Role of calcium in nitric oxide-induced programmed cell death in tobacco protoplasts

Y. WANG[1,2], J.S. LIN[2]* and G.X. WANG[3]

Key Laboratory of Hydrobiology in Liaoning Province's Universities, Dalian Fisheries University, Dalian, Liaoning Province 116023, P.R. China[1]
Key Laboratory of Arid Agroecology at Lanzhou University, Ministry of Education, Lanzhou, Gansu Province 730000, P.R. China[2]
College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang Province 310029, P.R. China[3]

Abstract

We tried to determine the mechanisms by which Ca^{2+} mediated NO-induced programmed cell death (PCD) in tobacco protoplasts. Treatment of tobacco protoplasts with the NO donor sodium nitroprusside (SNP) resulted in a rapid $[Ca^{2+}]_{cyt}$ accumulation and decrease in mitochondrial membrane potential ($\Delta\Psi_m$) before the appearance of PCD. NO-induced PCD could be largely prevented not only by NO scavenger c-PTIO, but also by EGTA (Ca^{2+} chelator), $LaCl_3$ (Ca^{2+}-channel blocker) or CsA (a specific mitochondrial permeability transition pore inhibitor, which also inhibit Ca^{2+} cycling by mitochondria). All results suggested that NO-induced PCD is mediated through mitochondrial pathway and regulated by Ca^{2+}.

Additional key words: mitochondria, mitochondrial permeability transition, sodium nitroprusside.

Introduction

Nitric oxide (NO) is a unique molecular messenger involved in the induction and execution of programmed cell death (PCD) in plants (Pedroso *et al.* 2000, Beligni *et al*. 2002, Casolo *et al.* 2005, Víteček *et al.* 2007). There are several NO-producing enzymatic or non-enzymatic systems in plants. Nitrite and L-arginine are main sources for enzymatic NO production (Lamotte *et al*. 2005, Besson-Bard *et al*. 2008). It is well known that the over-production of NO in plants is potentially toxic for lipids, proteins, DNA, *etc.*, resulting in damage and even the death of plants (Radi 2004, Hong *et al*. 2008). PCD or apoptosis, a genetically controlled programmed process, is an evolutionarily conserved cell death in eukaryotes operating in development and in cellular responses to stresses. In plants, PCD can be induced by a variety of stimuli including developmental signals, environmental factors and pathogen infection during the hypersensitive response (HR) (Van Breusegem and Dat 2006). NO can promote PCD in some cells, whereas it inhibits PCD in other cells (Casolo *et al.* 2005, Lopez-Carrion *et al.* 2008). This complexity is a consequence of the rate of NO production and the interaction with iron, thiols, proteins, and reactive oxygen species (Víteček *et al.* 2007). In the last decade, the role of NO in the induction of PCD and the mechanism of signal transduction in this process, have been subjects of extensive research.

Cell death pathways can be subdivided into two components, either involving death receptors or mitochondria (Robson and Vanlerberghe 2002). NO is seen as a signaling factor in the later. NO inhibits the activity of the last enzyme in the mitochondrial respiratory electron transport chain, the cytochrome *c* oxidase (COX) leading to the generation of superoxide O_2^- due to the dramatically reduced ubiquinone pool (Grun *et al.* 2006). NO can rapidly react with O_2^- to produce peroxynitrite ($ONOO^-$), whereas peroxynitrite can induce mitochondrial swelling,

Received 21 August 2008, *accepted* 5 May 2009.
Abbreviations: AO - acridine orange; cPTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; CsA - cyclosporin A; $\Delta\Psi_m$ - mitochondrial membrane potential; EGTA - ethyleneglycol(β-aminoethyether)-N,N'-tetraacetic acid; MPTP - mitochondrial permeability transition pore; PBS - phosphate buffer solution; PCD - programmed cell death; PI - propidium iodide; Rh123 - rhodamine 123; SNP - sodium nitroprusside.
Acknowledgements: This work was supported by the National Natural Science Fund of China (40806047) and the Youth Science Fund of Dalian (2006J23JH036).
* Corresponding author present address: Department of Biochemistry and Plant Science Innovation, University of Nebraska, Lincoln, NE 68503, USA; e-mail: linjiusheng@hotmail.com

