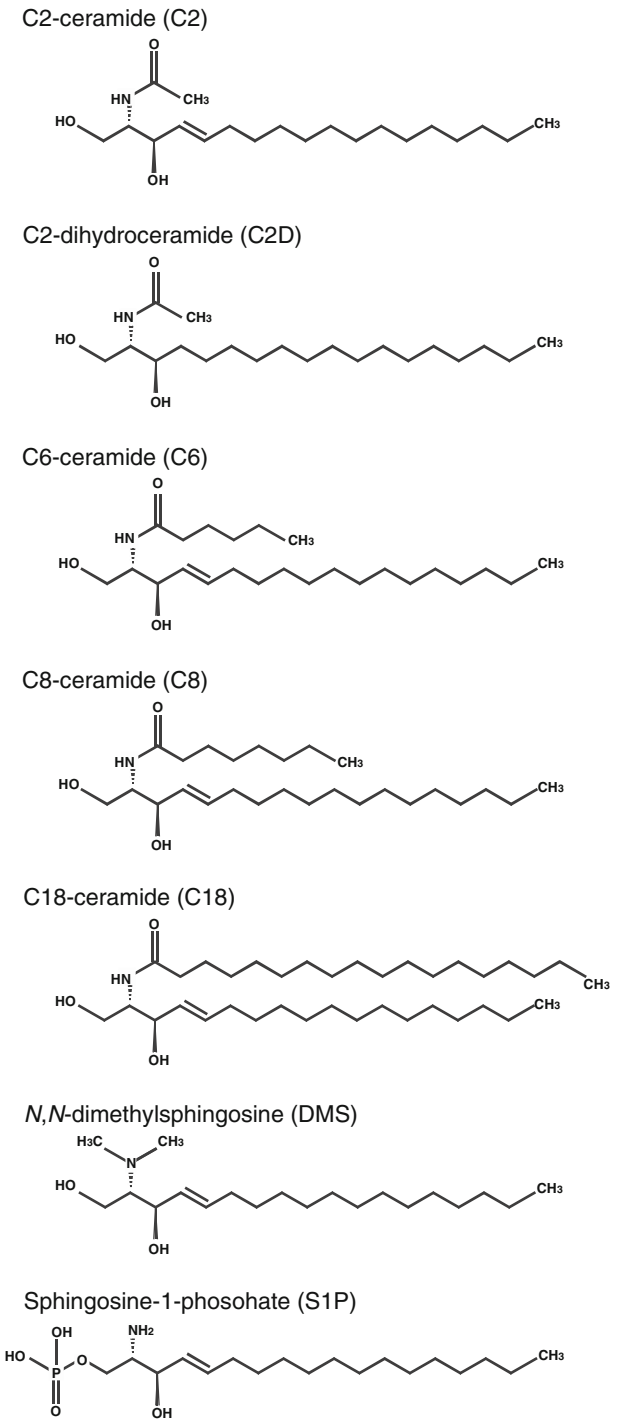

Fig. 1 Structure of ceramide-related chemicals used in this study

Virus-induced gene silencing (VIGS)

A. tumefaciens GV3101 carrying the binary TRV RNA 1 construct pBINTRA6 and the TRV RNA2 vector pTV00 or pTV00 derivatives were cultured to saturation in Luria–Bertani (LB) medium and bacterial suspensions were collected by centrifugation. The bacterial cells were resuspended in 10 mM MES-NaOH (pH 5.6), 10 mM $MgCl_2$ and 150 μM acetosyringone (final $OD_{600} = 0.5$), and incubated at room temperature for 2 h.

The cultures were mixed in a 1:1 ratio (RNA1/RNA2), to infiltrate leaves of *N. benthamiana* using a syringe without a needle. Three to four weeks after infiltration, the upper leaves of the inoculated plants were used for experiments.

RNA preparation and RT-PCR

Total RNA was isolated from frozen leaves or suspension-cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed, as previously described (Kato et al. 2008). Gene-specific amplifications of cDNA were performed under the same conditions as previously described (Kato et al. 2008). Gene-specific primer (Supplemental Table 1) combinations used for expression analysis were as follows: NbICS1-F and NbICS1-R2 (*NbICS1*), NbEIN2-F2 and NbEIN2-R2 (*NbEIN2*), NbNPR1-F and NbNPR1-R2 (*NbNPR1*), NbCOI1-F and NbCOI1-R2 (*NbCOI1*) and NbEF1α-F and NbEF1α-R (*NbEF-1α*).

