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Sulfated lentinan induced mitochondrial dysfunction leads to programmed cell death of tobacco BY-2 cells

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ABSTRACT

Sulphated lentinan (sLTN) is known to act as a resistance inducer by causing programmed cell death (PCD) in tobacco suspension cells. However, the underlying mechanism of this effect is largely unknown. Using tobacco BY-2 cell model, morphological and biochemical studies revealed that mitochondrial reactive oxygen species (ROS) production and mitochondrial dysfunction contribute to sLNT induced PCD. Cell viability, and HO/PI fluorescence imaging and TUNEL assays confirmed a typical cell death process caused by sLNT. Acetylsalicylic acid (an ROS scavenger), diphenylene iodonium (an inhibitor of NADPH oxidases) and protonophore carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (a protonophore and an uncoupler of mitochondrial oxidative phosphorylation) inhibited sLNT-induced H_2O_2 generation and cell death, suggesting that ROS generation linked, at least partly, to a mitochondrial dysfunction and caspase-like activation. This conclusion was further confirmed by double-stained cells with the mitochondria-specific marker MitoTracker RedCMXRos and the ROS probe H_2DCFDA. Moreover, the sLNT-induced PCD of BY-2 cells required cellular metabolism as up-regulation of the AOX family gene transcripts and induction of the SA biosynthesis, the TCA cycle, and miETC related genes were observed. It is concluded that mitochondria play an essential role in the signaling pathway of sLNT-induced ROS generation, which possibly provided new insight into the sLNT-mediated antiviral response, including PCD.

1. Introduction

An increasing demand for environmentally acceptable alternatives for traditional pesticides provides an impetus to conceive new bio-based strategies in crop protection. Applying elicitor-induced resistance is one such efficient strategy, consisting of priming the natural plant immunity [1–3]. Research has established that β-(1 → 3)-glucans (laminarin, lentinan) and sulfated polysaccharides (Fucans, carrageenans, ulvans) can trigger immune responses and act as efficient immunostimulating agents in several plant systems [4]. They can initiate subsequent complex cascades of signaling events that lead to synthesis of reactive oxygen species (ROS) [5,6], salicylic acid (SA), salicylic acid —2—O—β grape glycosides (SAG) and jasmonate [7–9], production of phytoalexin and accumulation of defense-related proteinase inhibitors [4,5,10–13], as well as induction of pathogenesis-related (PR) proteins [8,9,14,15].

Previous studies have given insight into the relationship between elicitor-induced cell death and plant immunity [16]. Chitosan, chitooligosaccharide and oligochitosan have been shown to prime micro hypersensitive response (HR) and induce a variety of programmed cell death (PCD) in soybean, *Arabidopsis thaliana* and tobacco suspension cells, which is linked with free cytosolic Ca^{2+} elevation, NO and H_2O_2 production [17–20]. Iriti et al. [21] showed that chitosan can activate plant defense by mimicking a localized virus infection, thus accelerating the subsequent HR when inoculation was challenged with tobacco necrosis necrovirus (TNV), ultimately impaired virus replication and translocation, reducing the number and size of virus lesions.

PCD is an important mechanism for the controlled elimination of targeted cells in both animals and plants [22]. In animals, lentinan (LNT) has been reported to induce apoptosis of tumor cells via mitochondrial pathways, which is characterized by a rapid stimulation of ROS production, the loss of mitochondrial membrane potential and an increase in intracellular concentration of Ca^{2+}, as well as the activation of caspase-3 [23,24]. Nevertheless, little information about the molecular regulation of LNT-induced plant PCD is available.

Sulphated lentinan (sLNT) is a sulphated derivative of lentinan isolated from the fruiting body of *Lentinus edodes* with the structure of β-(1 → 3)-D-glucose main chains and β-(1 → 6)-D-glucose side chains. It shares the backbone of β-(1, 3) linked glucopyranosyl residues with laminarin sulfates [9], and exhibits enhanced antiviral activities against TMV infections in tobacco in vivo [8,9]. In plants, PS3, laminarin sulfate increased the resistance in grapevine and tobacco against *Plasmopara viticola* and TMV through priming the SA- and ROS-dependent defenses

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including potentiated H_2O_2 production and HR-like cell death [5,6,25,26]. However, the question whether PCD was involved in PS3 and sLNT elicited defense events of tobacco infected with TMV and the underlying mechanism are largely unanswered. In this study, cell imaging technologies, and biochemical and genetic approaches were employed to understand whether sLNT-induced viral resistance truly is associated with HR-like cell death using the tobacco BY-2 cell model. The roles of mitochondria (behavior and function), several signaling events, as well as the induction of respiratory genes associated with disease resistance were assessed. The results provide strong evidence that sLNT induces programmed cell death through mitochondrial ROS production and dysfunction. This work contributes to the understanding of a mitochondria-dependent mechanism of sLNT-induced biological responses in plants, and provides new insights into the cellular signaling cascade in sLNT-mediated defense response.

2. Materials and methods

2.1. Chemicals and reagents

sLNT with sulfation degree of 0.98 was obtained by sulfation of purified LNT using the chlorosulfonic acid–pyridine method [9]. The sLNT was dissolved in distilled water and sterilized by filtration through a Millipore filter (0.22 μm) to be used in experiments.

All pharmacological compounds were purchased from Sigma-Aldrich unless specified otherwise, and dissolved in dimethyl sulfoxide (DMSO). When used, the final DMSO concentration did not exceed 0.25% (v/v).