uncoupling, depolarization and activation of mitochondrial permeability transition pore (MPTP) (Brown 2007). Opening of MPTP, the life-or-death switch of cells, triggers an increase of the permeability of mitochondrial membrane. Then apoptogenic factors, such as cytochrome *c*, Smac/Diablo, AIF and DNaseG, leak into the cytoplasm from the inner mitochondrial membrane space. In the presence of ATP (dATP), cytochrome *c* binds to Apaf-1 and triggers its oligomerization, after which pro-caspase-9 is recruited and undergoes autoactivation. The protein complex comprising cytochrome *c*, Apaf-1, and caspase-9 called the "apoptosome", begin a cascade of proteolytic activity that ultimately leads to nuclear damage (DNA fragmentation) and cell death (Green and Reed 1998, Saviani *et al.* 2002). Subsequently, 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). Plant MPTP are also a target of NO (Saviani *et al.* 2002, Zottini *et al.* 2002), which open the MPTP and induce the release of cytochrome *c*.

Ca^{2+} has been shown to be involved in the NO signal transduction in plant cells (Ali *et al*. 2007, Besson-Bard *et al*. 2008). The normal Ca^{2+} uptake and release from internal stores (endoplasmic reticulum, mitochondrion, mainly vacuoles in plants) or entry through the plasma membrane is under strict biochemical and physiological control (White and Broadley 2003). NO may stimulate the increases in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) by inducing both Ca^{2+} influx from the extracellular space and Ca^{2+} release from intracellular stores (Besson-Bard *et al*. 2008). Our previous studies have shown a role for Ca^{2+} in regulating the mitochondrial permeability transition in plant cells (Lin *et al.* 2005). These observations imply that Ca^{2+} is a signal that functions in the upstream of MPTP. It is reported that the NO donor diethylamine NONOate promoted an increase in $[Ca^{2+}]_{cyt}$, which was sensitive to intracellular Ca^{2+} channel inhibitors in plants (Lamotte *et al*. 2004). Nevertheless, how exactly these two second messengers are related is unknown in plants PCD. Furthermore, no single study has investigated the inter-relationship between Ca^{2+} and MPTP in NO-induced PCD.

In the present study, effects of Ca^{2+} on NO-induced PCD in tobacco protoplasts were studied. We investigated whether Ca^{2+} regulate mitochondrial permeability transition in NO-induced PCD in plants.

Materials and methods

Plants and protoplast isolation: Tobacco (*Nicotiana tabacum* L., cv. BY-2) seeds were surface sterilized with 70 % ethanol for 2 min and incubated for 5 min in sodium hypochlorite containing 0.1 % *Triton X-100*. Sterilized seeds were germinated on solid Murashige and Skoog (1962; MS) medium with 2 % sucrose and cultured at in a phytotron at 24 °C, 16-h photoperiod, irradiance of 100 μmol(photon) m^{-2} s^{-1}. Shoot tips were excised and subcultured every 4 - 5 weeks. For protoplast isolation only well-rooted plants that had undergone at least three subcultures were used. Protoplasts were isolated according to Ahad *et al* (2003). Fully expanded leaves were placed upside down in a sterile Petri dish on 10 cm^3 of CNT medium [KAO medium (Kao and Michayluk 1975) with 1 % cellulase (*Onozuka-R*, Yakult, Japan), 1 % macerozyme (*Serva*, Heidelberg, Germany) and 0.5 M glucose]. 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 carefully overlaid with 1 cm^3 PNT medium (KAO medium including 0.5 M glucose) on top of the suspension and then centrifuged for 5 min at low speed (80 *g*, 25 °C). Intact protoplasts were collected from the interphase and transferred in to a new tube. Fresh PNT medium was 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 haematocytometer. The supernatant was carefully removed and the isolated protoplasts resuspended in PNT medium to a density of 2×10^5 protoplasts cm^{-3}. Exogenous NO donor sodium nitroprusside (SNP) solutions were prepared just before the experiments.

Nuclear labeling: The ultrastructure of the protoplast nucleus was assessed using DNA-binding fluorochrome *Acridine Orange* (*Sigma*, St Louis, MO, USA) using a fluorescence microscope.

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 propidium iodide (PI) staining (Lin *et al.* 2005). Briefly, protoplasts were suspended in phosphate buffer solution (PBS) containing 10 μg cm^{-3} PI plus 0.1 % *Triton-X 100* (v/v) and 60 μg cm^{-3} RNase A for 30 min at room temperature in the dark. After incubation, the protoplasts were washed twice with PBS, pH 7.4. The volume was adjusted to 0.6 cm^{-3} and analyzed using a flow cytometer (*Epics XL*, *U.S Coulter*, Miami, USA; excitation 488 nm, emission 525 nm).

