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Oligochitosan induces programmed cell death in tobacco suspension cells

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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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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

5′-ATGCTGCTAGGAGCCAGTGC-3′. The cycle number of the PCRs was adjusted for each gene to obtain visible bands in agarose gels. Amplified PCR products (10 μL) were electrophoresed on a 1% (w/v) agarose gels and monitored using the FR-980 Bio-Electrophoresis Image Analysis System.

2.8. Cytochrome c extractions and western blotting

Tobacco suspension cells with different treatments were used to isolate cytochrome *c* (both from mitochondria and cytosolic fractions) using assay kit (Genmed Scientific Inc., USA). Protein concentration was determined using the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard. For western blotting, 16 μg of cytosolic proteins and 6 μg of mitochondrial proteins were loaded onto a 15% SDS–polyacrylamide gel, separated, and transferred to a nitrocellulose membrane (Schleicher and Schuell). After blocking in PBS-T (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20) with 5% nonfat dry milk 2.5 h at room temperature, the membranes were incubated with anti-cytochrome *c* antibody diluted with PBS-T at 4 °C overnight. After washing with PBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IGg antibody diluted with PBS-T for 1 h at room temperature. The protein bands were analyzed by ImageJ.

2.9. Statistical analyses

All data in this report were obtained from at least two independent experiments with two or three repeats and the mean values ± SD are presented. Statistical differences in mean values at each point were determined with the Duncan's multiple range test.

3. Results

3.1. Oligochitosan triggers PCD

To determine the nature of cell death induced by oligochitosan, some hallmark experiments recognized for plant PCD were analyzed, such as Annexin V-PE, HO/PI, TUNEL, cytochrome *c* release and PCD-related gene *hsr203J* expression assay, focusing our attention on the concentration of 500 μg ml^{-1} oligochitosan.

Firstly, cell death was examined by trypan blue staining after oligochitosan treatment for different times. It was observed that cells were dyed bright in the control sample, while blue in oligochitosan treatment sample with a retraction of the protoplast away from the cell wall (Fig. 1a), suggesting the protoplast condensation. After oligochitosan treatment for 5 d, the percentage of dead cell induced by oligochitosan increased to 93%.

A critical early event in PCD appears to be the acquisition of plasma membrane (PM) changes, including the translocation of phosphatidylserine (PS) from the cytoplasmic face of the plasma membrane to the cell surface. In this study, we used Annexin V-PE (Bidle & Bender, 2008) as a specific probe to detect redistribution of PS. As shown in Fig. 1b, cells without Annexin V-PE were invisible, and only the oligochitosan-treated cells loaded with Annexin V-PE showed dominantly red, suggesting oligochitosan induced the change of PM in tobacco cells. Same results were acquired in fluorescence microplate experiment (Fig. 1b).

To further characterize whether oligochitosan induces PCD in tobacco cells, nuclear morphology was analyzed by fluorescent DNA-binding dye HO and PI, and DNA breaks by endonucleases were performed by TUNEL assay. As shown in Fig. 1c, fluorescence from HO/PI of control cells was faint, whereas cells treated oligochitosan for 72 h showed an enhanced HO and PI fluorescence, meanwhile, some cells' nuclei were notable, due to chromatin condensation; some cells' nuclei (the arrow indicated) were ruptured into several parts, indicating the cells were undergoing PCD. In TUNEL assay, both positive and negative controls were included. The negative control was performed with the cells treated with oligochitosan and obtained by omitting TdT from the TUNEL reaction mixture. In negative controls and normal conditions, cells showed TUNEL-negative signals, while the positive control (pretreated with DNase I) and oligochitosan-treated cells presented TUNEL-positive signals (Fig. 1c). These data illustrated that oligochitosan induced damage to cellular DNA in tobacco suspension cells, which was a PCD process.

The release of cytochrome *c* has been shown a critical role both in mammalian and plant PCD. So cytochrome *c* release from mitochondria was investigated by immunoblot analysis using a monoclonal antibody against cytochrome *c*. Both cytosolic and mitochondrial fractions obtained from tobacco suspension cells were examined. Results of typical immunoblots were shown in Fig. 1d. Cells incubated with oligochitosan for different times led to the release of cytochrome *c* from mitochondria into cytosol with complete release at 8 h. Pretreatment of cyclosporin A, an inhibitor of mitochondrial permeability transition (MPT), led to the inhibition of cytochrome *c* release and cell death (Fig. 1d), implicating cytochrome *c* was involved and played a critical role in oligochitosan-induced cell death.

Hsr203J has been discovered to be a PCD-related gene in plant (Ma et al., 2010; Tronchet, Ranty, Marco, & Roby, 2001). To investigate the nature of the death events induced by oligochitosan, the expression of *hsr203J* gene was analyzed. Compared with untreated cells, the expression of *hsr203J* genes increased remarkably after oligochitosan treatment for 24 h (Fig. 1e). Taken together, above results indicated that oligochitosan triggered PCD in our model which was modulated by cytochrome *c*.

