Plant Growth Regulation (2005) 45:243–250
DOI 10.1007/s10725-005-5163-5

Salt stress-induced programmed cell death via Ca^{2+}-mediated mitochondrial permeability transition in tobacco protoplasts

Jiusheng Lin[1,2,†,*], Yuan Wang[2,†] and Genxuan Wang[1]
[1]*College of Life Sciences, Zhejiang University, Hanzhou, Zhejiang Province, 310029, China;* [2]*Key Laboratory of Arid Agroecology, Ministry of Education, Lanzhou University, Lanzhou, Gansu Province, 730000, China;* **Author for correspondence, Current address: Department of Biochemistry & Plant Science Initiative, University of Nebraska-Lincoln, Lincoln, NE, 68588, USA (e-mail: linjiusheng@hotmail.com; phone: +1-402-310-9853)*

Received 8 March 2005; accepted 7 April 2005

Key words: $[Ca^{2+}]_{cyt}$, Mitochondria, Programmed cell death, Salt stress, Tobacco

Abstract

The change in cytosolic free concentration of calcium ($[Ca^{2+}]_{cyt}$) plays a key role in regulating apoptosis in animal cells. In our experiment, we tried to investigate the function of Ca^{2+} in programmed cell death (PCD) in tobacco (*Nicotiana tobacum*, cultivar BY-2) protoplasts induced by salt stress. An obvious increase in $[Ca^{2+}]_{cyt}$ was observed a few minutes after treatment and the onset of a decrease in mitochondrial membrane potential ($\Delta\Psi_m$) was also observed before the appearance of PCD, pre-treatment of protoplasts with EGTA or $LaCl_3$ effectively retarded the increase in $[Ca^{2+}]_{cyt}$, which was concomitant with the decrease in the percentage of cell death and higher $\Delta\Psi_m$, pre-treatment with cyclosporine A (CsA) also effectively retarded the increase in $[Ca^{2+}]_{cyt}$, the decrease in $\Delta\Psi_m$ and the onset of PCD. All these results suggest that Ca^{2+} is a necessary element in regulating PCD and the increase in $[Ca^{2+}]_{cyt}$ and the opening of mitochondrial permeability transition pore (MPTP) could promote each other in regulating PCD in tobacco protoplasts induced by salt stress.

Abbreviations: AIF – apoptosis inducing factor; ANT – adenine nucleotide translocator; $[Ca^{2+}]_{cyt}$ – cytosolic free calcium concentration; CsA – cyclosporin A; DCFH-DA – 2,7-dichlorohydrofluoroscein diacetate; EB – ethidium bromide; HR – hypersensitive response; MPTP – mitochondrial permeability transition pore; PCD – programmed cell death; PI – propidium iodide; Rh123 – Rhodamine123; ROS – reactive oxygen species; $\Delta\Psi_m$ – mitochondrial membrane potential

Introduction

Programmed cell death (PCD) or apoptosis is a genetically defined cellular suicide process by which extraneous or damaged cells are eliminated from an organism (Steller 1995). In plants PCD can be induced by a variety of stimuli including developmental signals, environmental cues and the restriction of pathogen infection during the hypersensitive response (HR) (Greenberg et al. 1994; Rao and Davis 1999; Huh et al. 2002).

By regulating diverse aspects of cellular signal transduction in plants and animals, Ca^{2+} plays

[†]These authors contributed equally for this work.

an important role as a second messenger. Changes in cytosolic free concentration of calcium ($[Ca^{2+}]_{cyt}$) are necessary for adaptative responses to various stress conditions in plant cells (Knight et al. 1996; Kawano et al. 1998; Allen et al. 2001). In recent years, several lines of evidence have suggest that a change in $[Ca^{2+}]_{cyt}$ might be associated with plant PCD. Okadaic acid, a protein phosphatase inhibitor, blocks both calcium changes and gibberellin-induced programmed cell death in wheat aleurone (Kuo et al. 1996); PPF1, which encodes a putative calcium ion carrier, delays cytosolic Ca^{2+} elevation, and inhibits programmed cell death in apical meristems of both G2 pea and *Arabidopsis* plants (Li et al. 2004). The plant hypersensitive response is triggered by a signaling network. An increase in the cytosolic Ca^{2+} concentration, which occurs within seconds after elicitation, appears to be a master regulator that is required for many subsequent signaling (Scheel 1998), Ca^{2+} is also important for inducing defense gene activation (Ishihara et al. 1996; Levine et al. 1996), inhibition of $[Ca^{2+}]_{cyt}$ elevation can effectively retard the onset of HR (Xu and Heath 1998). Ca^{2+} is also involved in the cell death in pollen due to self-incompatibility in *Papaver* (Thomas and Franklin-Tong 2004).

