Endoplasmic reticulum stress-induced programmed cell death in soybean cells

Anna Zuppini*, Lorella Navazio and Paola Mariani

Dipartimento di Biologia, Università di Padova, via U. Bassi 58/B, 35131 Padova, Italy

*Author for correspondence (e-mail: zuppini@civ.bio.unipd.it)

Summary

In animal cells, the endoplasmic reticulum may participate in programmed cell death by sensing and transducing apoptotic signals. In an attempt to analyze the role of the endoplasmic reticulum in plant programmed cell death we investigated the effect of cyclopiazonic acid, a specific blocker of plant endoplasmic reticulum-type IIA Ca^{2+}-pumps, in soybean cells. Cyclopiazonic acid treatment elicited endoplasmic reticulum stress and a biphasic increase in cytosolic Ca^{2+} concentration, followed by the induction of a cell death program. Cyclopiazonic acid-induced programmed cell death occurred with accumulation of H_2O_2, cytochrome c release from mitochondria, caspase 9- and caspase 3-like protease activation, cytoplasmic shrinkage and chromatin condensation. Chelation of cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (acetoxymethil ester) failed to inhibit cyclopiazonic acid-induced cell death. Taken together, our results provide evidence for a role of the endoplasmic reticulum and mitochondria in regulating cyclopiazonic acid-induced programmed cell death in soybean cells, probably via a cross-talk between the two organelles.

Key words: Calcium, Cyclopiazonic acid, Endoplasmic reticulum, Programmed cell death, Soybean cells

Introduction

Programmed cell death (PCD) plays an essential role in many aspects of animal and plant development and in response to stresses (Beers and McDowell, 2001; Katoch et al., 2002). The main signaling pathways of PCD have been described mainly in animal cells. In plants, PCD has been observed during tracheary elements differentiation (Kuriyama and Fukuda, 2002), aerenchyma formation (Gunawardena et al., 2001), hypersensitive response (HR) (Heath, 2000) and as a consequence of several biological and chemical stresses (Beers and McDowell, 2001). Although there are few reports concerning the mechanisms of PCD in plant cells, recent studies have shown several morphological and biochemical similarities between apoptosis and plant PCD (Danon et al., 2000). In particular, similar to animal apoptosis, the involvement of calcium ions, production of reactive oxygen species, activation of caspase-like proteases, cytochrome c release from mitochondria, cytoplasmic shrinkage, chromatin condensation and DNA fragmentation have been observed to occur during plant PCD (Levine et al., 1996; Danon et al., 2000; McCabe and Leaver, 2000; Neill et al., 2002). On the whole, these data, even though not demonstrated simultaneously in the same plant system, suggest that PCD in plants and animals may be based on a common cell death process (Hoeberichts and Woltering, 2003).

The main PCD signaling pathways illustrated in animal cells involve plasma membrane receptors and mitochondria. Only recently has the importance of the endoplasmic reticulum (ER) in triggering a specific program of cell death been recognized (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). The ER is the major compartment for Ca^{2+} sequestration and signaling (Meldolesi and Pozzan, 1998). The high lumenal Ca^{2+} concentration acts as a pool for releasable Ca^{2+} during cell signaling (Berridge et al., 2000) and also regulates the functioning of numerous ER lumenal proteins (Brostrom and Brostrom, 1998). The main role of the ER, regulated by a number of Ca^{2+}-dependent proteins, is the control of synthesis, folding and export of proteins (Meldolesi and Pozzan, 1998). Prolonged ER Ca^{2+} depletion impaired ER functions causing the so called ER stress (Pahl, 1999). ER stress in animal cells has received increased attention for its involvement in pathologically relevant apoptosis (Aridor and Balch, 1999; Paschen and Doutheil, 1999). Stress in the ER has been shown to induce different apoptotic pathways involving a cross-talk between ER and mitochondria, the activation of caspase 12 and/or cytochrome c release by mitochondria, causing caspase 9 activation (Hacki et al., 2000; Zuppini et al., 2002; Jimbo et al., 2003). Procaspase 12 resides in the cytosolic side of the ER membrane and, upon its activation by ER stress, is released into the cytosol in an active form (caspase 12) (Nakagawa et al., 2000; Nakagawa and Yuan, 2000). The ER apoptotic pathway seems to be triggered by a specific ER stress: caspase 12 knockout mice undergo apoptosis induced by stimuli that do not involve ER stress, whereas they are resistant to ER stress-induced apoptosis (Nakagawa and Yuan, 2000).