Determination of $\Delta\Psi_m$: Mitochondrial membrane potential $\Delta\Psi_m$ was quantitatively measured by flow

cytometry as described (Braidot *et al*. 1998). Protoplasts were incubated with 10 μM *Rhodamine123* (*Rh123*), a specific $\Delta\Psi_m$-dependent fluorescence dye (Yamamoto *et al*. 2002), for 30 min at 26 °C and washed three times in PBS to remove free *Rh123* dye from the medium, suspended in a total volume of 0.6 cm^{-3} PBS and analyzed using the above flow cytometer .

Detection of $[Ca^{2+}]_{cyt}$ by laser confocal microscopy: The time-lapse change of $[Ca^{2+}]_{cyt}$ was detected after various treatments with Ca^{2+} probe *Fluo-3 AM* fluorescence dye. 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 (Lin *et al.* 2005). After incubation, the protoplasts were washed twice with PBS (pH 7.4) to remove free *Fluo-3 AM* dye from the medium, and 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.

Results

Typical nuclei morphological changes during PCD were shown in tobacco protoplasts after 1 mM SNP treatment. The nucleus condensation and peripheral distribution were observed. Normal viable protoplasts showed green round nuclei (Fig. 1*A*). Characteristic nuclei of PCD after SNP treatment showed orange fluorescence and nuclear marginalization onto the nuclear membrane with chromatin loss resulting in the nucleolus becoming prominent (Fig. 1*B*,*C*). These hallmarks of PCD were not seen in the control (untreated) protoplasts.

To investigate whether NO is able to trigger $[Ca^{2+}]_{cyt}$ increase, tobacco protoplasts were pretreated for 0.5 h with the Ca^{2+}-specific fluorophore *Fluo-3 AM* and subsequently incubated in the presence or absence of SNP. Treatment with 1 mM SNP led to significant increases in the fluorescence intensity of the protoplasts, which reached maximum values at 5 min and was 6.4-fold higher than that in the control (Fig. 2*A*). Application of the Ca^{2+} chelator EGTA or the Ca^{2+} channel blockers $LaCl_3$ substantially reduced the increases in fluorescence intensity induced by SNP (data not shown), suggesting that *Fluo-3*-fluorescence increases are associated with changes in $[Ca^{2+}]_{cyt}$. Fluorescence was abolished in the presence of the NO-specific scavenger cPTIO (Fig. 2*A*). This indicates that the $[Ca^{2+}]_{cyt}$ increase was caused by NO production.

SNP treatment inevitably induced the opening of MPTP. Mitochondrial membrane potential ($\Delta\Psi_m$) reflects the mitochondrial permeability transition as a result of opening of MPTP. $\Delta\Psi_m$ was determined by mean aggregate fluorescence of the fluorochrome *Rh123*. $\Delta\Psi_m$ decreased by 22.7, 46.3, 64.3 or 71.4 % when tobacco protoplasts were subjected to SNP treatment for 2, 4, 6 or 8 h, respectively. Treatment with cPTIO alone did not have any effect on $\Delta\Psi_m$ compared with control protoplasts (data not shown).The effect of NO induced $\Delta\Psi_m$ decrease could be partially reversed by the addition of l mM cPTIO (Fig. 2*B*). SNP treated protoplasts showed a gradual increase in PCD (Fig. 2*C*), which was measured by flow cytometry and the effect of NO on PCD followed a similar pattern to that observed on $\Delta\Psi_m$ (Fig. 2*B*).

In order to investigate whether NO-induced PCD is related to Ca^{2+} and mitochondrial permeability transition, the Ca^{2+} chelator EGTA, the Ca^{2+} channel blockers $LaCl_3$ and the mitochondrial permeability transition pore inhibitor CsA were used. Although pretreatment with EGTA, $LaCl_3$ or CsA was ineffective in protecting protoplasts from NO-mediated delayed (8 h) PCD, these inhibitors evidently prevented NO-induced PCD at 2 h (Table 1). These results suggest that Ca^{2+} and mitochondrial permeability transition are involved in NO-induced PCD.