In recent years, increasing evidence exists for the involvement of mitochondria in stress sensing and in the cell death pathway. Using plant cells, Yu et al. (2002) provided evidence supporting the involvement of mitochondria in PCD. There is also some evidence pointing to the importance of mitochondria in the expression of HR-associated PCD in plants (Lacomme and Santa Cruz 1999; Xie and Chen 2000).

Mitochondrial permeability transition pore (MPTP) protects the mitochondria from the loss of electrochemical potential by preventing nonspecific transfer of solutes less than 1500 D. In animals, functional and genetic experiments indicate that loss of mitochondrial membrane potential ($\Delta\Psi_m$) by MPTP and subsequent apoptosis cannot be dissociated (Kroemer et al. 1997). Consequently, cyclosporin A (CsA) blocks the opening of MPTP and prevents cell death (Scorrano et al. 2003). Existence of MPTP in plant mitochondria has been suggested by several reports (Arpagaus et al. 2002; Curtis and Wolpert 2002). MPTP in plant mitochondria has been shown to be sensitive to CsA (Arpagaus et al. 2002; Yu et al. 2002).

In animals, both raised $[Ca^{2+}]_{cyt}$ and the open of MPTP have been implicated as early events in apoptosis. Ca^{2+} overload can induce mitochondrial dysfunction and loss of ATP production (Budd and Nicholls 1996) and that is sensitive to CsA, thereby indicating that MPTP may be involved in this pathway (White and Reynolds 1996). Other evidence has also indicated that increases in $[Ca^{2+}]_{cyt}$ may induce opening of MPTP and loss of $\Delta\Psi_m$ (Vieira and Kroemer 1999). This can cause leakage of apoptogenic factors from the inner mitochondrial membrane space into the cytoplasm. Two such factors, cytochrome *c* and apoptosis inducing factor (AIF) are known to initiate cascade of proteolytic activity that ultimately leads to nuclear damage (DNA fragmentation) and cell death in animals (Jones 2000; Saviani et al. 2002). It is unclear whether a similar pathway also exists in plant cells. Mastoparan has been shown to stimulate increases in $[Ca^{2+}]_{cyt}$ in *Papaver* pollen leading to release of cytotochrome *c* into the cytosol (Thomas and Franklin-Tong 2004).

In animals mitochondria has been recognized as a key player of Ca^{2+} homeostasis and cellular Ca^{2+} signaling. Mitochondria are involved in uptake and release of Ca^{2+} thereby influencing local Ca^{2+} signals (Smaili et al. 2000). Inhibition of mitochondrial activity was found to drastically alter methacholine-evoked Ca^{2+} waves in glial cells (Simpson and Russell 1998). In similar studies it was found that both location of mitochondria and their functional integrity were important in Ca^{2+} signaling (Lawrie et al. 1996). However, there is no evidence for the occurrence of a similar mechanism in plant cells.

The main signaling pathways of PCD have been described primarily in animal cells, while recent studies have shown several morphological and biochemical similarities between animal and plant PCD (Danon et al. 2000), which suggests that PCD in plants and animals may be based on a common cell death. In our study, we investigated whether Ca^{2+} and MPTP participate in the regulation of the tobacco PCD induced by salt stress.

Materials and methods

Plant material and protoplasts

Seeds of tobacco (*Nicotiana tobacum*, cultivar BY-2) were sterilized with 70% ethanol for 2 min, followed by incubation for 5 min in sodium hypochloride containing 0.1% Triton X-100. They were then washed five times with sterile water and dried under sterile conditions. Sterilized seeds were germinated on solid MS medium (Murashige and Skoog 1962) with 2% sucrose and cultured at 24 °C in a 16/8 h photoperiod at 100 μmol photons m^{-2} s^{-1} in a phytotron. Shoot tips were excised and subcultured every 4–5 weeks. For protoplast isolation only well-rooted plants were used that had undergone at least three subcultures *in vitro* and then grown for 5–6 weeks. Protoplasts were isolated according to Ahad et al. (2003). Fully expanded leaves were placed upside down in a sterile petri-dish on 10 ml of CNT medium [KAO medium (Kao and Michayluk 1975) with 1% cellulase (Onozuka-R, Yakult, Japan), 1% macerozyme (Serva, Heidelberg, Germany) and 0.4 M sucrose], the midrib was removed and the leaf blade cut into pieces of 0.5–1 cm^2 in the liquid medium. Then the leaves were incubated overnight at 26 °C in the dark. Subsequently, this mixture was passed through a sterile stainless steel mesh sieve (mesh size 100 μm). This filtered protoplast suspension was very carefully overlaid with 1 ml PNT medium (KAO medium including 0.4 M glucose) on top of the suspension and then centrifuged for 5 min at low speed (1000 rpm, 25 °C). Intact protoplasts were collected from the interphase and transferred into a new tube. Ten microliters of fresh PNT medium were added and mixed gently followed by a second centrifugation under the same conditions. This washing step was repeated and a small aliquot of the washed protoplasts was used for the estimation of cell density in a hematocytometer. The supernatant was carefully removed and the isolated protoplasts resuspended in PNT medium to a density of 2×10^5 protoplast ml^{-1}.