3.2. Effects of oligochitosan on $[Ca^{2+}]_{cyt}$ elevation, NO and H_2O_2 generation

Changes in $[Ca^{2+}]_{cyt}$ was investigated using Ca^{2+} specific fluorescent probe Fluo-3AM, which has been successfully used to monitor cytosolic Ca^{2+} fluxes in plants (Bouranis et al., 2006). A molecular device assay was developed to monitor Ca^{2+} elevation. It was observed that, the resting $[Ca^{2+}]_{cyt}$ was no obvious change during the assay period in control cells. Whereas oligochitosan induced tobacco cells a rapid elevation of $[Ca^{2+}]_{cyt}$ in a dose-depended trend (Fig. 2a-1). Although these data did not reflect the real concentration of Ca^{2+} in cells, it indicated that the $[Ca^{2+}]_{cyt}$ elevation elicited by oligochitosan was most probably transient and reached a peak within 20 min. In Fig. 2a-2, the addition of Ca^{2+} chelator EGTA suppressed the $[Ca^{2+}]_{cyt}$ increase induced by oligochitosan, indicating that $[Ca^{2+}]_{cyt}$ elevations depended on the Ca^{2+} influx from the extracellular medium. The oligochitosan-triggered $[Ca^{2+}]_{cyt}$ elevation was reduced by almost 50% by $LaCl_3$, suggesting plasma membrane Ca^{2+}-permeable channels was involved in this process. The possible involvement of intracellular Ca^{2+} stores in oligochitosan-evoked $[Ca^{2+}]_{cyt}$ changes were determined using ruthenium red (RR), which inhibits Ca^{2+} release via ryanodine receptor (RYR)-like channels when used at low concentration (Sanders, Muir, & Allen, 1995). RR inhibited the increase of $[Ca^{2+}]_{cyt}$ by 30%, suggesting that the oligochitosan-induced $[Ca^{2+}]_{cyt}$ elevation was partly related to intracellular Ca^{2+} release through RYR-like Ca^{2+} permeable channels. The contribution of NO and H_2O_2 to oligochitosan-induced Ca^{2+} influx variation was also analyzed. cPTIO pretreatment reduced oligochitosan-induced $[Ca^{2+}]_{cyt}$ elevation, assuming that NO could regulate the oligochitosan-triggered Ca^{2+} influx. CAT and Vc had no effect on Ca^{2+} flux, indicating H_2O_2 was downstream of Ca^{2+} flux.

NO production in tobacco cells was determined using the specific probe DAF-FMDA as described previously (Foresi et al.,

2010). It was observed that oligochitosan-treated cells led to an increase in fluorescence indicative of NO production (Fig. 2b-1), which reached a plateau at 50 min after oligochitosan treatment. Simultaneously, the green fluorescence which was presented NO occurred in the majority of cells after oligochitosan treatment, while in control cells, it was negligible. Results also showed that oligochitosan-induce NO production was in a dose-depended trend (Fig. 2b-2). The addition of NO scavenger cPTIO completely