Several studies have suggested that many stimuli that alter the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and the storage of Ca^{2+} in the intracellular organelles induce apoptosis in a variety of cells (Arnaudeau et al., 2002; Grebenová et al., 2003). Molecules that inhibit the sarcoplasmic/ER Ca^{2+}-ATPase (SERCA) such as thapsigargin (TG) and cyclopiazonic

acid (CPA) cause depletion of intracellular Ca^{2+} stores with consequent increase in $[Ca^{2+}]_{cyt}$ and induce apoptosis (Thastrup et al., 1990; Mason et al., 1991; Nguyen et al., 2002). The data so far available on ER stress-induced apoptosis by TG or CPA are from animal cells. There is no evidence for a similar mechanism in plant cells.

We have used a soybean cell line stably expressing the Ca^{2+}-sensitive photoprotein aequorin, to analyze the pathway of cell death induced by a definite ER stress. Although the ER has a smaller Ca^{2+} storage capacity than the vacuole, its importance in Ca^{2+} release during signaling has recently been suggested (Navazio et al., 2000; Navazio et al., 2001). In plant cells two distinct subtypes of Ca^{2+}-ATPases have been found in the ER: ECA1, a type IIA ER-type Ca^{2+}/Mn^{2+} pump, which is sensitive to CPA (Sze et al., 2000; Pittman and Hirschi, 2003); and ACA2, a CaM-regulated type IIB Ca^{2+} pump, which is insensitive to CPA (Harper et al., 1998; Pittman and Hirschi, 2003). We investigated the role of CPA, a specific blocker of plant ER-type IIA Ca^{2+} pumps (Sze et al., 2000), in triggering cell death. We found that inhibition of ER Ca^{2+}-ATPase by CPA causes PCD involving a $[Ca^{2+}]_{cyt}$ increase. Our findings indicate that in soybean cells CPA induces an ER stress, which triggers a PCD program involving cytochrome c release from mitochondria and caspase-like activities. The participation of both ER and mitochondria suggests the existence of a cross-talk between these two compartments to achieve the program of cell death.

Materials and Methods

Chemicals
CPA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (acetoxymethil ester) (BAPTA-AM) and bafilomycin A_1 were obtained from Sigma-Aldrich (St Louis, USA). Coelenterazine was purchased from Molecular Probes Inc. (Eugene, OR).

Cell culture
Soybean (*Glicine max* L.) cell-suspension culture, transformed with the Ca^{2+}-sensitive photoprotein aequorin (Mithöfer et al., 1999; kindly provided by G. Neuhaus, Freiburg, Germany), were grown in a Murashige-Skoog liquid medium supplemented with 5% sucrose, 10 µg/ml naphthlaleneacetic acid, 2 µg/ml kinetin, 1 µg/ml thiamine-HCl and 10 µg/ml kanamycin. Cells were subcultured every 3 weeks by making a 1:10 dilution in 40 ml of fresh medium and maintained at 24°C with a 16-hour photoperiod with constant shaking. Treatments with CPA were performed 3 weeks after reinoculation, during the exponential growth phase of the cells.

SDS-PAGE and western blot analysis
Cells were harvested by centrifugation (5 minutes, 1600 rpm) and solubilized with a solution containing 0.1 M Tris, 0.2 M NaCl, 0.2% Triton X-100, 1 mM EDTA, 1 µM leupeptin and 0.5 mM phenylmethylsulphonylfluoride (PMFS). Protein extracts were separated by SDS-PAGE (10% polyacrylamide) (Laemmli, 1970) and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) for chemiluminescence detection, according to the method of Towbin et al. (Towbin et al., 1979). Filters were incubated with 5% skimmed milk in TBS for 1 hour and immunoblotted with the specific antibodies for 2 hours. Membranes were probed with polyclonal antisera raised against the tobacco binding protein (BiP) (1:5000, from A. Vitale, Milano, Italy) and spinach calreticulin (CRT) (1:2000, Navazio et al., 1995). Moreover, a polyclonal antibody against murine caspase 12 was used (1:5000, Oncogene, Boston, MA). After incubation, filters were washed twice with 0.05% Tween/TBS followed by one wash in TBS before incubation with the secondary antibody. Labeled proteins were detected using CDP-star™ (Biolabs, Hitchin, UK) and autoradiography films (Sigma-Aldrich, St Louis, USA). The exposed films were quantified by densitometric analysis using Quantity One and Molecular Analyst software (BioRad, Oakland, CA).

