Carbohydrate Polymers 87 (2012) 2270–2278

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Oligochitosan induces programmed cell death in tobacco suspension cells

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ARTICLE INFO

Article history:
Received 8 September 2011
Received in revised form 25 October 2011
Accepted 25 October 2011
Available online 31 October 2011

Keywords:
Oligochitosan
PCD
Ca^{2+}
NO
H_2O_2
Cytochrome *c*

ABSTRACT

Oligochitosan has been proved to trigger plant cell death. To gain some insights into the mechanisms of oligochitosan-induced cell death, the nature of oligochitosan-induced cell death and the role of calcium (Ca^{2+}), nitric oxide (NO) and hydrogen peroxide (H_2O_2) were studied in tobacco suspension cells. Oligochitosan-induced cell death occurred in cytoplasmic shrinkage, phosphatidylserine externalization, chromatin condensation, TUNEL-positive nuclei, cytochrome *c* release and induction of programmed cell death (PCD)-related gene *hsr203J*, suggesting the activation of PCD pathway. Pretreatment cells with cyclosporin A, resulted in reducing oligochitosan-induced cytochrome *c* release and cell death, indicating oligochitosan-induced PCD was mediated by cytochrome *c*. In the early stage, cells undergoing PCD showed an immediate burst in free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation, NO and H_2O_2 production. Further study showed that these three signals were involved in oligochitosan-induced PCD, while Ca^{2+} and NO played a negative role in this process by modulating cytochrome *c* release.

1. Introduction

Oligochitosan is the fragment of chitosan which is produced by deacetylation of chitin. Oligochitosan is well-known elicitor in plant and has been widely used to mimic pathogen attack and shown to induce plant defense responses (Hamel & Beaudoin, 2010; Muzzarelli et al., 2011). PCD is a basic and vital cellular process for plants both under normal and abnormal conditions (Greenberg, 1996). Especially, adverse abiotic and biotic stresses, such as drought or pathogen attack, can promote plant PCD pathway (Reape & McCabe, 2008). Chitosan has been reported to induce PCD in soybean cells in a Ca^{2+}-mediated pathways (Zuppini et al., 2003). Chitosan also induced hypersensitive response in tobacco leaves to inhibit the tobacco necrosis necrovius replication and translocation (Iriti et al., 2006). Cabrera showed that chitooligosaccharide elicitors induced cell death in *Arabidopsis* suspension cells depending on its different size, acetylation and concentration (Cabrera, Messiaen, Cambier, & Van Cutsem, 2006). Similarly, Wang found that exposure of tobacco suspension cells to oligochitosan led to cell death whose process was independent of H_2O_2 pathway (Wang, Li, Zhao, Du, & Lin, 2007).

The mechanisms of PCD in animals have been widely investigated and well documented (Hedrick, Ch'en, & Alves, 2010), whereas little is acquired about the control of plant PCD. Over the past decade, reports found that early calcium flux, mitochondrial release of apoptogenic proteins (such as cytochrome *c*), activation of caspase-like proteases or metacaspases, are key factors in the utmost destruction of the plant cell (Reape & McCabe, 2010). Others reported that NO and H_2O_2 could trigger PCD, either separately or together with each other, or mediate elicitor-induced PCD process (De Pinto, Paradiso, Leonetti, & De Gara, 2006; Delledonne, Xia, Dixon, & Lamb, 1998; Torres, Jones, & Dangl, 2005).

Ca^{2+} influx is early event in plant PCD. Changes in $[Ca^{2+}]_{cyt}$ have been suggested to be involved in plant cell death induced by several elicitors (Errakhi et al., 2008; Zhu, Caplan, Mamillapalli, Caymmek, & Dinesh-Kumar, 2010; Zuppini et al., 2003). Some reports found that the addition of Ca^{2+} induced mitochondrion swelling and cytochrome *c* release that may mediate PCD in plant cells (Virolainen, Blokhina, & Fagerstedt, 2002). Recent reports find that early Ca^{2+} influx is a prerequisite to thaxtomin A-induced cell death in *Arabidopsis thaliana* cells (Errakhi et al., 2008). Zhu showed that endoplasmic reticulum Ca^{2+}-ATPase is a component of the calcium efflux pathway that controls PCD during plant innate immune response (Zhu et al., 2010).