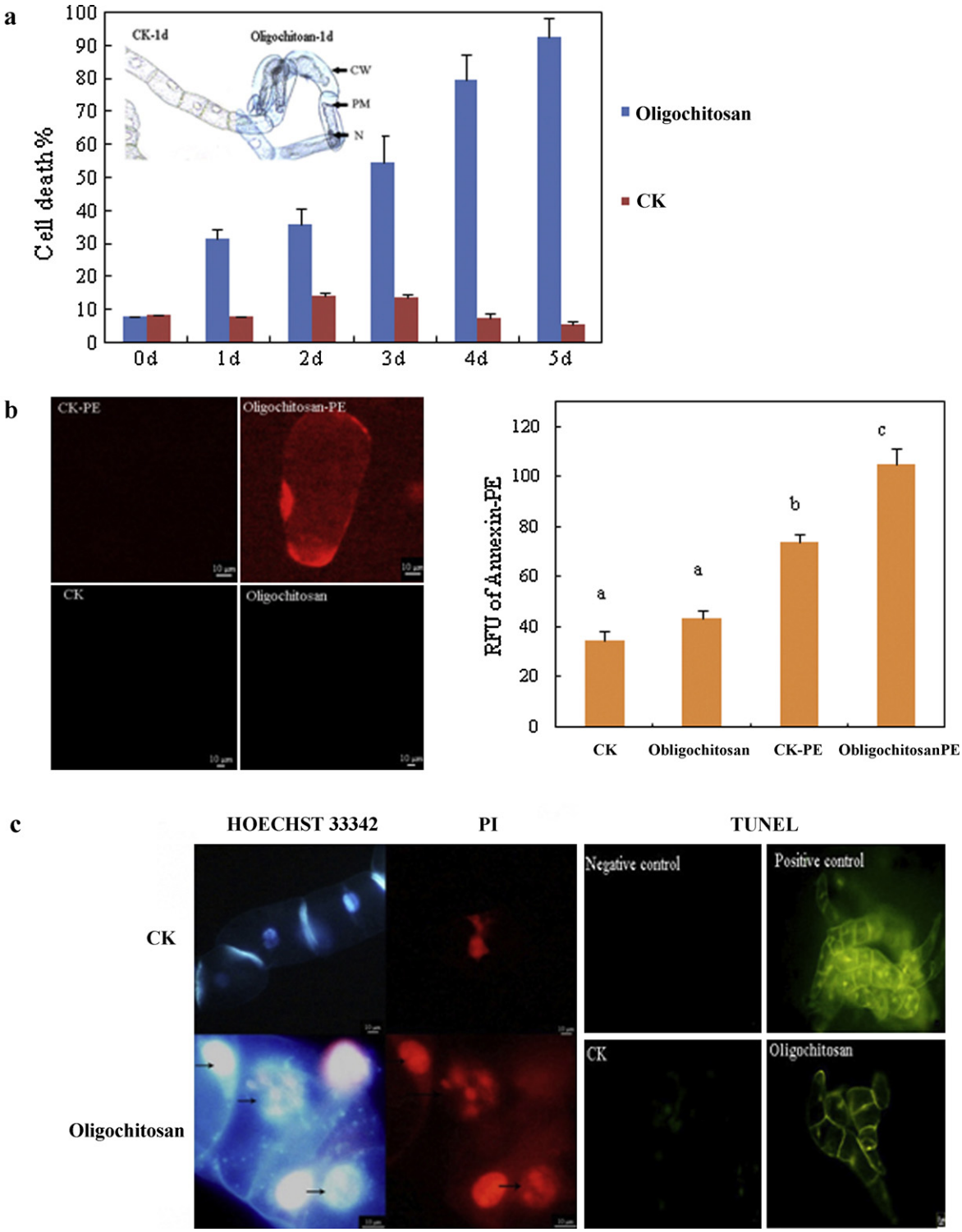

Fig. 1. Oligochitosan triggers PCD in tobacco suspension cells. (a) Effects of oligochitosan on viability of tobacco cells for different times using trypan blue staining. CK, control; CW, cell wall; PM, plasma membrane; N, nucleus. (b) Tobacco cells were treated with oligochitosan 24 h for Annexin V-PE assay. *Left column* fluorescence images, *right column* the relative fluorescence unit of Annexin V-PE. CK and oligochitosan: without fluorescence probe; CK-PE and oligochitosan-PE: with fluorescence probe. (c) Tobacco cells after treatment with oligochitosan 72 h were co-stained with Hoechst 33342 and propidium iodide or for TUNEL assay, and examined by fluorescence microscope. Arrows indicate the cells undergoing PCD with bright blue and red fluorescence. Bar 10 μm. (d) The release of cytochrome *c* and its function in oligochitosan-induced cell death. Cytochrome *c* in each fraction was determined by western blot analysis using anti-cytochrome *c* antibody. Cell death was examined by trypan blue staining. (e) Expression of *hsr203J* in tobacco cells treated with oligochitosan for 24 h by semi-quantitative RT-PCR. The expression level of actin was used as a positive internal control.