Protein quantification
Protein content in extracts was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as standard.

Aequorin-dependent Ca^{2+} measurements
Reconstitution of the Ca^{2+}-sensitive photoprotein aequorin was performed in vivo by incubating soybean cells with 5 µM coelenterazine overnight in darkness. Treatments with CPA were carried out by injecting 50 µl of twofold concentrated CPA solution (dissolved in the basal cell culture medium) into the same volume of reconstituted cell-suspension culture (about 3 mg fresh weight) using a light-tight syringe. For experiments carried out in the absence of extracellular Ca^{2+}, cells were extensively (10 vol) washed three times with Ca^{2+}-free culture medium and then resuspended in the same medium containing 100 µM EGTA. The residual aequorin was completely discharged by adding 0.33 M $CaCl_2$ in 10% ethanol. Luminescence data were collected and converted into $[Ca^{2+}]_{cyt}$ by a computer algorithm based on the Ca^{2+} response curve of aequorin (Brini et al., 1995).

Cell viability and treatments
The viability of cells was determined by incubation with 0.05% Evans Blue (Levine et al., 1996). Cells were then extensively washed with deionized water to remove excess and unbound dye. Dye bound to dead cells was solubilized in 50% methanol/1% SDS for 30 minutes at 50°C and quantified by absorbance at 600 nm. CPA was applied dissolved in dimethyl sulfoxide (DMSO, final solvent concentration, 0.1% v/v). DMSO had no effect on the viability of cells. The caspase inhibitors Ac-DEVD-CHO and Ac-LEHD-CHO (BioSource International, Inc., Camarillo, CA, USA) were dissolved in DMSO and applied either to cell suspension together with CPA or to cytosolic protein extracts.

Measurements of hydrogen peroxide
Hydrogen peroxide (H_2O_2) release by soybean cells was measured as described by Wolff (Wolff, 1994). Cells were treated and harvested at different time points to measure H_2O_2 release. The assay is based on a colorimetric reaction caused by the peroxide-mediated oxidation of Fe^{2+} followed by the reaction of Fe^{3+} with xylenol orange. 0.5 ml assay solution (0.5 mM ammonium ferrous sulfate, 50 mM H_2SO_4, 0.2 mM xylenol orange, 200 mM sorbitol) was added to 0.5 ml of control and treated soybean cells and the absorbance at 560 nm was detected after 45 minutes incubation.

Detection of cytochrome c release
Cytosolic protein extracts were prepared by incubating cells (10 minutes on ice) with 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose and 190 µg/ml digitonin. Cytosolic fractions were then separated by centrifugation (5 minutes, 13,000 *g*, 4°C), loaded (10 µg) onto a 12% acrylamide gel, transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) and probed with a rabbit polyclonal cytochrome c antibody (1 µg/ml dilution, Oncogene, Boston, MA) as described previously.

Caspase activity assays

Caspase 3 and caspase 9 activities were measured using the 'caspase 3 colorimetric activity assay kit' and the 'caspase 9 colorimentric activity assay kit' (Chemicon International, Inc., Temecula, CA), respectively, as described by the manufacturer. The assays are based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after the caspase-dependent cleavage from the labeled substrates DEVD-pNA or LEHD-pNA by active caspase 3 or 9, respectively. 15 µg of cytosolic protein extracts from soybean cells were incubated for 2 hours at 37°C with DEVD-pNA or LEHD-pNA, in the presence or absence of 20 µM caspase inhibitors Ac-DEVD-CHO (for caspase 3) or Ac-LEHD-CHO (for caspase 9). Caspase-like activity was determined by spectrophotometric quantification of the free pNA (λ_{em}=405 nm).