Microscopy

To monitor plant cell death, leaves were stained as described by Takemoto et al. (2003). Infected leaves were cleared in methanol for more than 24 h and boiled for 3 min in lactophenol trypan blue stain (10 ml H_2O, 10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, and 10 mg of trypan blue). After the leaves had cooled to room temperature for 1 h, the stain was replaced with 1 g ml/1 chloral hydrate. Stained leaves were decolorized overnight and viewed using an Olympus microscope BX51 (Olympus, Tokyo, Japan). Chromatin condensation is visualized by DAPI (4′,6-diamidino-2-phenylindole) staining. Suspension-cultured cells were washed with PBS (137 mM NaCl, 8.1 mM $Na_2HPO_4{\cdot}12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO, pH 7.4) and stained with 1 mg/ml DAPI solution for 15 min at room temperature. Stained cells were

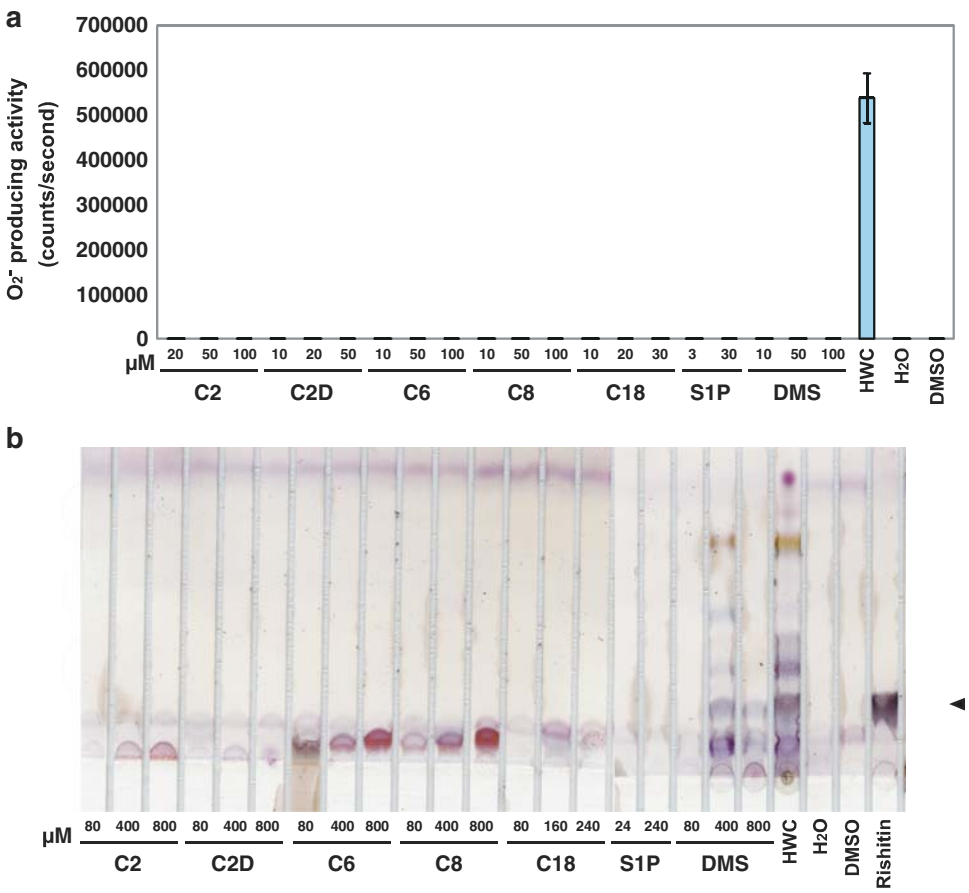

Fig. 2 **a** Ceramide-related chemicals do not induce O_2^- production of suspension-cultured potato cells. Cells were treated with chemicals at indicated concentrations or 1 mg/ml HWC for 3 h, then O_2^- was measured with L-012 using a chemiluminescence plate reader. **b** DMS induces phytoalexin production in potato tubers. Tubers were treated with compounds as in **a**, and phytoalexins were extracted 48 h after treatment. Extracted samples and 25 μg purified rishitin (*arrowhead*) were separated and developed on a TLC plate. *C2* C2-ceramide, *C2D* C2-dihydroceramide, *C6* C6-ceramide, *C8* C8-ceramide, *C18* C18-ceramide, *S1P* sphingosine-1-phosphate

monitored with a fluoresce microscope (BX51, Olympus) equipped with a UV-filter system providing an excitation wavelength of 365 nm.