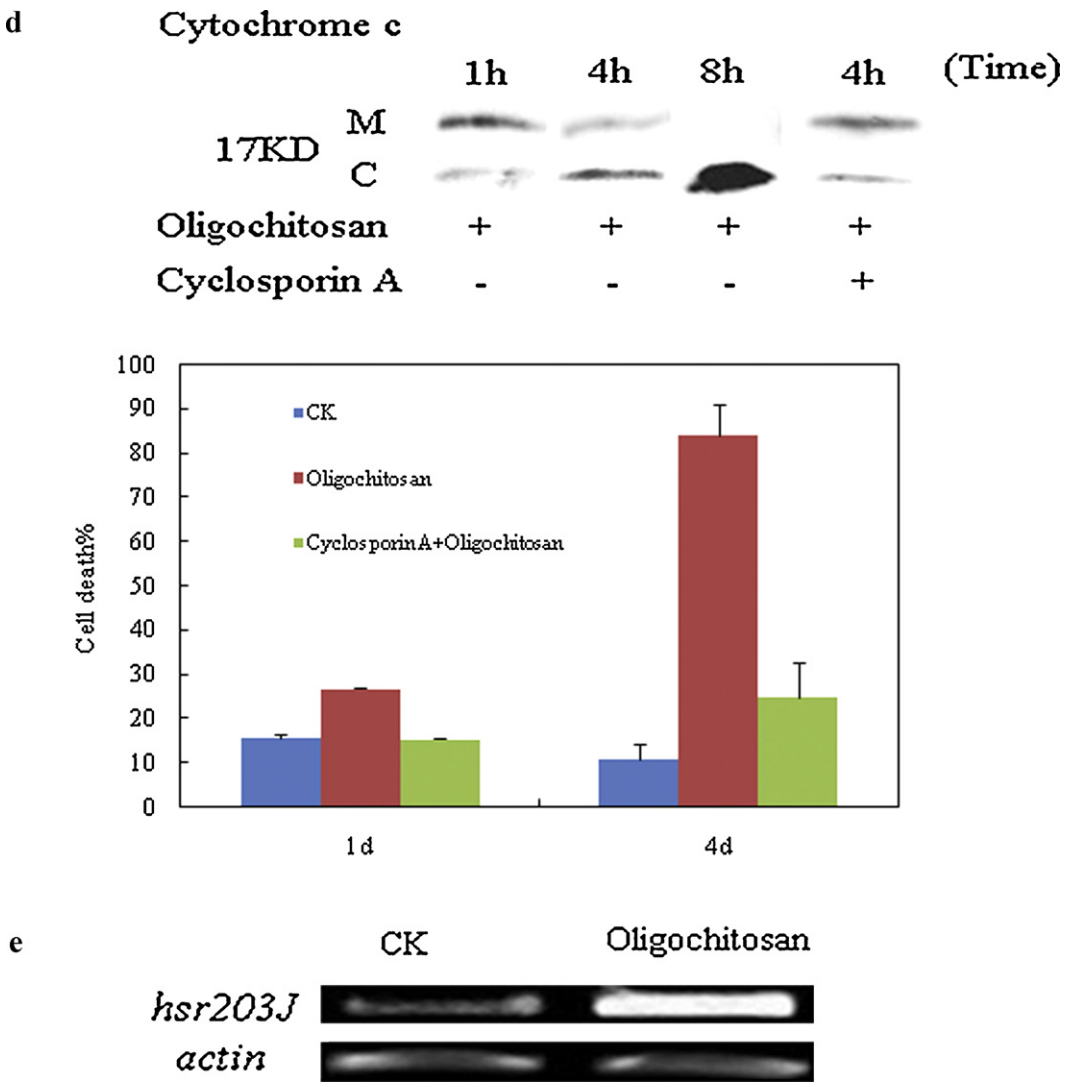

Fig. 1. (*Continued*).

suppressed oligochitosan-induced NO burst, confirming that the increase of fluorescence was due to NO production (Fig. 2b-3). NO can be synthesized in plant by different routes, both enzymatically and nonenzymatically (Gupta, Fernie, Kaiser, & Dongen, 2011). Most work focuses on nitric oxide synthase (NOS)-like enzyme (Foresi et al., 2010) and nitrate reductase (NR) (Yamasaki & Sakihama, 2000). To test whether NOS-like enzyme and/or NR are involved in oligochitosan-induced NO generation, we assessed the effects of NOS inhibitor L-NAME and NR inhibitor Tu and NaN_3. NO production can be inhibited by L-NAME (not by Tu and NaN_3), suggesting that a NOS-Like enzyme may be responsible for NO synthesis in tobacco cells. Treatment cells with CAT, Vc, EGTA, $LaCl_3$ and RR all reduced NO generation, indicating NO production was regulated by H_2O_2 and Ca^{2+}.

H_2O_2 production was measured using another fluorescent probe H_2DCF-DA as the same method with NO. Results showed that oligochitosan could induce H_2O_2 production (oligochitosan-treated cells showed green fluorescence), which reached peak value at 16 min after oligochitosan treatment (Fig. 2c-1); this H_2O_2 increments induced by oligochitosan were in a dose-depended trend (Fig. 2c-2); experiments showed that H_2O_2 generation was reduced by CAT and Vc. cPTIO, L-NAME, EGTA, $LaCl_3$ and RR all reduced H_2O_2 production (Fig. 2c-3), suggesting H_2O_2 production was also regulated by NO and Ca^{2+}.

3.3. Effects of Ca^{2+}, NO and H_2O_2 on oligochitosan-induced PCD

In order to test the role of Ca^{2+}, NO and H_2O_2 in oligochitosan-induced PCD, we firstly measured the effects of those three signals-related inhibitors on cell death induced by oligochitosan. Pretreatment with three signals-related inhibitors all separately reduced counterpart's accumulation induced by oligochitosan (Fig. 2a-2, b-3, and c-3), but these inhibitors had no obvious effect on the cell death induced by oligochitosan in Fig. 1d (Fig. 3a). After 3 d, the cell death induced by oligochitosan was accelerated by pretreatment with Ca^{2+}- and NO-related inhibitors, suggesting Ca^{2+} and NO may prevent oligochitosan-induced cell death. Scavenging H_2O_2 with CAT or Vc had no significant effect on oligochitosan-induced cell death both at 1d and 3d (Fig. 3a), indicating H_2O_2 may not take part in oligochitosan-induced cell death. Similar results were obtained in HO/PI assay (data not shown). These results suggested that NO and Ca^{2+} may play a negative role in oligochitosan-induced PCD.