NO is another important second messenger involved in multiple physiology process such as plant–pathogen response (Hong et al., 2008) and PCD (De Michele et al., 2009). Reports have indicated that abiotic and biotic elicitor-induced NO production can mediate the induction of cell death (Delledonne et al., 1998; Ma et al., 2010). Others found that NO can act as an antioxidant or antiapoptotic modulator to prevent cell death (Chung, Pae, Choi, Billiar, & Kim, 2001). Some other work implicated that NO can affect

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doi:10.1016/j.carbpol.2011.10.059

mitochondrial functionality and induce cytochrome *c* release in plant cell death (Zottini et al., 2002).

In addition to NO, reactive oxygen species (ROS), mainly superoxide anion $O_2^{\bullet -}$ and H_2O_2, is a common molecular in plant exposed to elicitors and generally regarded as the cause of hypersensitive response (Torres et al., 2005). Moreover, ROS plays pivotal roles in plant PCD (Dat et al., 2003; De Pinto et al., 2006). Reports proved that cytochrome *c* was released in a ROS-dependent manner in heat shock-induced cell death (Vacca et al., 2006). Cross talk between NO and H_2O_2 in plant PCD has been extensively studied in past several years. Consistent reports show that PCD requires the simultaneous presence of NO and H_2O_2 (De Pinto, Tommasi, & De Gara, 2002; Delledonne et al., 1998), while contrasting data are also present in other papers (De Pinto et al., 2006; Houot et al., 2001).

In our laboratory, oligochitosan was produced from chitosan by enzymatic hydrolysis and separated with membrane. It has been applied as effective biopesticide for plant disease control. Our previous work found that oligochitosan treatments increase intracellular NO levels (Zhang et al., 2011), accelerate production of H_2O_2 (Li et al., 2009), induce changes in protein phosphorylation (Feng et al., 2006), trigger defense-related gene expression (Chen et al., 2009), and strengthen cell wall. We also found that oligochitosan induced cell death in tobacco suspension cells (Wang et al., 2007). Nevertheless, the mechanisms that modulate oligochitosan-induced cell death are still limited and the role of Ca^{2+}, NO and H_2O_2 in oligochitosan-induced cell death remain unclear.

In this paper, to obtain further information on oligochitosan-induced cell death, the features of cell death induced by oligochitosan and the early signal about Ca^{2+}, NO and H_2O_2 were investigated.

2. Materials and methods

2.1. Chemicals

Oligochitosan with 95% N-deacetylation and polymerization degree from 3 to 9 was produced by enzymatic hydrolysis method, and dissolved in distilled water (Zhao, She, Du, & Liang, 2007). Oligochitosan were sterilized by filtration through a Millipore filter (0.22 μm).

Hoechst 33342 (HO), propidium iodide (PI), 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA), vitamin C (Vc), catalase (CAT, from bovine liver), N^G-nitro-L-arginine methyl ester (L-NAME), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and lanthanum chloride ($LaCl_3$) were obtained from Sigma. 3-Amino,4-aminomethyl-2′,7′-difluorescein (DAF-FMDA), cyclosporin A and Fluo-3AM were obtained from Beyotime Institute of Biotechnology. All other reagents were obtained from Alfa Aesar, Tianjin Kermel Chemical Development Centre, or Beijing Chemical Plant.

2.2. Cells culture and treatments

Tobacco suspension cells (*Nicotiana tabacum* var. *samsun* NN) were routinely propagated and cultured as described previously (Wang et al., 2007). For the experiments, cells during the exponential growth phase were reinoculation (1% w/v inoculum). At the second day of culture, oligochitosan was added to the medium. Where indicated, the inhibitors were added to the culture medium 30 min before oligochitosan treatment.