Detection of DNA laddering

Total plant DNA was extracted as described by Kim et al. (2008). Suspension-cultured cells were homogenized using liquid nitrogen, and samples were incubated for 30 min at 25°C in DNA extraction buffer (0.1 M glycine, 50 mM NaCl, 10 mM EDTA, 2% SDS, and 1% sodium lauryl sarcosine), then mixed with an equal volume of Tris–saturated phenol. The mixture was centrifuged for 15 min at 10,000×*g* and the supernatant was treated with chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated with a two-fold volume of 100% ethanol, washed with 70% ethanol, and dissolved in Tris–EDTA buffer containing RNase A (40 μg/ml). Laddering of DNA was analyzed by ethidium bromide staining on 1.8% (w/v) agarose gel.

Real time PCR

Expression of potato genes after the treatment of elicitors was analyzed by real time PCR. Total RNA from suspension-cultured potato cells was extracted and cDNA was prepared using ReverTra-Plus (TOYOBO, Osaka, Japan) with Oligo(dT) primes as described previously (Kato et al. 2008). Real time PCR was performed with SYBR® *Premix Ex* Taq (TaKaRa, Otsu, Shiga, Japan) and Thermal Cycler Dice Real Time System Model TP800 (TaKaRa) according to the manufacturer's instructions with conditions of one cycle of 95°C for 10 s, then 40 cycles of 95°C for 5 s and 60°C for 30 s. The gene-specific primer (Supplemental Table 1) combinations used for real time PCR were as follows: StRbohB-F and StRbohB-R (*StRbohB*), PVS3-F and PVS3-R (*PVS3*) and StEF1α-F and StEF1α-R (*StEF-1α*).

Results and discussion

DMS, but not other ceramide-related chemicals, can induce phytoalexin production of potato tuber

Hyphal wall components (HWC) elicitor is a crude fraction extracted from mycelia of *Phytophthora infestans*, which induces various defense responses of potato, such as ROS production, expression of defense genes, phytoalexin accumulation and hypersensitive cell death (Doke and Tomiyama 1980; Takemoto et al. 1999). We previously aimed to purify elicitors of *P. infestans*, and identified a ceramide-related compound as an elicitor, which induced ROS production of suspension-cultured potato cells (Nozawa T, Ito Y and Kawakita K, unpublished data). In this study, we tested commercially available ceramide-related chemicals, including C2-ceramide, C2-dihydroceramide, C6-ceramide, C8-ceramide, C18-ceramide, DMS and sphingosine-1-phosphate (Fig. 1) for their activity as elicitor of ROS production. Five-day-old suspension-cultured