Next, cytochrome *c* release was identified in tobacco suspension cells by pretreating with different inhibitors. In Fig. 3b, oligochitosan treatment led to the release of cytochrome *c* from mitochondria into cytosol, clearing NO with cPTIO, or inhibiting $[Ca^{2+}]_{cyt}$ elevation with $LaCl_3$ and RR resulted in accelerating the release of cytochrome *c*, which may be the reason

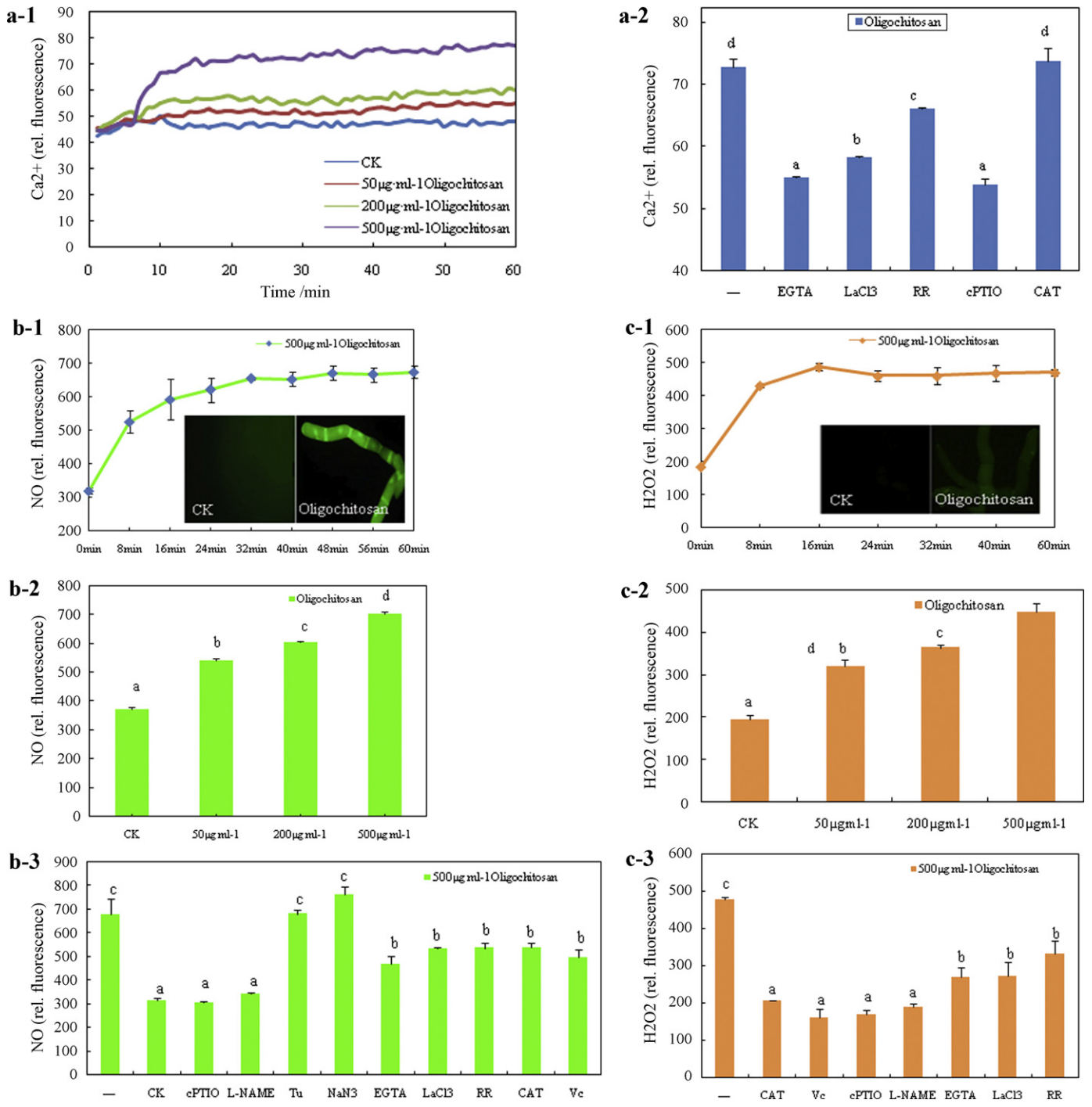

Fig. 2. Oligochitosan induces $[Ca^{2+}]_{cyt}$ elevation, NO and H_2O_2 generation. (a) Oligochitosan induces $[Ca^{2+}]_{cyt}$ elevation in tobacco cells. Effects of different concentrations of oligochitosan on $[Ca^{2+}]_{cyt}$ variation during 1 h of treatment (a-1). And effects of Ca^{2+}, NO and H_2O_2-related inhibitors on oligochitosan-induced $[Ca^{2+}]_{cyt}$ elevation at 30 min (a-2). (b) Oligochitosan induces NO accumulation in tobacco suspension cells. (b-1) Time course of oligochitosan-induced NO accumulation during 1 h of treatment. The values are obtained by subtraction of NO accumulation in control cells from NO accumulation in cells treated by oligochitosan. (b-2) Effects of different concentrations of oligochitosan on NO production at 30 min. (b-3) Effects of mammalian NOS inhibitors, NR inhibitor, Ca^{2+} and H_2O_2 inhibitors on oligochitosan-induced NO burst in tobacco suspension cells at 30 min. (c) Oligochitosan induces H_2O_2 accumulation in tobacco cells. (c-1) Time course of oligochitosan-induced H_2O_2 accumulation during 1 h of treatment. The values are obtained by subtraction of H_2O_2 accumulation in control cells from H_2O_2 accumulation in cells treated by oligochitosan. (c-2) Effects of different concentrations of oligochitosan on H_2O_2 production at 15 min. (c-3) Effects of H_2O_2, NO and Ca^{2+}-related inhibitors on oligochitosan-induced H_2O_2 burst in tobacco suspension cells at 15 min.