Nuclei staining

To analyze nuclear morphological changes induced by CPA, cells were stained with the fluorescent dyes Hoechst 33342 (HO) and propidium iodide (PI). Cells were incubated in darkness with 8 µg/ml HO (Sigma-Aldrich, St Louis, USA) and 5 µg/ml PI (Sigma-Aldrich, St. Louis, USA) at room temperature for 10 minutes and observed by fluorescence microscopy using an excitation light of 350 nm and 570 nm, respectively for the two dyes.

Statistical analysis

Data are presented as mean±s.d. of three independent experiments and the differences between groups were assessed with Student's t-test; statistically significant at $P<0.05$ (one asterisk) and $P<0.005$ (two asterisks).

Results

Induction of ER stress by CPA in soybean suspension cells

Previous reports indicate that the ER stress response produces an increase in the expression of some ER chaperones, such as BiP (Rao et al., 2001) and CRT (Llewellyn et al., 1996). Moreover caspase 12, the most recently characterized member of the caspase family, is activated only by ER stress inducers (Nakagawa et al., 2000). We investigated whether CPA treatment causes an ER stress by analyzing both the rise in protein level of the ER chaperones BiP and CRT and the activation of ER-resident caspase 12.

As shown in Fig. 1, incubation of cells (24 hours) with CPA (50 µM) increased both BiP [(1.5±0.1)-fold] (Fig. 1A) and CRT [(2.0±0.03)-fold] (Fig. 1B) expression levels, compared with untreated cells.

Fig. 1. Expression of ER proteins in response to CPA treatment. Protein extracts from untreated and CPA-treated soybean cells were analyzed by western blotting using anti-BiP (A), anti-CRT (B) and anti-caspase 12 (C) antibodies. Quantitative analyses were carried out as described in Materials and Methods and are represented by histograms: white bars, untreated cells (Co); grey bars, CPA-treated cells (CPA). In C the quantitative data refers to the procaspase 12-like (proc 12) protein band. Cells were treated with 50 µM CPA for 24 hours. The values for untreated cells were taken as 100%. Data are mean±s.d. of three independent experiments. Statistically significant at *$P<0.05$ and **$P<0.005$.

Moreover, although up to now no functional homologs of animal caspases have been identified in plants, by using an antibody against the murine caspase 12 we found a very specific cross-reacting protein band in protein extracts from soybean cells. This protein has an expected molecular mass of about 53 kDa and its expression is augmented by (1.7±0.2)-fold after CPA treatment (Fig. 1C). In addition, the procaspase 12-like seems to be processed after the CPA treatment as indicated by the presence of proteolytic fragments (Fig. 1C) of comparable molecular mass to active animal caspase 12 (Nakagawa et al., 2000). Taken together the data highlight the occurrence of an ER stress in soybean cells caused by CPA treatment.

Effect of CPA on $[Ca^{2+}]_{cyt}$

We investigated whether CPA treatment affects $[Ca^{2+}]_{cyt}$. Addition of CPA (50 µM) to soybean suspension cells was found to induce, in temporal terms, a biphasic Ca^{2+} signal: an immediate sharp elevation in $[Ca^{2+}]_{cyt}$, which dissipated within 5 minutes, was followed by a second flattened Ca^{2+} increase (Fig. 2A). The first Ca^{2+} rise peaked at 0.44±0.05 µM 100 seconds after CPA administration, whereas the second Ca^{2+} elevation reached 0.30±0.03 µM after 10 minutes and did not return to basal level in the time evaluated (15 minutes).