To test if Ca^{2+} regulate mitochondrial permeability transition in NO-induced PCD, we pre-treated protoplasts with the Ca^{2+} chelator EGTA or the Ca^{2+} channel blocker $LaCl_3$ prior to NO induction. Treatment with either EGTA or $LaCl_3$ prevented the decrease of $\Delta\Psi_m$ in tobacco protoplasts under SNP stress (Table 1).

To further determine the interrelationship between Ca^{2+} and mitochondrial permeability transition in plant

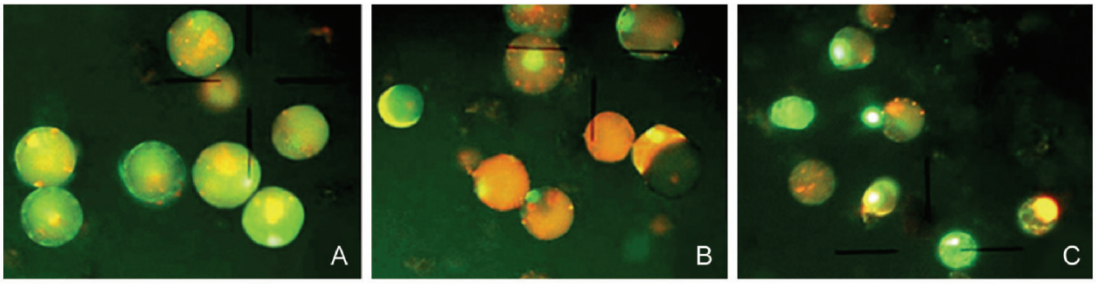

Fig. 1. AO stained nuclei of normal and PCD tobacco protoplasts induced by NO. NO donor SNP was added into suspended tobacco protoplasts at 1 mM final concentration. *A* - Normal nucleus. *B*, *C* - Different phases of PCD. Nuclear dense mass and apoptotic bodies induced by SNP. Original magnification 400×.

PCD, the protoplasts were pretreated with the Ca^{2+} chelator EGTA, the Ca^{2+} channel blockers $LaCl_3$ or the mitochondrial permeability transition pore inhibitor CsA, and then exposed to $CaCl_2$ treatment. The pretreatments with these Ca^{2+} inhibitors or mitochondrial permeability transition pore inhibitor significantly inhibited the increases in PCD and the activities of the open state of MPTP induced by $CaCl_2$ treatment (Table 1). These results suggest that Ca^{2+} and mitochondrial permeability transition is required for $CaCl_2$-induced PCD. Moreover, pretreatment with these Ca^{2+} inhibitors also substantially blocked the opening of MPTP induced by $CaCl_2$ treatment, indicating that Ca^{2+} is also involved in $CaCl_2$-induced up-regulation of the PCD and the open of MPTP in plants.

Discussion

Previous papers showed that Ca^{2+} and MPTP are required for NO signaling in plants (Ali *et al*. 2007, Besson-Bard *et al*. 2008, Saviani *et al.* 2002). However, it was not clear how Ca^{2+} and MPTP regulate the plants PCD induced by NO. An increase of outer mitochondrial membrane permeability is one of the key events in apoptotic or necrotic death, although the details of the mechanism involved remain to be elucidated. NO can induce transient elevations of $[Ca^{2+}]_{cyt}$ in plants (Lamotte *et al.* 2004), and the information encoded in transient Ca^{2+} signals may regulate MPTP. Here we provide evidence that Ca^{2+} is involved in NO-induced up-regulation of mitochondrial permeability transition in tobacco protoplasts.

We found two lines of evidence for an essential role of NO in PCD through the regulation of $[Ca^{2+}]_{cyt}$. First, a rapid increase of $[Ca^{2+}]_{cyt}$ was observed at the early stage (from 0 to 5 min) of NO-induced PCD in tobacco protoplasts (Fig. 2*A*). Secondly, the Ca^{2+} chelator EGTA or the Ca^{2+} channel blocker $LaCl_3$ significantly retarded the increase in $[Ca^{2+}]_{cyt}$ and inhibited the onset of cell death (Table 1). NO modulates the activity of plasma membrane as well as intracellular Ca^{2+}-permeable channels. Strictly, almost all types of Ca^{2+} channels appear to be regulated by NO (Clementi 1998). NO impacts on their activity directly through S-nitrosylation (the reversible formation of a covalent bound between a cysteine residue and an NO group) or indirectly involving cGMP, produced by NO-induced activation of soluble guanylate cyclase, and/or cyclic ADP-ribose (cADPR; a Ca^{2+}-mobilizing metabolite that is synthesized from NAD^+ by ADP-ribosyl-cyclase). Accumulating evidence suggest that cADPR mediates Ca^{2+} release by activating the intracellular Ca^{2+} channels ryanodine receptors (RYR) not only in mammals but also in plants (Besson-Bard *et al*. 2008). NO might also influence the activity of inositol 1,4,5-triphosphate receptors (Orrenius *et al*. 2003, Courtois *et al*. 2008). Thus our experiments show that NO might promote an influx of Ca^{2+} from the extracellular space and/or mobilization of Ca^{2+} sequestered in intracellular Ca^{2+} stores.