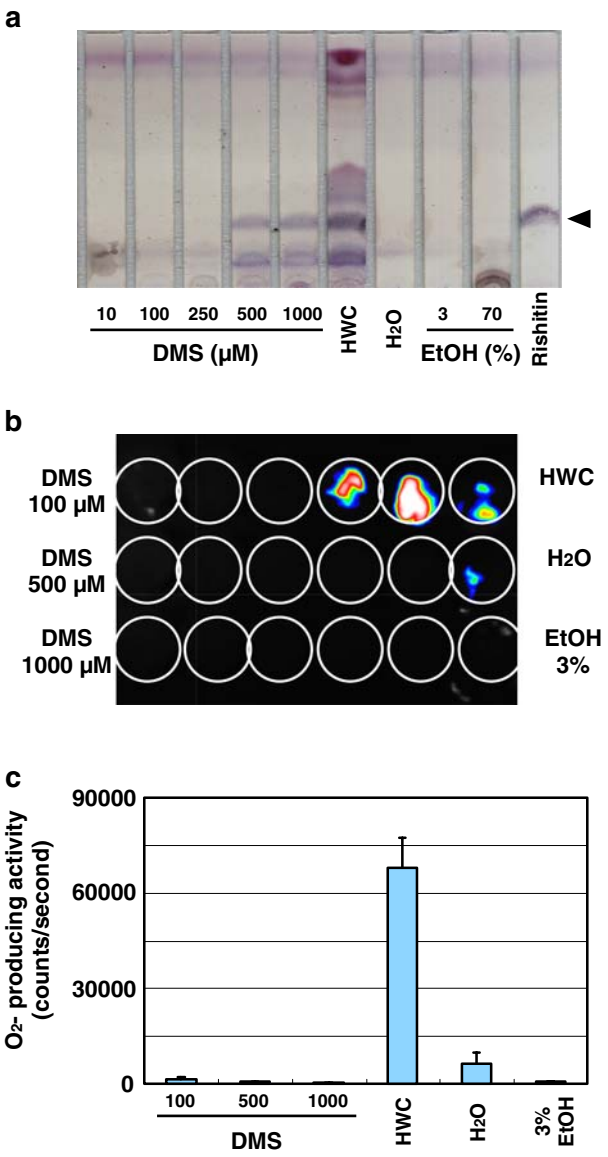

Fig. 3 DMS induces phytoalexin production in potato tuber without ROS production. **a** Potato tubers were treated with various concentrations of DMS or HWC and phytoalexins were extracted 48 h after treatment. Extracted samples and 25 μg purified rishitin (arrowhead) were separated and developed on the TLC plate. **b** Potato tuber disks were treated with various concentrations of DMS or 1 mg/ml HWC and production of O_2^- was detected with L-012 using CCD camera as chemiluminescence 1 h after treatment. Outline of 3 potato tubers for each treatment are shown as *circles*. **c** Intensities of chemiluminescence shown in **b** were quantified with photon image processor

potato cells were treated with water, HWC or ceramide-related chemicals, and ROS production of potato cells was analyzed 3 h after the treatment of elicitors. Only HWC elicitor had strong activity in inducing ROS production whereas none of the ceramide-related chemicals were active for ROS production of potato cells (Fig. 2a).

Potato tubers were treated with water, HWC or ceramide-related chemicals and accumulation of the potato phytoalexin, rishitin, was analyzed by TLC. Twenty-four hours after the treatment, HWC induced production of rishitin, as described previously (Fig. 2b, Sakai et al. 1981). Of the tested ceramide-related chemicals, only DMS was active in eliciting phytoalexin production (Fig. 2b). DMS induced rishitin production at a concentration of 500–1000 μM (Fig. 3a). However, same concentration of DMS did not elicit ROS production of potato tuber (Fig. 3b, c) indicating that DMS has activity to induce phytoalexin production without ROS generation. To eliminate the possibility that DMS can induce ROS production during earlier stages of the treatment, ROS production of DMS-treated potato suspension-cultured cells was monitored at 15 min, 30 min, 1 and 2 h after treatment. However, ROS production was not induced by any concentration of DMS at any time, confirming that DMS dose not induce ROS (Supplemental Fig. 1).

DMS induces HR-like programmed cell death

Cell viability of suspension-cultured potato cells was investigated after treatment with either HWC elicitor or DMS using Evans blue staining. Because Evans blue is excluded from living cells but remains in dead cells, it can be used to monitor the progress of cell death (Yano et al. 1998). Six hours after the treatment with HWC, the treated potato cells were stained by Evans blue 5.5 times more compared with untreated cells, indicating that the HWC elicitor induced cell death (Fig. 4a). Treatment with 50–100 μM DMS induced death of potato cells within 1 h, and at 6 h after the DMS treatment at 100 μM, cell death was six-fold higher than that of HWC-treated cells (Fig. 4a). Fifty μM of C2 ceramide also had weak but significant activity for inducing plant cell death, whereas C2-dihydroceramide, C6-ceramide, C8-ceramide, C18-ceramide, and sphingosine-1-phosphate did not induce cell death of potato cells (Supplemental Fig. 2a). Concomitant treatment with HWC elicitor and 50 μM DMS only slightly enhanced of cell death compared with a single treatment with 50 μM DMS, suggesting that HWC elicitor and DMS share similar signaling pathway to induce cell death (Supplemental Fig. 2b). Within 1 day after the treatment with HWC, treated area of potato tubers developed browning, inductive of hypersensitive cell death (Fig. 4b). DMS-treated potato