2.2. Cell culture

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cells (kindly provided by Prof. Xuefeng Yuan of Department of Plant pathology, Shandong Agricultural University, Tai'an, China) were grown in the liquid Murashige-Skoog medium (Cat. no. M5524) at pH 5.8 (with KOH) as described previously with minor revision [27]. Cells were cultured in 100 mL flasks at 27 °C on a light-protected shaker at 123 rpm. Subcultures were made every 4 days by transferring the cultured cells into fresh media (1:40, v/v). All experiments were performed using 4-day-old cultures (in the exponential growth phase).

2.3. Cell apoptosis assays

At 24 h after subculture, sLNT solution was added at the concentrations of 25, 50, 100, 200, 400 and 600 μg/mL cell suspension culture. Cell viability was measured at 0, 3, 6, 9, 12 and 24 h after the sLNT treatments using Evans blue staining as previously described [27]. Cell morphology was visualized at 24 h after sLNT treatment using a light microscope. To characterize nuclear morphology, cells were stained with the Hoechst 33342 and propidium iodide (HO/PI) (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions and visualized using a confocal laser scanning microscope (Leica TCS SP8; Leica Microsystems IR GmbH) with an excitation filter of 345 nm and 570 nm.

One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) was used to analyze nuclear DNA fragmentation according to the manufacturer's instructions. Briefly, cell samples were harvested at 24 h after sLNT treatment at 200 μg/mL and fixed in 4% formaldehyde for 1 h at 25 °C followed by three washes in PBS. The cell samples were then permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate for 2 min in ice bath. Finally, the samples were incubated in 50 μL TUNEL reaction mixture for 1 h at 37 °C in total darkness. Fluorescence imaging was obtained using a fluorescent microscope with an excitation of 488 nm and an emission of 515 nm. Blank controls were performed with an equal volume of PBS. A negative control was conducted by omitting TdT from the TUNEL reaction mixture. A positive control was conducted by pre-treating with DNase I. There were three independent replicates, and the whole experiment was repeated twice.

Mitochondrial and cytosolic Cytochrome *c* were isolated from BY-2 suspension cells treated with sLNT at 200 μg/mL of different durations using the Cytochrome *c* Assay Kit (Genmed Scientific Inc., USA). The protein concentration was determined using the Bio-Rad Protein Assay Kit (Genmed Scientific Inc., USA) with bovine serum albumin (BSA) as a standard. Cytosolic proteins (15 μg) and mitochondrial proteins (8 μg) were loaded for western blotting. The protein bands were analyzed by ImageJ.

2.4. Mitochondria isolation and H_2O_2 production

Mitochondria were isolated and purified from 250 mg of BY-2 cells using Tissue Mitochondria Isolation Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. H_2O_2 production was measured using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen Corp.) according to the manufacturer's recommendations, and H_2O_2 accumulation was determined by monitoring the fluorescent probe H_2DCF-DA with a LS 55 Luminescence Spectrophotometer (PerkinElmer, UK). The assay was performed for BY-2 cells treated with 0, 50, 100 and 200 μg/mL sLNT.

In order to elucidate the role of mitochondria in sLNT induced PCD; diphenylene iodonium (DPI, an inhibitor of a NADPH oxidase inhibitor or NOX), acetylsalicylic acid (ASA, a hydrogen peroxide scavenger) and the protonophore carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP, an inhibitor of the opening of mPTP) were added 30 min before sLNT treatment. The effects of the added agents on H_2O_2 generation and cell death induced by sLNT treatment were evaluated.

2.5. Mitochondrial membrane potential ($\Delta\Psi_m$) and mitochondrial permeability transition pore (MPTP) assays

Mitochondrial swelling were measured by decrease in absorbance at 540 nm using a 96-well plate reader (Bio-Tek ELX800 spectrophotometer) [28,29]. The measurement was repeated at least 3 times for each treatment. The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using the JC-1 Mitochondrial Membrane Potential Assay Kit (Beyotime) as described previously [30]. Luminescence intensity was assessed with the LS 55 Luminescence Spectrophotometer at emission wavelength 535 and 580 nm. The ratio of fluorescence ($F\lambda_{580}/F\lambda_{535}$) was calculated.

MPTP was measured according to the method described previously [31]. Briefly, isolated mitochondria were double-stained with 4 μM calcein/acetoxymethyl ester and 800 nM MitoTracker (Invitrogen, Carlsbad, CA, USA) for 15 min before washing. 1 mM $CoCl_2$ was used to quench the fluorescence of cytosolic calcein during calcein and MitoTracker loading. The fluorescence intensity of calcein (excitation 488 nm, emission at 530 nm) and MitoTracker (excitation 543 nm, emission at 595 nm) were detected using the LS 55 Spectrophotometer. The value of MPTP was calculated as the ratio of mitochondrial calcein and MitoTracker fluorescence.

2.6. Measurements of caspase-like activities

Caspase 9- and caspase 3-like activities were determined using the Caspase-9 and Caspase-3 Colorimetric Activity Assay Kits (Beyotime Institute of Biotechnology, Shanghai, China), respectively, according to the manufacturer's instructions. The assays are based on spectrophotometric detection of the chromophore pNA after the caspase-dependent cleavage from the labeled substrates DEVDpNA or LEHD-pNA by active caspase 3 or 9, respectively. Cytosolic protein extracts from tobacco cells (0.1 g) were incubated for 2 h at 37 °C with DEVD-pNA or LEHD-pNA, in the presence or absence of 3 μM caspase inhibitors Ac-DEVD-CHO (for caspase 3) or Ac-LEHD-CHO (for caspase 9) before