It was proposed that an increase of outer mitochondrial membrane permeability is central to apoptosis (Scorrano *et al*. 2003). The relationship between Ca^{2+} and MPTP in plants exposed to NO has been investigated. On the one hand, in the present study, time-course analysis of Ca^{2+} and $\Delta\Psi_m$ showed that the increase of $[Ca^{2+}]_{cyt}$ preceded loss of $\Delta\Psi_m$ (Fig. 2), and pretreatments with the Ca^{2+} chelator EGTA, the Ca^{2+} channel blocker $LaCl_3$ blocked NO-induced loss of $\Delta\Psi_m$ (Table 1). Furthermore,

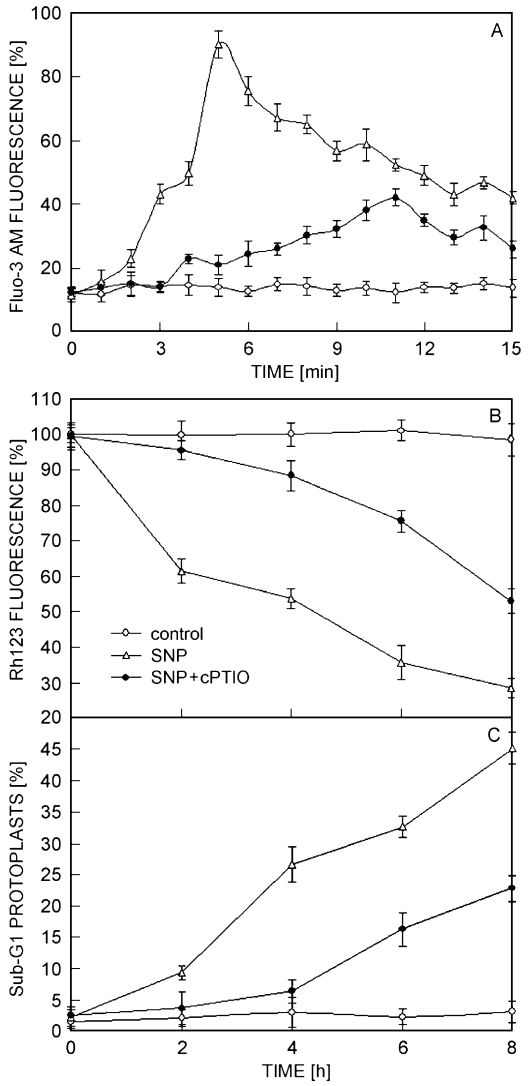

Fig. 2. The protoplasts loaded with fluorochrome were treated with 1 mM SNP or SNP + 1 mM cPTIO. *A* - Changes in Fluo-3 AM fluorescence measured by laser scanning confocal microscopy (means ± SE obtained by scanning over 60 protoplasts from 5 different experiments. *B* - Changes in Rh123 fluorescence measured by flow cytometry. *C* - Protoplasts viability in NO-stressed tobacco protoplasts measured by propidium iodide staining and flow cytometry (n = 6).

Table 1. Effects of pretreatments (0.5 h) with Ca^{2+} inhibitors and MPTP blockers (2 mM EGTA, 2 mM $LaCl_3$, 100 μM CsA) on PCD (sub-G_1 protoplasts) and mitochondrial membrane potential (Rh123 fluorescence) in tobacco exposed to NO donor 1 mM SNP for 2 or 8 h or 6 mM $CaCl_2$ for 6 h. Means ± SE, $n = 6$.