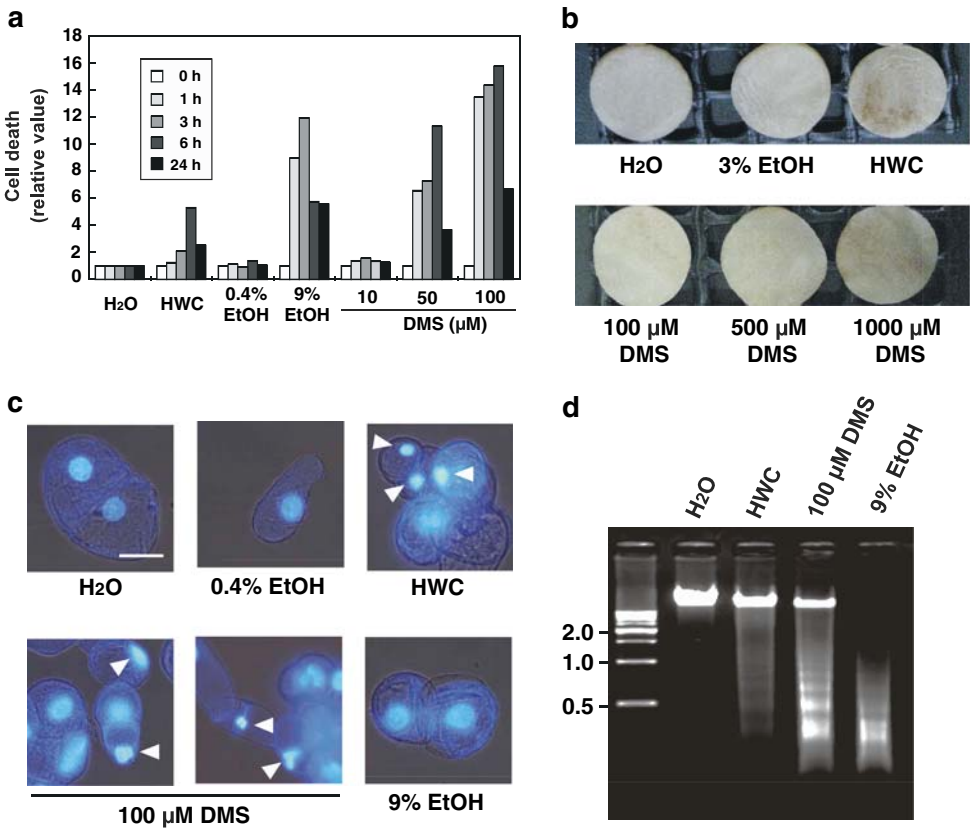

Fig. 4 DMS induces HR-like programmed cell death of potato cells. **a** Suspension-cultured potato cells were treated with HWC, ethanol or DMS, and induction of cell death was measured using Evans blue staining as described in "Materials and methods". Dye that bound to dead cells was solubilized and quantified by monitoring the A_{600}. Values are relative to water-treated control cells. **b** Potato tuber disks were treated with HWC or DMS at indicated concentrations and photographed 48 h after treatment. HR-like cell death is seen as browning of tissue. **c** Suspension-cultured potato cells were treated with HWC, ethanol or DMS, and chromatin condensation was visualized by DAPI staining. **d** Suspension-cultured potato cells were treated with HWC, ethanol or DMS, and genomic DNA was extracted. DNA was analyzed by ethidium bromide staining on 1% (w/v) agarose gel

tubers also developed dead cells (Fig. 4b). For potato tubers, a higher concentration of DMS, compare with that for suspension cells, was needed to induce cell death, perhaps due to the poorer accessibility of ceramides, which are a family of lipid molecules, to intact potato cells.

Chromatin condensation paralleled by DNA fragmentation is one of the most important criteria to identify apoptotic cells (Cohen et al. 1992). To investigate the nature of cell death induced by DMS, chromatin condensation and DNA fragmentation of DMS-treated potato cells was investigated. Potato cells treated with a high concentration (9%) of ethanol developed necrotic cell death, and this treatment was used as a negative control (Fig. 4a). Nuclear DNA of potato cells was visualized by DAPI staining. Although potato cells treated with 9% ethanol had no morphological change in chromatin compared with water-treated cells, HWC-treated cells often had a dense and strong blue fluorescence, indicating induction of chromatin condensation by HWC elicitor. Treatment with 100 μM DMS also induced chromatin condensation similar to HWC-treated cells (Fig. 4c).