of why those inhibitors accelerated oligochitosan-induced cell death (Fig. 3a). CAT or Vc still had no significant effect on oligochitosan-induced cytochrome *c* release. However, in the PCD-related *hsr203J* gene expression assay, these three signals-related inhibitors all had obvious and different effects on oligochitosan-induced *hsr203J* gene expression (Fig. 3c), indicating that these three signals all participated in oligochitosan-induced PCD.

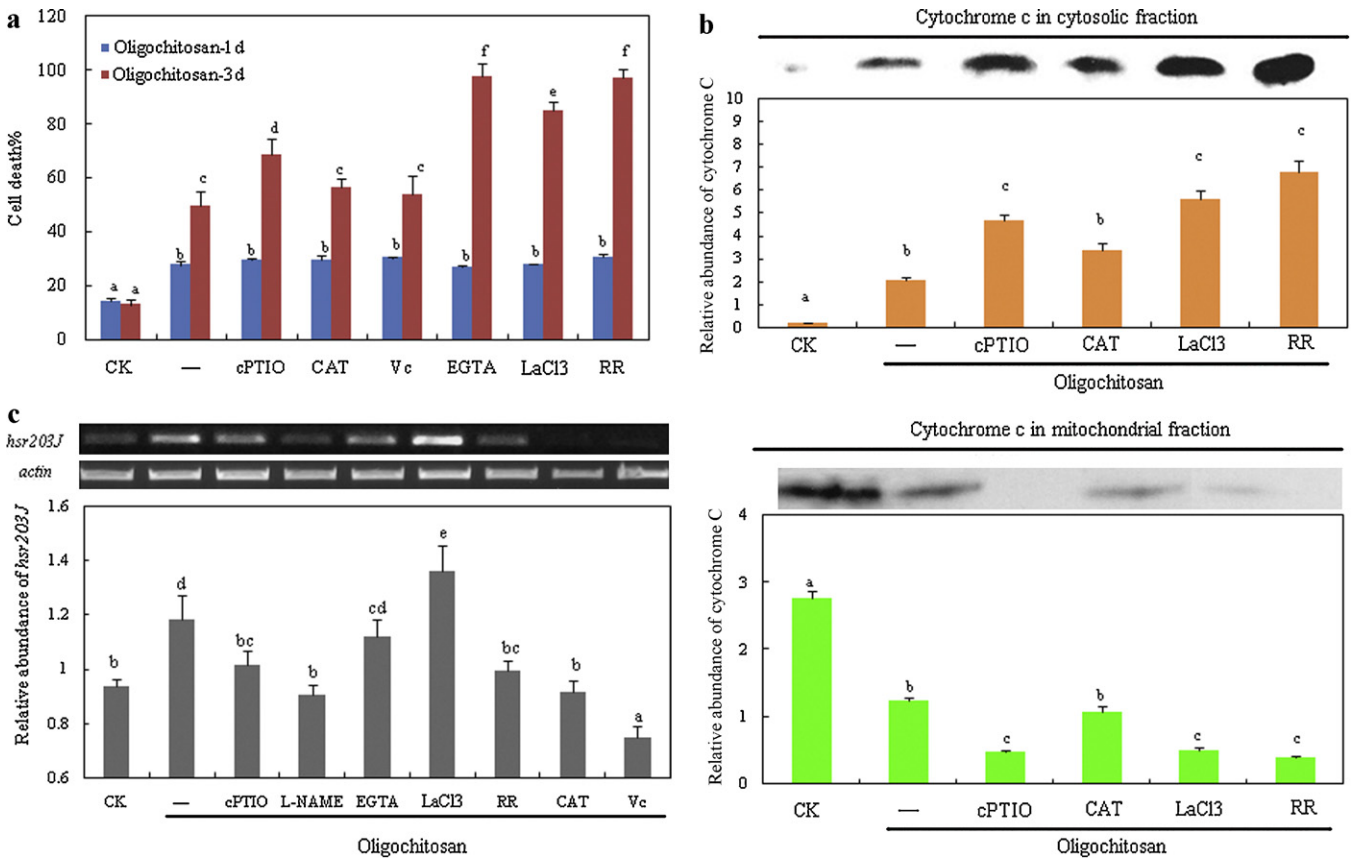

Fig. 3. The role of Ca^{2+}, NO and H_2O_2 in oligochitosan-induced PCD. (a) Effects of different inhibitors on oligochitosan-induced cell death. Cell viability was examined by trypan blue staining at 1 d and 3 d after treatment. (b) Effects of different inhibitors on cytochrome *c* release. Both cytosolic and mitochondrial fractions were determined by western blot analysis using anti-cytochrome *c* antibody. Histograms are the quantitative result of western blot. (c) Effects of different inhibitors on expression of *hsr203J*. mRNA was extracted and analyzed by RT-PCR and actin gene was used as internal control. Histograms are the quantitative result of RT-PCR.