Parameter		Time [h]	Control	SNP/$CaCl_2$	+EGTA	+$LaCl_3$	+CsA
Sub-G_1 protoplasts [%]	SNP	2	2.1 ± 1.3	9.4 ± 1.1	3.6 ± 1.8	4.5 ± 0.7	2.7 ± 1.6
	SNP	8	3.1 ± 1.7	45.2 ± 2.5	24.7 ± 2.7	35.1 ± 2.2	15.6 ± 2.5
Rh123 fluorescence [%]	SNP	2	99.8 ± 4.1	61.5 ± 3.4	88.3 ± 2.1	75.6 ± 2.9	96.7 ± 3.5
	SNP	8	98.6 ± 4.5	28.6 ± 2.7	51.7 ± 2.8	38.1 ± 4.8	58.3 ± 2.3
Sub-G_1 protoplasts [%]	$CaCl_2$	6	99.6 ± 1.7	38.4 ± 3.0	67.3 ± 3.6	55.5 ± 4.3	74.1 ± 3.1
Rh123 fluorescence [%]	$CaCl_2$	6	2.3 ± 1.2	42.2 ± 2.5	21.6 ± 2.8	32.7 ± 2.1	13.3 ± 1.5

pre-treatment with CsA also effectively retarded the increase in $[Ca^{2+}]_{cyt}$ and the onset of PCD induced by salt stress (Lin *et al.* 2005) and NO (Table 1). CsA can not only specifically inhibit the MPTP opening but also prevent the endogenous Ca^{2+} cycling by mitochondria (Richter 1998). These results clearly suggest that Ca^{2+} acts upstream of MPTP in NO signaling. On the other hand, when challenged with high $[Ca^{2+}]_{cyt}$, the mitochondria can contribute to Ca^{2+} homeostasis by providing an intracellular sink for Ca^{2+}. Moreover, the uptake of Ca^{2+} by mitochondria has a powerful impact on cellular Ca^{2+} signaling, affecting the generation and propagation of Ins (1,4,5) P_3-triggered Ca^{2+} waves and the modulation of store-operated Ca^{2+} currents (Coelho *et al*. 2002). Taken together, it is implied that there exists cross-talk between Ca^{2+} and MPTP in plant cells in response to stresses or stimuli.

Our data show that NO treatment induced increase in the concentration of $[Ca^{2+}]_{cyt}$ and loss of $\Delta\Psi_m$, and that pretreatments with the Ca^{2+} chelator EGTA, the Ca^{2+} channel blocker $LaCl_3$ or the MPTP blocker CsA, which have been shown to prevent loss of $\Delta\Psi_m$ (Table 1), substantially blocked the increases in PCD induced by NO (Table 1). Moreover, exogenous $CaCl_2$ has been shown to induce loss of $\Delta\Psi_m$ and PCD (Table 1). Although the absolute specificities of the inhibitors used in this study can always be questioned, it has been shown that these inhibitors in the concentration range used in the present study should be suitable for studying the role of Ca^{2+} and MPTP in plant cells (Larkindale and Knight 2002, Li *et al*. 2004, Zottini *et al.* 2002). Our results clearly suggest that Ca^{2+} and mitochondrial permeability transition is required for NO-induced PCD in tobacco protoplasts.

References

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

Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsaltas, D., Leng, Q., Von Bodman, S., Berkowitz, G.A.: Death don't have no mercy and neither does calcium: *Arabidopsis* CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. - Plant Cell **19**: 1081-1095, 2007.

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

Beligni, M.V., Fath, A., Bethke, P.C., Lamattina, L., Jones, R.L.: Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. - Plant Physiol. **129**: 1642-1650, 2002.

Besson-Bard, A., Courtois, C., Gauthier, A., Dahan, J., Dobrowolska, G., Jeandroz, S., Pugin, A., Wendehenne, D.: Nitric oxide in plants: production and cross-talk with Ca^{2+} signalling. - Mol. Plant. **1**: 218-228, 2008.

Braidot, E., Petrussa, E., Macri, F., Vianello, A.: Plant mitochondrial electrical potential monitored by fluorescence quenching of rhodamine 123. - Biol. Plant. **41**: 193-201, 1998.

Brown, G.C.: Nitric oxide and mitochondria. - Front Biosci. **12**: 1024-1033, 2007.