Genomic DNA was isolated from potato cells 3 h after treatment with the elicitors, and DNA degradation was monitored. Six hours after treatment with HWC or 100 μM DMS, smearing of genomic DNA was obvious and laddering of DNA was detected in DMS-treated cells (Fig. 4d). In contrast, DNA isolated from potato cells treated with 9% ethanol was completely degraded, probably indicating passive degradation of genomic DNA as a result of necrotic cell death (Fig. 4d). Altogether, treatment with HWC and DMS induced chromatin condensation of potato cells paralleled by fragmentation of genomic DNA, indicating that DMS can induce HR-like programmed cell death.

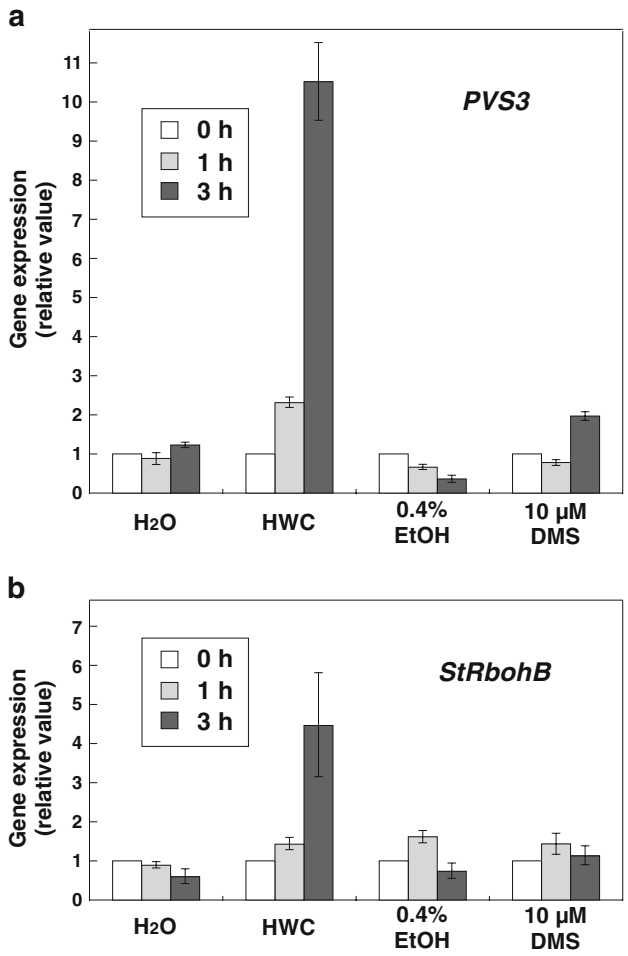

Fig. 5 Expression of *PVS3*, but not *StRbohB*, was induced by the treatment of DMS in suspension-cultured potato cells. Expression of *PVS* potato vetispiradiene synthase (**a**), and *StRbohB*, a potato NADPH oxidase (**b**) in suspension-cultured potato cells after treatment with 1 mg/ml HWC, 0.4% ethanol (EtOH) or 10 μM DMS was calculated using real time PCR relative to expression of EF1α as internal standard

Expression of *PVS* and St*RbohB* genes after DMS treatment

Potato vetispiradiene synthase (PVS) is a key enzyme required for the synthesis of potato phytoalexin. *StRbohB* gene encodes a NADPH oxidase that is required for ROS production in response to elicitor treatment. It was previously reported that expression of *PVS3* and *StRbohB* is induced in potato tuber treated with HWC elicitor (Yoshioka et al. 1999, 2001). We investigated whethere these genes were induced by DMS. Expression of *PVS3* and *StRbohB* in potato cells treated with elicitors was analyzed by real time PCR using the elongation factor (*EF1α*) gene as an internal standard. In comparison to untreated cells, the HWC-treated cells showed 10 times higher expression of *PSV3* after 3 h of the treatment. Potato cells were treated with 10 μM DMS for expression analysis despite this concentration of DMS is not enough to induce cell death (Fig. 4a), because of extremely poor yield of RNA extracted from cells treated with 50–100 μM DMS probably due to the induction of rapid cell death. DMS (10 μM) treatment also induced expression of *PVS3* though it was only two-fold higher than that of the control cells (Fig. 5a). Expression level of *StRbohB* in HWC-treated cells was four-fold higher than the cells treated with water, whereas there was no induction of *StRbohB* expression by DMS treatment (Fig. 5b). These results were consistent with earlier results that DMS induces phytoalexin production but not ROS production.