4. Discussion

In this study, we confirmed that oligochitosan induced PCD by Annexin V-PE, HO/PI, TUNEL assay and the expression of PCD-related gene *hsr203J*. At the same time, we found cytochrome *c* was involved in and played a key role in this process for the first time. Then, three signal moleculars about Ca^{2+}, NO and H_2O_2 were investigated, and results showed that oligochitosan could induce these three signals changes. Further investigation showed that these three signals were involved in oligochitosan-induced PCD, and Ca^{2+} and NO may regulate cytochrome *c* release in turn to modulate oligochitosan-triggered PCD.

Several papers reported that some species of plant suspension cells and leaves in response to chitosan led to PCD. Zuppini reported that low concentration of chitosan was able to induce PCD in soybean suspension and Ca^{2+} flux was involved in this process (Zuppini et al., 2003). Iriti et al. (2006) reported that tobacco plants treated with 0.1% chitosan induced breaks of genomic DNA in a distinct DNA-laddering pattern. In this study, the cell death induced by oligochitosan shared some hallmark of morphological PCD with chitosan such as cytoplasmic shrinkage, chromatin condensation, besides oligochitosan treatment led to upregulation of a PCD-related gene *hsr203J* (Tronchet et al., 2001) and induction of the release of cytochrome *c* (Fig. 1d and e). In plant cell culture models, it has been shown that cytochrome *c* is released from mitochondria following death stimuli such as heat shock or harpin treatment (Krause & Durner, 2004; Vacca et al., 2006). In our study, oligochitosan-induced the release of cytochrome *c* and cell death, both of which was blocked by cyclosporin A, an inhibitor of MPT (Fig. 1d), suggesting cytochrome *c* released through MPT hole played a significant role in oligochitosan-induced PCD. Similar phenomenon was also found in *Arabidopsis* protoplasts when treated with protophorphyrin IX (Yao, Eisfelder, Marvin, & Greenberg, 2004).

Ca^{2+} played an important role in plant PCD. We have introduced above that chitosan induced Ca^{2+}-mediated PCD in soybean cells (Zuppini et al., 2003), in their research, elevation of Ca^{2+} induced by chitosan was prevented by Ca^{2+} chelator EGTA. In our model, oligochitosan induced a fast elevation of $[Ca^{2+}]_{cyt}$ in a dose-dependent trend (Fig. 2a-1), which was opposite to Ca^{2+} change trend induced by chitosan in soybean cells. This may be due to the difference of degree of acetylation or DP between chitosan and oligochitosan. Next, we learned that the $[Ca^{2+}]_{cyt}$ elevation induced by oligochitosan can be via the activation of extracellular Ca^{2+}, plasma membrane and intracellular Ca^{2+}-permeable channels related to RYR (Fig. 2a-2). Moreover, we found NO took part in regulating $[Ca^{2+}]_{cyt}$ elevation induced by oligochitosan, while H_2O_2 was downstream of Ca^{2+} influx. In the cell death experiment, EGTA, $LaCl_3$ and RR all accelerated cell death triggered by oligochitosan (Fig. 3a), suggesting that Ca^{2+} may prevent oligochitosan-induced PCD. This phenomenon can be explained by those inhibitors all accelerated cytochrome *c* release from mitochondria into cytosol (Fig. 3b). However, EGTA and RR reduced the expression of *hsr203J* induced by oligochitosan, while $LaCl_3$ operated the reverse function (Fig. 3c), indicating different origins of Ca^{2+} influx may regulate oligochitosan-induce PCD in different pathways.

NO and H_2O_2 are considered to be early events in PCD. Strong evidence indicates that NO and H_2O_2 play important roles in plant