To analyze whether extracellular Ca^{2+} influx contributed to CPA-induced Ca^{2+} changes, cells were extensively washed and resuspended in Ca^{2+}-free medium supplemented with 100 µM EGTA to chelate any residual Ca^{2+} ions. Removal of the external Ca^{2+} pools did not markedly affect the biphasic response (Fig. 2A). The observed limited reduction of the first
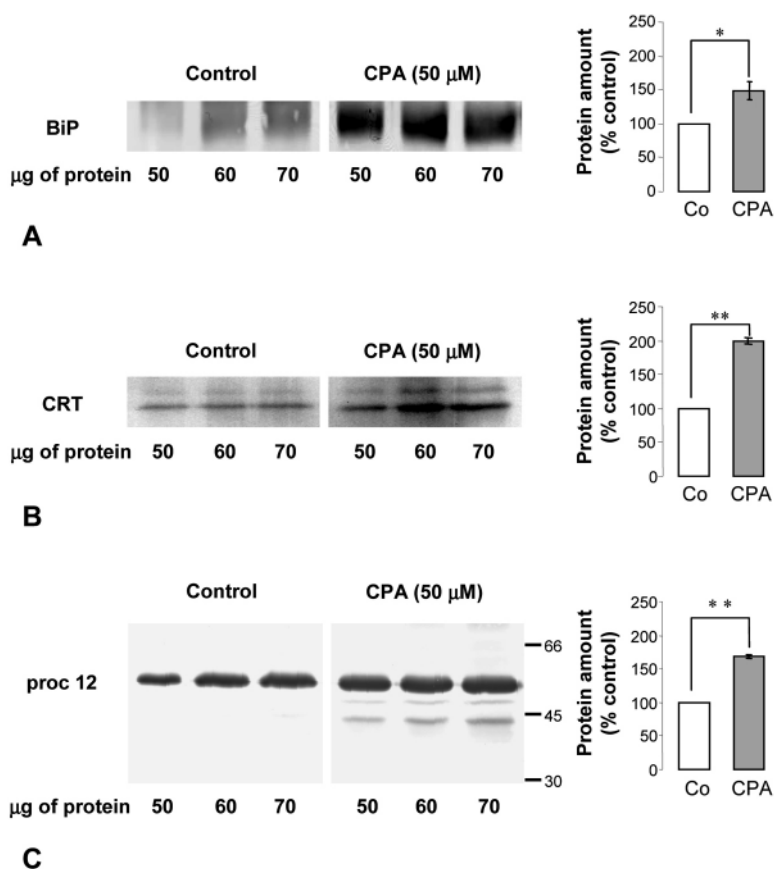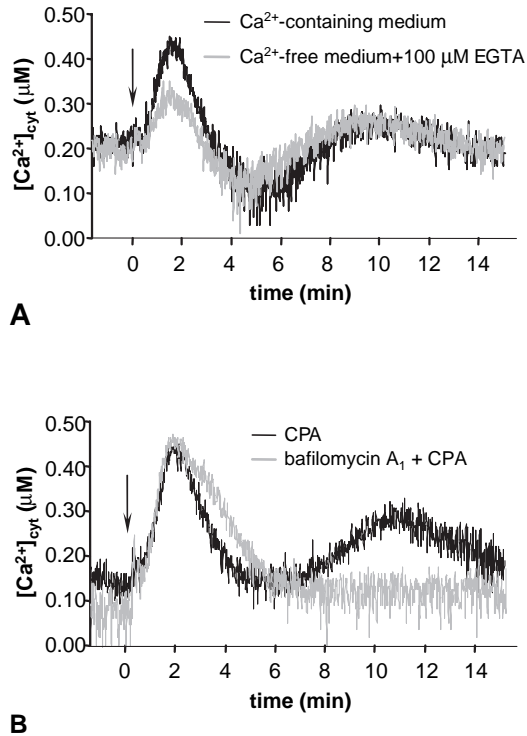
Fig. 2. Ca^{2+} responses of soybean cells to 50 μM CPA treatment. (A) Black trace, $[Ca^{2+}]_{cyt}$ elevations induced by CPA in soybean cells grown in normal culture medium; grey trace, $[Ca^{2+}]_{cyt}$ elevations induced by CPA in soybean cells incubated in Ca^{2+}-free medium containing 100 μM EGTA. (B) Black trace, $[Ca^{2+}]_{cyt}$ elevations induced by CPA in soybean cells grown in normal culture medium; grey trace, $[Ca^{2+}]_{cyt}$ elevations induced by CPA in soybean cells pre-treated for 1 hour with 200 nM bafilomycin A_1. Traces are representative of three independent experiments, which gave very similar results. Arrow, time of CPA administration.