DNA extraction and analysis

DNA was extracted from protoplasts after various treatments. Briefly, protoplasts were lysed with a lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.4% SDS, 0.2 g L^{-1} protease K) for 30 min at 37 °C. Purified DNA (1–3 μg) was electrophoresed in 1.8% agarose gel, and DNA-laddering was visualized by staining with 0.5 μg ml^{-1} ethidium bromide (EB) and UVB illumination.

The quantitative assessment of programmed cell death

Flow cytometric analysis was performed to identify sub-G_1 cells/programmed death cells and to measure the percentage of sub-G_1 cells after PI staining. Briefly, protoplasts were suspended in PBS containing 50 μg PI ml^{-1} plus 0.1% Triton X-100 (v/v) and 60 μg ml^{-1} RNase (w/v) and the protoplasts were analyzed by the use of a flow cytometer (ESP Elite, Coulter). Cells undergoing PCD appeared in the cell cycle distribution as cells with a DNA content of less than that of G_1 cells and were estimated with Listmode software (Wang et al. 2001).

Assessment of $\Delta\Psi_m$

$\Delta\Psi$m was quantitatively measured by flow cytometry as described (Chen 1988; Bergounioux et al. 1992). Protoplasts were incubated with 10 μM Rhodamine123 (Rh123), a specific mitochondrial membrane potential-dependent fluorescence dye (Yamamoto et al. 2002), for 30 min at 27 °C and washed three times in PBS to remove free Rh123 from the medium, suspended in a total volume of 350 μl and analyzed using a flow cytometer (EPICS XL, U.S COULTER; excitation 488 nm, emission 525 nm). Rh123 exhibits fluorescence emission depending on $\Delta\Psi_m$. Therefore retention of the dye in the protoplasts can be monitored through the intensity of fluorescence.

Detection of $[Ca^{2+}]_i$ *by laser confocal microscopy*

The time-lapse change of $[Ca^{2+}]_{cyt}$ in the apoptosis was detected with Ca^{2+} probe Fluo-3 AM. The dye was loaded into tobacco protoplasts at a final concentration of 10 μM at 25 °C for 30 min in the dark, as described previously (Subbaiah et al. 1998). After incubation, the protoplasts were washed twice with PBS, pH 7.4 to remove free Fluo-3 AM from the medium, analyzed using a laser confocal microscopy. Fluorescence images

were collected with the laser confocal system mounted on an inverted microscope and equipped with an argon–krypton laser. Fluorescence of fluo-3 AM, excited at 488 nm, was collected through a 515-nm longpass barrier filter. $[Ca^{2+}]_{cyt}$ was measured at desired times after treatment.

Results

The degradation of genomic DNA and the formation of a DNA ladder

No genomic DNA degradation was observed at 0 h time point. The degradation of DNA appeared after incubation for 4 h; the 'DNA ladder' could also be seen after incubation for 6 or 8 h (Figure 1). DNA ladder has been described as an important biochemical marker of PCD (Ryerson and Heath 1996; Liljeroth and Bryngelsson 2001). So the result indicates that NaCl treatment could effectively induce PCD.

Effect of $LaCl_3$ and EGTA on PCD in tobacco protoplasts

Treated and untreated protoplasts were studied by flow cytometry light scatter analysis. The cellular DNA content was determined by PI labeling. The percentage of cellular fragments with low DNA content, which is consistent with a PCD process, was calculated. Control cultures contained a low percentage of cellular fragments with low content of DNA. No difference was observed in the percentage of PCD between protoplasts pre-treated by $LaCl_3$ or EGTA and control without stress. The percentage of PCD was not significantly different from control when protoplasts were treated by salt stress for 2 h. A time-dependent increase in percentage of sub-G1 population appeared after treatment for 4 h. While the protoplasts that were pre-treated by EGTA or $LaCl_3$, the percentage of PCD did not increase significantly even when protoplasts were treated by salt for 4 h. A significant increase was observed after stress treatment for 6 h and also showed a time-dependent increase with prolonged stress (Figure 2).