DMS induces HR-like cell death of *Nicotiana* plants without ROS production

To investigate whether DMS can induce HR-like cell death of other Solanaceae plants, the suspension-cultured tobacco

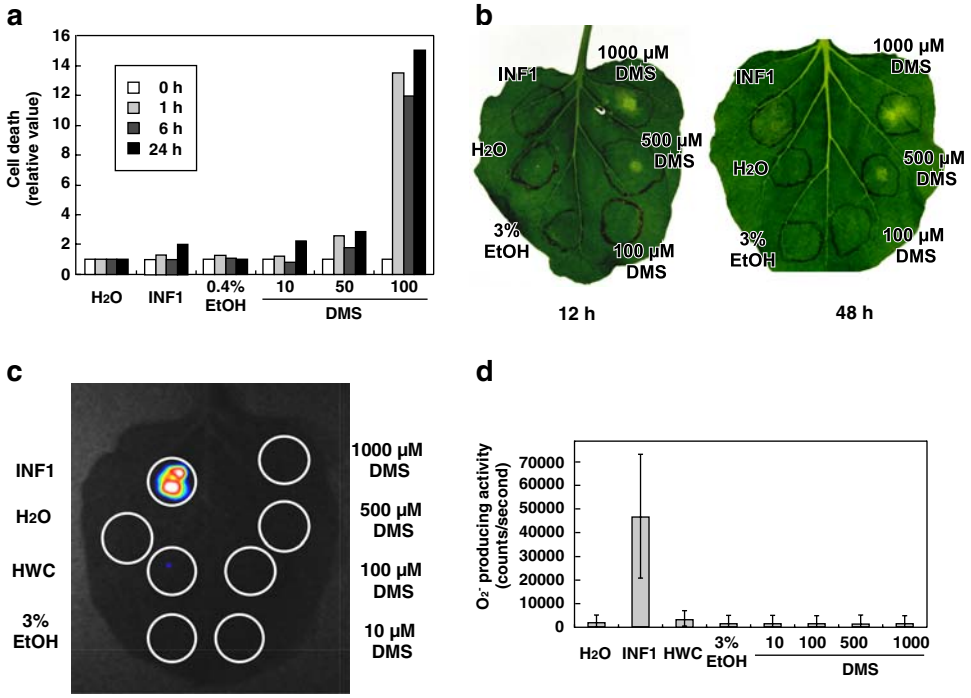

Fig. 6 DMS induced HR-like programmed cell death of *Nicotiana* species. **a** Suspension-cultured tobacco cells (BY-2) were treated with 150 nM INF1 or various concentrations of DMS, and induction of cell death was measured using Evans blue staining as described in "Materials and methods". Values are relative to water-treated control cells. **b** Leaves of *N. benthamiana* were treated with 150 nM INF1 or DMS at indicated concentrations and photographed at 12 and 48 h after treatment. **c** Leaves of *N. benthamiana* were treated with 1 mg/ml HWC, 150 nM INF1 or DMS at indicated concentrations and production of O_2^- was detected with L-012 using CCD camera as chemiluminescence 1 h after treatment. **d** Intensities of chemiluminescence shown in c were quantified with photon image processor

cell line Blight Yellow-2 (BY-2) was used. Twenty-four hours after treatment, 10 µM DMS induced comparable level of cell death with cells treated with 150 nM INF1, a secreted elicitor protein of *P. infestans* (Fig. 6a). Fifty to 100 µM DMS induced rapid and strong cell death within 1 h, and laddering of DNA was detected for DMS-treated tobacco cells (Fig. 6a, data not shown). Leaves of *N. benthamiana* treated with various concentrations of DMS also developed cell death (Fig. 6b), but induction of ROS was not detected at all (Fig. 6c, d). These results confirmed that DMS has activity to induce HR-like cell death of *Nicotiana* species without ROS production.