Ca^{2+} peak (from 0.44 to 0.33 μM) is probably due to a partial depletion of intracellular Ca^{2+} stores following the EGTA treatment (Cessna and Low, 2001), rather than a participation of extracellular Ca^{2+} in the Ca^{2+} rise induced by CPA. Thus, the increase in $[Ca^{2+}]_{cyt}$ seems to be mainly due to Ca^{2+} release from intracellular stores.

Addition of 200 nM bafilomycin A_1, a highly specific inhibitor of vacuolar-type H^+-ATPase inducing a reduction of the storage capacity of the vacuole (Takahashi et al., 1997), resulted in the complete abolition of the second Ca^{2+} peak (Fig. 2B). This suggests the possibility that Ca^{2+} release from the vacuole may account for the second Ca^{2+} transient.

Effect of CPA on viability of cultured soybean cells

Soybean suspension cells were treated with 50 μM CPA and cell viability was assessed with Evans Blue staining (Levine et al., 1996), as lethally damaged cells are unable to exclude the dye. Exposure of cells for 24 hours to CPA significantly decreased cell viability: CPA induces a rise in cell death of about (1.7±0.08)-fold compared to untreated cells (Fig. 3). Thus a continuous exposure of cells to CPA triggers cell death within 24 hours.

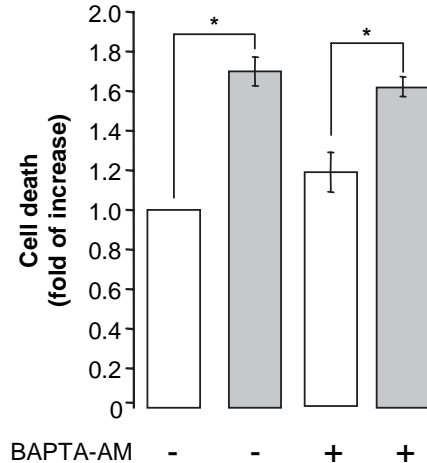Treatment of cells with the intracellular Ca^{2+} chelator

Fig. 3. Effect of CPA on viability of soybean cells. Exponentially growing cells were treated with CPA (50 μM) for 24 hours (grey bars). Pre-treatment with 10 μM BAPTA-AM was carried out 1 hour before the 24-hour CPA treatment. Cells cultured without CPA were taken as a control (white bars). Data are means±s.d. of three independent experiments. Statistically significant at *$P<0.05$.

BAPTA-AM (10 μM for 1 hour) prior to CPA treatment (24 hours), although causing an effective chelation of $[Ca^{2+}]_{cyt}$ (data not shown), did not produce any significant change in the percentage of cell death compared with CPA-treated cells (Fig. 3).

H_2O_2 production in response to ER stress

To examine the events that link ER stress to cell death in soybean cells challenged with CPA we monitored some of the recognized hallmarks of PCD such as the accumulation of H_2O_2, cytochrome c release, caspase-like activity and chromatin condensation.

A tightly controlled generation of reactive oxygen species is crucial in the apoptotic pathway (Jabs, 1999). In particular, H_2O_2 functions as a signaling molecule, which could drive PCD induced by various stimuli in plant cells (Beers and McDowell, 2001). Treatment of soybean cells with CPA resulted in a transient accumulation of H_2O_2 in the culture medium. The H_2O_2 concentration started to increase rapidly within 2 minutes after the treatment, reaching a maximum (169 μM ±1.66 μM) at about 25 minutes, and declined more slowly without getting back to the baseline level within the considered time lapse (Fig. 4). Untreated cells show a baseline production of H_2O_2 of less than 16 μM in the medium (Fig. 4).