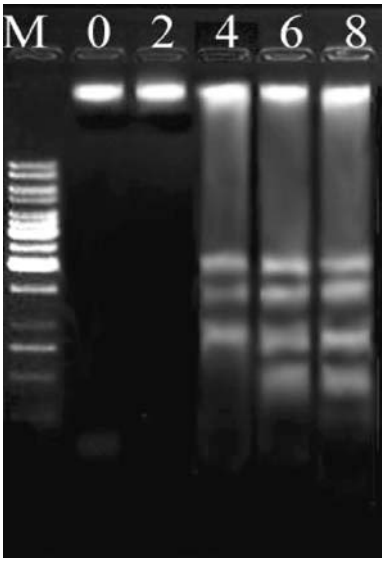

Figure 1. Agarose gel analysis of tobacco protoplasts for DNA fragmentation. NaCl was added into suspended tobacco protoplasts at 200 mM final concentration. Samples were taken from 0 to 8 h at 2-h intervals. The number on the top is the number of hours after NaCl stress; M, 1 kb DNA marker.

Effect of EGTA or $LaCl_3$ on $[Ca^{2+}]_{cyt}$ in tobacco protoplasts

We measured $[Ca^{2+}]_{cyt}$ changes in tobacco protoplasts after NaCl treatment by Ca^{2+} probe Fluo-3 AM using a laser confocal microscopy. Figure 3 illustrated that protoplasts treated with NaCl generated an initial rapid increase $[Ca^{2+}]_{cyt}$

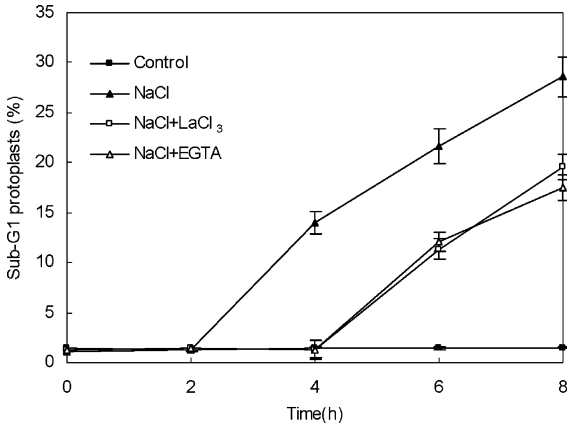

Figure 2. Effect of $LaCl_3$ or EGTA on PCD in tobacco protoplasts induced by NaCl. NaCl was added into suspended tobacco protoplasts at 200 mM final concentration. Samples were taken from 0 to 8 h at 2-h intervals. Two millimolar EGTA or 2 mM $LaCl_3$, were added into the system, respectively, 0.5 h before treated by NaCl. The data are means ± SE from $n = 6$ from three experiments.

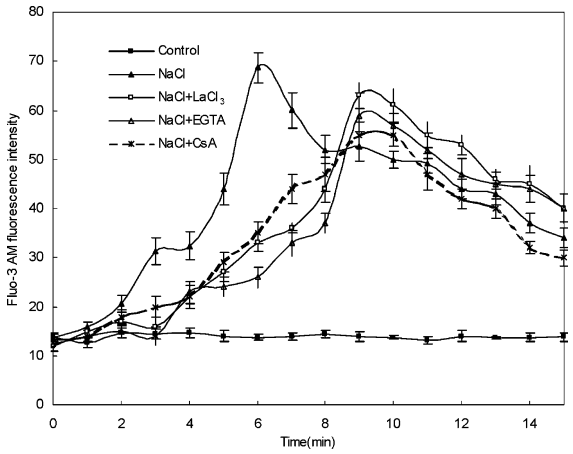

Figure 3. Effect of $LaCl_3$, EGTA or CsA on $[Ca^{2+}]_{cyt}$ in tobacco protoplasts induced by NaCl. NaCl was added into suspended tobacco protoplasts at 200 mM final concentration. Samples were taken from 0 to 15 min at 1 min intervals. Two millimolar EGTA, 2 mM $LaCl_3$ or 50 μM of CsA were added into the system, respectively, 0.5 h before treated by NaCl. The data are means ± SE from $n = 64$ from three experiments.

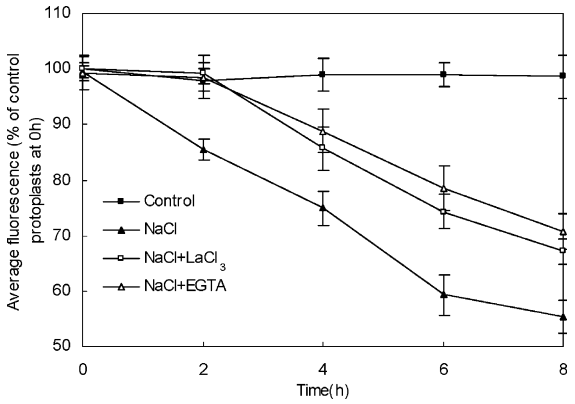

Figure 4. Effect of $LaCl_3$ or EGTA on the $\Delta\Psi_m$ in tobacco protoplasts induced by NaCl. NaCl was added into suspended tobacco protoplasts at 200 mM final concentration. Samples were taken from 0 to 8 h at 2-h intervals. Two millimolar EGTA or 2 mM $LaCl_3$, were added into the system, respectively, 0.5 h before treated by NaCl. The data are means ± SE from $n = 6$ from three experiments.