Casolo, V., Petrussa, E., Krajnakova, J., Macri, F., Vianello, A.: Involvement of the mitochondrial K^+-ATP channel in H_2O_2- or NO-induced programmed death of soybean suspension cell cultures. - J. exp. Bot. **56**: 997-1006, 2005.

Clementi, E.: Role of nitric oxide and its intracellular signalling pathways in the control of Ca^{2+} homeostasis. - Biochem. Pharmacol. **55**: 713-718, 1998.

Coelho, S.M., Taylor, A.R., Ryan, K.P., Sousa-Pinto, I., Brown, M.T., Brownlee, C.: Spatiotemporal patterning of reactive oxygen production and Ca^{2+} wave propagation in fucus rhizoid cells. - Plant Cell **14**: 2369-2381, 2002.

Courtois, C., Besson, A., Dahan, J., Bourque, S., Dobrowolska, G., Pugin, A., Wendehenne, D.: Nitric oxide signalling in plants: interplays with Ca^{2+} and protein kinases. - J. exp. Bot. **59**: 155-163, 2008.

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

Green, D.R., Reed, J.C.: Mitochondria and apoptosis. - Science **281**: 1309-1312, 1998.

Grun, S., Lindermayr, C., Sell, S., Durner, J.: Nitric oxide and gene regulation in plants. - J. exp. Bot. **57**: 507-516, 2006.

Hong, J.K., Yun, B.W., Kang, J.G., Raja, M.U., Kwon, E., Sorhagen, K., Chu, C., Wang, Y., Loake, G.J.: Nitric oxide function and signalling in plant disease resistance. - J. exp. Bot. **59**: 147-154, 2008.

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

Lamotte, O., Courtois, C., Barnavon, L., Pugin, A., Wendehenne, D.: Nitric oxide in plants: the biosynthesis and cell signaling properties of a fascinating molecule. - Planta **221**: 1-4, 2005.

Lamotte, O., Gould, K., Lecourieux, D., Sequeira-Legrand, A., Lebrun-Garcia, A., Durner, J., Pugin, A., Wendehenne, D.: Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein. - Plant Physiol. **135**: 516-529, 2004.

Larkindale, J., Knight, M.R.: Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene, and salicylic acid. - Plant Physiol. **128**: 682-695, 2002.

Li, B., Liu, H.T., Sun, D.Y., Zhou, R.G.: Ca^{2+} and calmodulin modulate DNA-binding activity of maize heat shock transcription factor *in vitro*. - Plant Cell Physiol. **45**: 627-634, 2004.

Lin, J.S., Wang, Y., Wang, G.X.: Salt stress-induced programmed cell death via Ca^{2+}-mediated mitochondrial permeability transition in tobacco protoplasts. - Plant Growth Regul. **45**: 243-250, 2005.

Lopez-Carrion, A.I., Castellano. R., Rosales, M.A., Ruiz, J.M., Romero, L.: Role of nitric oxide under saline stress: implications on proline metabolism. - Biol. Plant. **52**: 587-591, 2008.

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

Orrenius, S., Zhivotovsky, B., Nicotera, P.: Regulation of cell death: the calcium-apoptosis link. - Nat. Rev. mol. cell. Biol. **4**: 552-565, 2003.

Pedroso, M.C., Magalhaes, J.R., Durzan, D.: A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm callus cells and foliar tissues. - J. exp. Bot. **51**: 1027-1036, 2000.

Radi, R.: Nitric oxide, oxidants, and protein tyrosine nitration. - Proc. nat. Acad. Sci. USA **101**: 4003-4008, 2004.

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

Robson, C.A., Vanlerberghe, G.C.: Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. - Plant Physiol. **129**: 1908-1920, 2002.

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

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

Van Breusegem, F., Dat, J.F.: Reactive oxygen species in plant cell death. - Plant Physiol. **141**: 384-390, 2006.

Víteček, J., Wünschová, A., Petřek, J., Adam, V., Kizek, R., Havel, L.: Cell death induced by sodium nitroprusside and hydrogen peroxide in tobacco BY-2 cell suspension. - Biol. Plant. **51**: 472-479, 2007.

White, P.J., Broadley, M.R.: Calcium in plants. - Ann. Bot. **92**: 487-511, 2003.

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

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

Zottini, M., Formentin, E., Scattolin, M., Carimi, F., Lo Schiavo, F., Terzi, M.: Nitric oxide affects plant mitochondrial functionality *in vivo*. - FEBS Lett. **515**: 75-78, 2002.