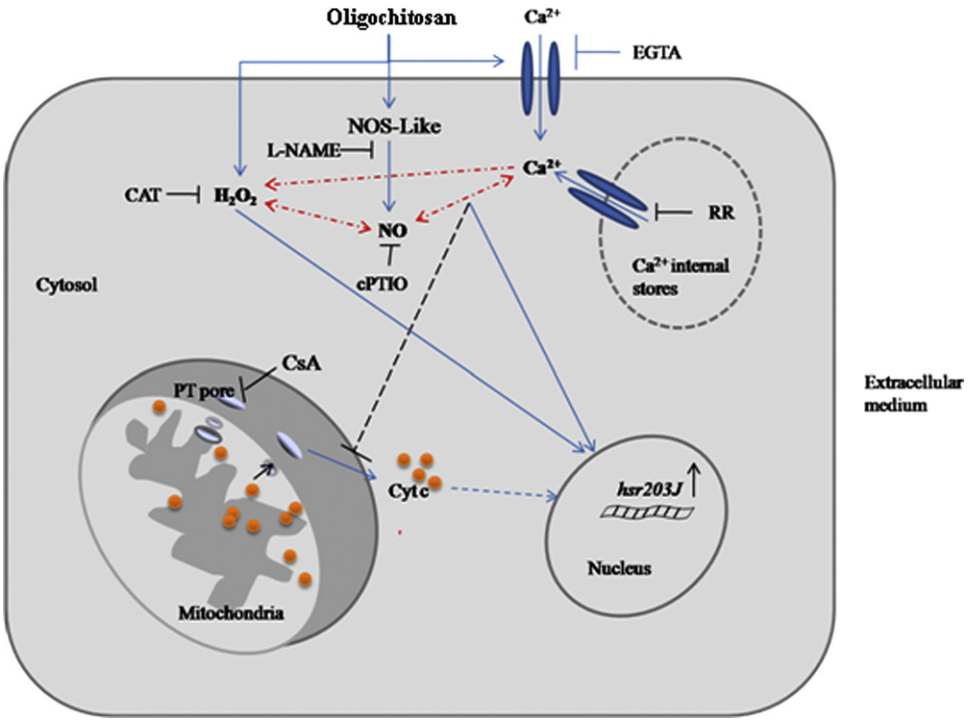

Fig. 4. Schematic model of signaling pathways activated in oligochitosan-triggered PCD in tobacco suspensions cells. Oligochitosan activates $[Ca^{2+}]_{cyt}$ elevation both from the external medium and internal stores, simultaneously, oligochitosan induces NO and H_2O_2 generation. NO and Ca^{2+} are interacted with each other, NO and H_2O_2 are also interacted with each other, but H_2O_2 is downstream of Ca^{2+} influx. Oligochitosan-treated cells result in cytochrome *c* release which mediated oligochitosan-induced PCD. NO and Ca^{2+} participated in regulating the expression of PCD-related gene *hsr203J*, inhibiting cytochrome *c* release to prevent oligochitosan-induced PCD, while H_2O_2 could regulate *hsr203J* gene expression, but has no effect on oligochitosan-induced PCD.

PCD. Each of them alone is able to induced cell death, and simultaneous presence of NO and H_2O_2 also reduced cell viability (De Pinto et al., 2002; Delledonne et al., 1998). Lots of reports show that elicitors induce both of NO and H_2O_2 in plant PCD (De Michele et al., 2009; Vacca et al., 2004). In the present study, we showed that oligochitosan induced a fast and transient NO production (Fig. 2b), probably through activating a NOS-like enzyme. Accompany by NO generation, oligochitosan elicited large amount of H_2O_2 synthesis (Fig. 2c). Interestingly, each of the two molecules was able to inhibit the production of the other, and this result is the same with previous report (Zhao, Fujita, & Sakai, 2007). How they mutually regulated each other is still unexplained in our model.

Scavenging NO accelerated cell death and cytochrome *c* release induced by oligochitosan (Fig. 3a and b), suggesting NO may play the same role with Ca^{2+} in oligochitosan-induced PCD. Beligni, Fath, Bethke, Lamattina, and Jones (2002) have reported that NO can act as an antioxidant and delayed PCD in barley aleurone layers. This result was opposite to the report by Zottini et al. (2002), who reported that the addition of NO donor SNP induced cytochrome *c* release. The reason may be because of the different concentrations and lasting time of NO as previously reported (Chung et al., 2001).

Scavenging H_2O_2 appeared to have no effect on oligochitosan-induced cell death and cytochrome *c* release (Fig. 3a and b), but CAT or Vc severely suppressed the expression of *hsr203J* induced by oligochitosan (Fig. 3c), illuminating that H_2O_2 was involved in oligochitosan-induced PCD. Reports indicated that the low concentration of H_2O_2 was not enough to induce cell death (Houot et al., 2001). Lots of others papers reported that the release of cytochrome *c* was dependent of ROS and CAT or SOD treatment led to protect cell death (Gao, Xing, Li, & Zhang, 2008; Vacca et al., 2006). However, in our system, H_2O_2 seems to act different roles and its function should be further studied. Simultaneously scavenging NO and H_2O_2 also failed to prevent oligochitosan-induced cell death (data not shown).

Taken together, the results presented here enable us to describe a part of the signaling network induced by oligochitosan in tobacco suspension cells (Fig. 4). According to these data, we could postulate that, in our model, oligochitosan-induced PCD in tobacco suspension cells is mediated by cytochrome *c* which may be regulated by NO and Ca^{2+} pathway. In the future study, we will try to investigate the role of cascades pathway and H_2O_2 in oligochitosan-induced PCD. It will be helpful to understand the oligochitosan-induced PCD and amplify the oligochitosan signaling.

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