within the first 6 min, followed by a slow decrease between 6 and 15 min. However, such an increased $[Ca^{2+}]_{cyt}$ did not appear in the control (untreated protoplasts). Therefore, NaCl stress may increase $[Ca^{2+}]_{cyt}$ in early stage of apoptosis in tobacco protoplasts. Figure 3 also showed that EGTA or $LaCl_3$ retarded the increase in $[Ca^{2+}]_{cyt}$, but the pattern of change was similar to that of protoplasts only when treated with NaCl stress.

Effect of EGTA or $LaCl_3$ on mitochondrial membrane potential in tobacco protoplasts

As shown in Figure 4, pre-treatment with EGTA or $LaCl_3$ had no significant effect on the $\Delta\Psi_m$ compared with that of control. $\Delta\Psi_m$ decreased significantly and gradually after stress, while no significant change was observed between protoplasts pre-treated by EGTA or $LaCl_3$ and control after stressed for 2 h, with prolonged stress, $\Delta\Psi_m$ of protoplasts pre-treatment with EGTA or $LaCl_3$ also declined.

Effect of CsA on PCD, mitochondrial membrane potential and $[Ca^{2+}]_{cyt}$ in tobacco protoplasts

Pre-treatment with CsA had no effect on PCD, $\Delta\Psi_m$ and $[Ca^{2+}]_{cyt}$ in un-stressed protoplasts (Figures 3, 5, 6), after treatment with NaCl for 8 h, compared to the protoplast treated with NaCl, pre-treated with CsA significantly decreased the percentage of PCD (Figure 5), and inhibited the decrease in $\Delta\Psi_m$ (Figure 6); Figure 3 showed that pre-treatment with CsA effectively delayed the increase in $[Ca^{2+}]_{cyt}$.

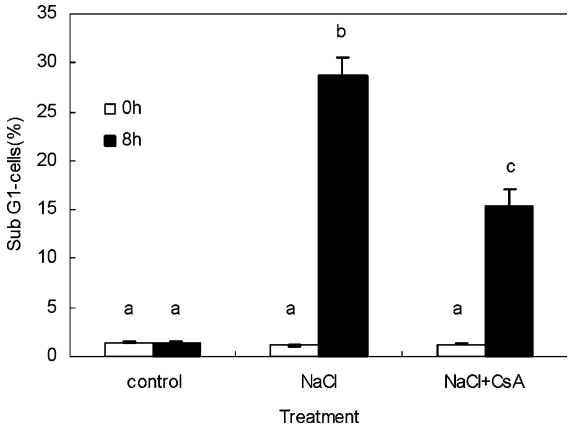

Figure 5. Effect of CsA on PCD in tobacco protoplasts. NaCl was added into suspended tobacco protoplasts at 200 mM final concentration. Samples were taken after salt treatment for 0 and 8 h. Fifty μM of CsA was added into the system, respectively, 0.5 h before treated by NaCl. The data are means ± SE from $n = 6$ from three experiments. Different letters indicate significant difference at $p = 0.05$ (LSD).

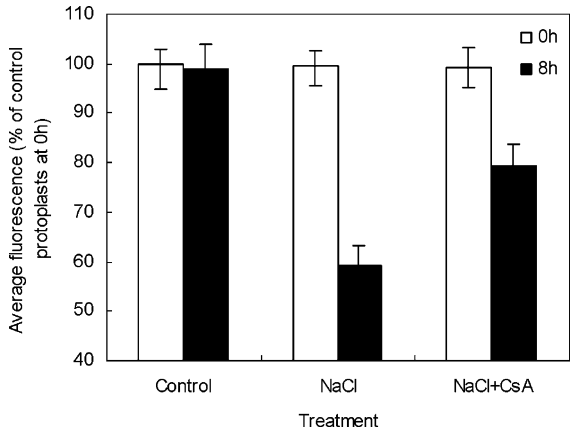

Figure 6. Effect of CsA on $\Delta\Psi_m$ in tobacco protoplasts. NaCl was added into suspended tobacco protoplasts at 200 mM final concentration. Samples were taken after salt treatment for 0 and 8 h. Fifty μM of CsA was added into the system, respectively, 0.5 h before treated by NaCl. The data are means ± SE from $n = 6$ from three experiments. Different letters indicate significant difference at $p = 0.05$ (LSD).