In *N. benthamiana*, we used VIGS to investigate the involvement of phytohormone signaling on DMS-induced cell death. Expression of genes for *EIN2* (a regulator of ethylene responses), *COI1* (an F-box protein for JA signaling), *ICS1* (isochorismate synthase1 for SA biosynthesis) or *NPR1* (a regulatory protein for SA-dependent resistance) was suppressed by TRV-based VIGS, and the response of these silenced plants to DMS was analyzed (Supplemental Fig. 3, Alonso et al. 1999; Cao et al. 1994; Wildermuth et al. 2001; Xie et al. 1998). All silenced plants treated with DMS showed comparable induction of cell death with control plants, although INF1-induced cell death was affected by silencing of *ICS1* (Shibata Y and Takemoto D, unpublished data). These results indicate that phytohormone signaling has no role in cell death induced by DMS.

Silencing of *NbRbohB*, an NADPH oxidase required for ROS production, delays the development of cell death induced by INF1 treatment (Yoshioka et al. 2003). Treatment with an ROS scavenger inhibited production of tobacco phytoalexin, capsidiol, induced after inoculation with an incompatible race of *P. nicotianae* (Perrone et al. 2003). These reports indicate that ROS production is an upstream regulator of HR-like cell death and production of phytoalexin during the induction of defense responses. Given DMS induces accumulation of phytoalexin and HR-like cell death of plant without ROS, DMS is perhaps not recognized as an exogenous elicitor but acts later in the signaling pathway, inside the plant cell, leading to the induction of defense reactions. This possibility is consistent with the fact that suspension-cultured cells were relatively sensitive to low concentration of DMS, whereas induction of HR-cell death of plant tissue needed high concentration of DMS (Fig. 4), probably due to the great difference between cultured cells and normal cells in the permeability of their cell walls. Suspension-cultured potato cells treated

with both HWC elicitor and DMS had only a slight enhancement of cell death compared with single treatment of DMS (Supplemental Fig. 2b). This result also supports the idea that the HWC elicitor and DMS induce HR-like cell death through a similar signaling pathway.

We originally tested DMS as a candidate pathogen-derived elicitor to induce defense response of plant. DMS, however, is a competitive inhibitor of sphingosine kinase, which is involved in the production of sphingosine-1-phosphate (Edsall et al. 1998). Ceramide and related sphingolipids are important bioactive lipids that act as second messengers for signal transduction and regulators of the induction of programmed cell death in animals (Hannun and Obeid 2002). Sphingosine-1-phosphate is reported to act as a negative regulator of programmed cell death (Spiegel and Milstien 2003). Several reports implicate the involvement of ceramide and sphingolipid derivatives in programmed cell death in plant. In *Arabidopsis*, knockout of *ACD5* (Accelerated cell death 5, encodes ceramide kinase, CERK) and *ACD11* (encodes sphingosine transfer protein) enhances activation of cell death (Brodersen et al. 2002; Liang et al. 2003). Interestingly, *acd5* mutant is more sensitive to C2 ceramide compared with wild type, and C2 ceramide-1-phosphate partially blocks cell death induction by C2 ceramide. Takahashi et al. (2009) reported that overexpression of LCB2, an enzyme for sphingolipid biosynthesis, induces HR-like cell death of *N. benthamiana*. Toxins produced by plant pathogens, such as AAL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* and fumonisin B1 produced by *Fusarium* species, induce cell death of plant cells by inhibiting sphinganine N-acyltransferase (Spassieva et al. 2002). These reports indicate that dihydrosphingosine and 3-ketosphinganine are pro-apoptotic factors in plant cells.

Accumulation of phytoalexin is induced only by DMS and not by other ceramide-related chemicals including C2 ceramide, although treatment of C2 ceramide can induce plant cell death of *Arabidopsis* (Liang et al. 2003), potato suspension cells (Supplemental Fig. 2a) and *N. benthamiana* (data not shown). Thus, HR-like cell death itself is not a cue for the accumulation of phytoalexin. Production of phytoalexin induced by DMS could be the result of an abnormal balance between pro-apoptotic and anti-apoptotic ceramides in the plant cell, but the pathway leading to the production of phytoalexin is perhaps distinct from that for HR-cell death.

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