2.3. Cell viability and nuclear morphology

Cell viability was measured using trypan blue staining as described previously (Wang et al., 2007), and cell morphology was investigated using light microscope at 24 h after oligochitosan treatment. For the analysis of nuclear morphology, Tobacco cells with different treatments for 72 h were stained with HO for 30 min and PI for 15 min as described previously (Ma et al., 2010) and visualized using a fluorescence microscope (Nikon, Japan) with an excitation filter of 345 nm and 570 nm.

2.4. Phosphatidylserine externalization assay

Annexin V-PE (Beyotime Institute of Biotechnology) was used as an in vivo staining test for PS externalization. Cells with different treatments for 24 h were harvested via centrifugation (300 g) and washed with PBS for one time, then re-suspended in 195 μl of Annexin binding buffer and stained with 5 μl of Annexin V-PE for 20 min at room temperature in the dark. After being stained, cells were collected via centrifugation, washed twice with PBS, re-suspended in PBS, and stored at 4 °C until further analysis. The fluorescence was observed using a fluorescent microscope (Nikon, Japan) and Microplate (GENIMI EM) with an excitation of 500 nm and an emission of 575 nm.

2.5. TUNEL assay

One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) was used according to the manufacturer's instructions, with minor modifications. Tobacco suspension cells were subjected to 500 μg ml^{-1} oligochitosan and harvested at 72 h. The samples were fixed in 4% formaldehyde for 1 h and incubated in 50 μl TUNEL reaction mixture for 1 h in the dark at 37 °C. The fluorescence was observed using a fluorescent microscope (Nikon, Japan) with an excitation of 488 nm and an emission of 515 nm.

2.6. Measurement of NO, H_2O_2 and Ca^{2+}

NO accumulation was determined using the fluorophore probe DAF-FMDA as described previously (Foresi et al., 2010). Briefly, the tobacco suspension cells were incubated with 5 μM DAF-FMDA for 1 h in the dark at 25 °C on a rotary shaker (120 rpm) and then rinsed twice with fresh suspension buffer to wash off excessive fluorophore probe. Cells were then transferred into 96-well plates (Brand) (200 μl of cells per well), and treated with oligochitosan or inhibitors in the dark. NO production was measured using a 96-well Gemini EM Fluorescence Microplate Reader with 488-nm excitation and 510-nm emission filters. Fluorescence was expressed as relative fluorescence units. H_2O_2 and Ca^{2+} accumulation were determined by corresponding fluorescent probe H_2DCF-DA (2 μM) and Fluo-3AM (5 μM) using the same method with NO except that the Ca^{2+} experiment was operated at 37 °C, the excitation and emission for H_2O_2 and Ca^{2+} is 488-nm and 530-nm. For each treatment, measurements of NO, H_2O_2 and Ca^{2+} production over time were performed on the same batch of cells.

2.7. Total RNA extraction and semi-quantitative RT-PCR

The expression of *hsr203J* in tobacco cells was analyzed by semi-quantitative RT-PCR. Tobacco suspension cells with different treatments for 24 h were collected and stored at −80 °C. Total RNA was isolated from the frozen cells using TRIZOL Reagent according to the manufacturer's protocol, and the RNA quality was validated using electrophoresis and spectrophotometer measurements. RNA isolated from tobacco cells was reverse transcribed to first strand cDNA using oligo(dT) primer in a total volume of 10 μL according to the supplier's instruction (TaKaRa RNA PCR kit VER3.0). Resulting cDNA was amplified by PCR using the following primers, the specific primers for *Hsr203J* were 5′-CGTCTCCGCATCTACTTACC-3′ and 5′-CCTTGTTGCTCCCTACTGG-3′ and primers for actin were 5′-GATGGTGTCAGCCACACTGTC-3′ and