Discussion

Ca^{2+} participated in programmed cell death in tobacco protoplasts

More and more data suggest that increased Ca^{2+} is an essential mediator of PCD both in animals and in plants (Orrenius et al. 2003; Li et al. 2004). However, in animals, many results indicate that an increased $[Ca^{2+}]_{cyt}$ does not always induce cell death, on some occasions it can inhibit apoptosis, there are also data which indicate that a decrease in Ca^{2+} can also induce apoptosis (Kass and Orrenius 1999). In the present study, we have investigated whether intracellular Ca^{2+} accumulation regulates cell death in tobacco protoplasts under NaCl stress. A rapid increase of $[Ca^{2+}]_{cyt}$ was observed at the early stage (few minutes from 0 to 6 min) of PCD in tobacco protoplasts. Both depletion of Ca^{2+} with Ca^{2+}–chelator EGTA, and using plasma voltage-dependent Ca^{2+}-channel blocker ($LaCl_3$) significantly retarded the increase in $[Ca^{2+}]_{cyt}$ and inhibited the onset of cell death. All these results suggested that an increase in $[Ca^{2+}]_{cyt}$ is a pre-requisite for regulation of PCD in tobacco protoplasts.

A number of different mechanisms exist to achieve $[Ca^{2+}]_{cyt}$ rise. One of them is opening of the voltage-operated Ca^{2+} channels on the membrane surface. Another one is release of Ca^{2+} from intracellular stores. There are many specialized cellular structures in plant cells, such as endoplasmic reticulum (ER) and the vacuole that serve not only by sequestering Ca^{2+} ions from the cytosol but also by providing additional sources of Ca^{2+} by releasing the stored Ca^{2+} ion for signaling purposes (Navazio et al. 2001). Ca^{2+} channels in both membrane and intracellular Ca^{2+} stores contribute to PCD in animal (Orrenius et al. 2003). Yasuhiro et al. (2004) found involvement of putative voltage-dependent Ca^{2+} permeable channels in cryptogein-induced Ca^{2+} transients and defense responses in tobacco cells, while Zuppini et al. (2004) suggested that ER play an important role in PCD in soybean cells. In the current experiment, both EGTA and $LaCl_3$ only retarded, instead of inhibition, the increase in $[Ca^{2+}]_{cyt}$ and cell death. Results indicate that both interior and exterior Ca^{2+} participate in inducing cell death in tobacco, but when the Ca^{2+} channel is blocked by $LaCl_3$ or exterior Ca^{2+} is depleted by EGTA, then Ca^{2+} from other stores is enough to induce PCD.

Increased Ca^{2+} regulated PCD by affecting the open state of MPTP in tobacco protoplast

In 1996, Wang's group found that the activity of caspase-3 was unleashed by cytochrome *c*, a mitochondrial protein then thought to be dedicated solely to energy production (Liu et al. 1996). Then many experiments shifted the focus to the role of mitochondria in PCD. Cyclosporines can bind to cyclophilin D that is associated with the adenine nucleotide translocator (ANT), thus inhibiting the formation of MPTP. To test the role of MPTP in the salt induced PCD, we took advantage of the demonstrated inhibition of MPTP by CsA (Arpagaus et al. 2002; Scorrano et al. 2003). The results showed that pre-treatment of cells with CsA effectively reduced the amount of cell death and the decreases in $\Delta\Psi_m$. The results above indicate that similar to some of the studies with animal systems, the MPTP played an important role in regulating PCD in tobacco protoplasts treated with NaCl stress. The decrease in $\Delta\Psi_m$ prior to the appearance of cell death but after stress was also observed. This result indicates that the change in $\Delta\Psi_m$ precedes

the appearance of PCD and the decrease in $\Delta\Psi_m$ was a pre-requisite of the induction of PCD.

Classical work on isolated mitochondria from animals has shown that an increase in Ca^{2+} concentration in the cytosol results in Ca^{2+} uptake into mitochondria. This uptake when it exceeds a threshold, results in MPTP opening (Hoek et al. 1995; Zoratti and Szabo 1995). While mitochondria were found to release Ca^{2+} via the MPTP during agonist-evoked Ca^{2+} signals in tumor cells (Ichas et al. 1997). Another group showed that mitochondrial Ca^{2+} uptake triggers mitochondrial Ca^{2+} release via transient openings of PTP leading to an increase in $[Ca^{2+}]_{cyt}$. CsA not only specifically inhibits the MPTP formation but also prevents the Ca^{2+} cycling by mitochondria (Richter 1998). Our experiments clearly showed that (a) additional EGTA or $LaCl_3$ not only inhibited the increase in Ca^{2+}, but also effectively prevented the decrease in $\Delta\Psi_m$ and (b) additional CsA not only inhibited the decrease in $\Delta\Psi_m$ but also inhibited the increase in Ca^{2+} at an early stage. This suggests that $[Ca^{2+}]_{cyt}$ and the open state of MPTP could promote each other. Thus an increase in $[Ca^{2+}]_{cyt}$ can lead to a self-amplifying process to induce PCD.

Acknowledgements

The work was supported by National Natural Science Foundation of China (90102015, 30170161) and Cooperation Project of International in China and Greece (05-46).

References

Ahad A., Wolf J. and Nick P. 2003. Activation-tagged tobacco mutants that are tolerant to antimicrotubular herbicides are cross-resistant to chilling stress. Transgenic Res. 12: 615–629.

Allen G.J., Chu S.P., Harrington C.L., Schumacher K., Hoffmann T., Tang Y.Y., Grill E. and Schroeder J.I. 2001. A defined range of guard cell calcium oscillation parameters encodes stomatal movements. Nature 411: 1053–1057.

Arpagaus S., Rawyler A. and Braendle R. 2002. Occurrence and characteristics of the mitochondrial permeability transition in plants. J. Biol. Chem. 277: 1780–1787.

Bergounioux C., Brown S.C. and Petit P.X. 1992. Flow cytometry and plant protoplast cell biology. Physiol. Plant. 85: 374–386.

Budd S.L. and Nicholls D.G. 1996. A re-evaluation of the role of mitochondria in neuronal calcium homeostasis. J. Neurochem. 66: 403–411.

Chen L.B. 1988. Mitochondrial membrane potential in living cell. Annu. Rev. Cell Biol. 4: 155–181.

Curtis M.J. and Wolpert T.J. 2002. The oat mitochondrial permeability transition and its implication in victorin binding and induced cell death. Plant J. 29: 295–312.

Danon A., Delorme V., Mailhac N. and Gallois P. 2000. Plant programmed celll death: a common way to dye. Plant Physiol. Biochem. 38: 647–655.

Greenberg J.T., Guo A., Klessig D.F. and Ausubel F.M. 1994. Programmed cell death in plants: a pathogen-triggered response activitied coordinately with multiple defense function. Cell 77: 551–563.

Hoek J.B., Farber J.L., Thomas A.P. and Wang X. 1995. Calcium ion-dependent signalling and mitochondrial dysfunction: mitochondrial calcium uptake during hormonal stimulation in intact liver cells and its implication for the mitochondrial permeability transition. Biochim. Biophys. Acta 1271(1): 93–102.

Huh G.H., Damsz B., Matsumoto T.K., Reddy M.P., Rus A.M., Ibeas J.I., Narasimhan M.L., Bressan R.A. and Hasegawa P.M. 2002. Salt causes ion disequilibrium-induced programmed cell death in yeast and plants. Plant J. 29: 649–659.

Ichas F., Jouaville L.S. and Mazat J.P. 1997. Mitochondria are excitable organelles capable of generating and conveying electrical and calcium signals. Cell 89: 1145–1153.

Ishihara A., Miyagawa H., Kuwahara Y., Ueno T. and Mayama S. 1996. Involvement of Ca^{2+} ion in phytoalexin induction in oats. Plant Sci. 115: 9–16.

Jones A. 2000. Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? Trends Plant Sci. 5: 225–230.

Kao R.N. and Michayluk M.R. 1975. Nutritional requirements for growth of Vicia hajastana cells at very low population density in liquid medium. Planta 126: 105–110.

Kass G.E. and Orrenius S. 1999. Calcium signaling and cytotoxicity. Environ. Health Perspect. 107((1): 25–35.

Kawano T., Sahashi N., Takahashi K., Uozumi N. and Muto S. 1998. Salicylic acid induces extracellular superoxide generation followed by an increase in cytosolic calcium ion in tobacco suspension culture: the earliest events in salicylic acid signal transudation. Plant Cell Physiol. 39: 721–730.

Knight H., Trewavas A.J. and Knight M.R. 1996. Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. Plant Cell 8(3): 489–503.

Kroemer G., Zamzami N. and Susin S.A. 1997. Mitochondrial control of apoptosis. Immunol. Today 18: 44–51.

Kuo A., Cappellutti S., Cervantes-Cervantes M., Rodriquez M. and Bush D.S. 1996. Okadaic acid, a protein phosphatase inhibitor, blocks calcium changes, gene expression and cell death induced by gibberellin in wheat aleurone. Plant Cell 8: 259–269.

Lacomme C. and Santa Cruz S. 1999. Bax-induced cell death in tobacco is similar to the hypersensitive response. Proc. Natl Acad. Sci. USA 96: 7956–7961.

Lawrie A.M., Rizzuto R., Pozan, T. and Simpson A.W.M. 1996. A role for calcium influx in the regulation of mitochondrial calcium in endothelial cells. J. Biol. Chem. 271: 10753–10759.

Levine A., Rennell R.I., Alvarez M.E., Palmer R. and Lamb C. 1996. Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. 6(4): 427–437.

Li J., Wang D.Y., Li Q., Xu Y.J., Cui K.M. and Zhu Y.X. 2004. PPF1 inhibits programmed cell death in apical meristems of both G2 pea and transgenic *Arabidopsis* plants possibly by delaying cytosolic Ca^{2+} elevation. Cell Calcium 35(1): 71–77.

Liljeroth E. and Bryngelsson T. 2001. DNA fragmentation in cereal roots indicative of programmed root cortical cell death. Physiol. Plant. 111: 363–372.

Liu X., Kim C.N., Yang J., Jemmerson R. and Wang X. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 86: 147–157.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473–497.

Navazio L., Mariani P. and Sanders D. 2001. Mobilization of Ca^{2+} by cyclic ADP-ribose from the endoplasmic reticulum of cauliflower florets. Plant Physiol. 125: 2129–2138.

Orrenius S., Zhivotovsky B. and Nicotera P. 2003. Regulation of cell death: the calcium-apoptosis link. Nature 4: 552–565.

Rao M.V. and Davis K.R. 1999. Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. Plant J. 6: 603–614.

Richter C. 1998. Nitric oxide and its congeners in mitochondria: implications for apoptosis. Environ. Health Perspect. 106: 1125–1128.

Ryerson D.E. and Heath M.C. 1996. Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell 8: 393–402.

Saviani E.E., Orsi C.H., Oliveira J.F., Pinto-Maglio C.A. and Salgado I. 2002. Participation of the mitochondrial permeability transition pore in nitric oxide-induced plant cell death. FEBS Lett. 510: 136–140.

Scheel D. 1998. Resistance response physiology and signal transduction. Curr. Opin. Plant Biol. 1(4): 305–310.

Scorrano L., Oakes S.A., Opferman J.T., Cheng E.H., Sorcinelli M.D., Pozan T. and Korsmeyer S.J. 2003. BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300: 135–139.

Simpson P.B. and Russell J.T. 1998. Role of mitochondrial Ca^{2+} regulation in neuronal and glial cell signalling. Brain Res. Rev. 26: 72–82.

Smaili S.S., Hsu Y., Youle R.J. and Russell J.T. 2000. Mitochondria in Ca^{2+} signaling and apoptosis. J. Bioenerg. Biomembr. 32: 35–46.

Steller H. 1995. Mechanisms and genes of cellular suicide. Science 267: 1445–1449.

Subbaiah C.C., Bush D.S. and Sachs M.M. 1998. Mitochondrial contribution to the anoxic Ca^{2+} signal in maize suspension-cultured cells. Plant Physiol. 118: 759–771.

Thomas S.G. and Franklin-Tong V. 2004. Self-incompatibility triggers programmed cell death in *Papaver* pollen. Nature 429: 305–309.

Vieira H.L.A. and Kroemer G. 1999. Pathophysiology of mitochondrial cell death control. Cell. Mol. Life Sci. 56: 971–976.

Wang C.Y., Bowen J.H., Weir I.E., Allan A.C. and Perguson I.B. 2001. Heat-induced protection against death of suspension-cultured apple fruit cells exposed to low temperature. Plant Cell Environ. 24: 1199–1207.

White R.J. and Reynolds I.J. 1996. Mitochondrial depolarization in glutamate-stimulated neurons: an early signal specific to excitotoxin exposure. J Neurosci. 16: 5688–5697.

Xie Z. and Chen Z. 2000. Harpin-induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. Mol. Plant–Microbe. Interact. 13: 183–190.

Xu H.X. and Heath M.C. 1998. Role of calcium in signal transudation during the hypersensitive response caused by basidiospore infection of the cowpea rust fungus. Plant Cell. 10: 585–597.

Yamamoto Y., Kobayashi Y., Rama Devi S., Rikiishi S. and Matsumoto H. 2002. Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol. 128: 63–72.

Yasuhiro K., Takuya F., Yoko O., Tatsuaki G., Katsumi H., Shoshi M. and Kazuyuki K. 2004. Identification of putative voltage-dependent Ca^{2+}-permeable channels involved in cryptogein-induced Ca^{2+} transients and defense responses in tobacco BY-2 cells. Biochem. Biophys. Res. Commun. 317: 823–828.

Yu X.H., Perdue T.D., Heimer Y.M. and Jones A.M. 2002. Mitochondrial involvement in tracheary element programmed cell death. Cell Death Differ. 9: 189–198.

Zoratti M. and Szabo I. 1995. The mitochondrial permeability transition. Biochim. Biophys. Acta 1241: 139–176.

Zuppini A., Navazio L. and Mariani P. 2004. Endoplasmic reticulum stress-induced programmed cell death in soybean cells. J. Cell Sci. 117(12): 2591–2598.