Involvement of mitochondria in CPA-induced cell death

Mitochondria play a central role in the initiation of apoptosis in animal cells by releasing cytochrome c into the cytosol with the resulting activation of caspase 9 (Desagher and Martinou, 2000). We evaluated the release of cytochrome c on cytosolic protein extracts by western blot analysis. Fig. 5 shows CPA-induced release of cytochrome c in soybean cells. Cytosolic extracts from untreated cells do not contain any detectable cytochrome c (Fig. 5). The presence of caspase 9-like activity was monitored using the synthetic substrate LEHD conjugate
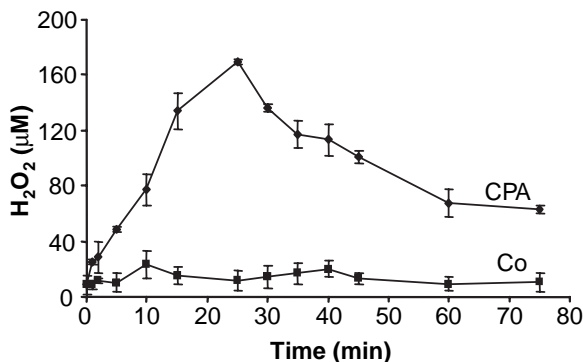
Fig. 4. H₂O₂ accumulation in the culture medium in response to CPA treatment. Co, untreated cells; CPA, CPA-treated cells (50 µM). H₂O₂ concentration is normalized per g fresh weight. Traces represent the mean±s.d. of three independent experiments.

Fig. 5. Cytochrome c release. Cytochrome c accumulation in the cytosol was assessed by western blot analysis: control, cytosolic extracts from untreated cells; CPA, cytosolic extracts from cells incubated with 50 µM CPA for 24 hours.

Fig. 6. Caspase 9-like activity in soybean cells treated with CPA. White bar, untreated cells; grey bar, cells treated for 24 hours with 50 µM CPA; black bar, cells treated for 24 hours with 50 µM CPA in the presence of the caspase 9 inhibitor Ac-LEHD-CHO (20 µM). Caspase 9-like activity in untreated cells was taken as 100%. Data are means±s.d. of three independent experiments. Statistically significant at **$P<0.005$.

Fig. 7. Assay for caspase 3 enzymatic activity in soybean cell-suspension culture. White bar, untreated cells; grey bar, cells treated for 24 hours with 50 µM CPA; black bar, cells treated for 24 hours with 50 µM CPA in the presence of the caspase 3 inhibitor Ac-DEVD-CHO (20 µM). Caspase 3-like activity in untreated cells was taken as 100%. Data are means±s.d. of three independent experiments. Statistically significant at **$P<0.005$.

with the chromophore pNA. We found that cytosolic extracts from CPA-treated cells show about twofold increase in caspase 9-like activity compared with the control (Fig. 6). In addition, application of the caspase 9 inhibitor Ac-LEHD-CHO (20 µM) together with CPA, blocked the protease activation (Fig. 6).

CPA induces the activity of a caspase 3-like protease

The release of cytochrome c from mitochondria leads to the formation of a cytoplasmic complex with Apaf-1 and the caspase 9 protease, which in turn promotes a cascade of caspase activation, including caspase 3 (Cain et al., 2002). In our study a synthetic tetrapeptide sequence (DEVD), based on the caspase 3 cleavage site and conjugated to pNA, was used for the assay of caspase 3-like protease activity in soybean cells. As shown in Fig. 7, caspase 3-like activity was significantly activated after 24 hours of incubation with CPA. Cytosolic fractions of CPA-treated soybean cells show about twofold increase in caspase 3-like activity compared to untreated cells (Fig. 7); moreover, this activity was blocked in the presence of 20 µM of the caspase 3 inhibitor Ac-DEVD-CHO (Fig. 7).

In an attempt to further define the involvement of caspase-like activities in CPA-induced cell death, the same tetrapeptide inhibitors of caspase 9 and caspase 3 (20 µM) were administered to cell cultures 30 minutes prior to CPA treatment. Inhibition of caspases failed to block CPA-induced cell death, as tested with the Evans Blue cell viability assay (data not shown). Similar results have been previously obtained in different animal experimental systems, and interpreted as a redirection of cells towards necrosis when the apoptotic pathway is blocked by caspase inhibitors (Vercammen et al., 1998a; Vercammen et al., 1998b; Hetz et al., 2002).
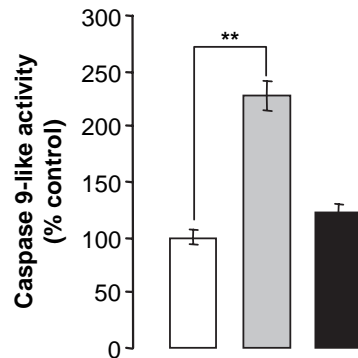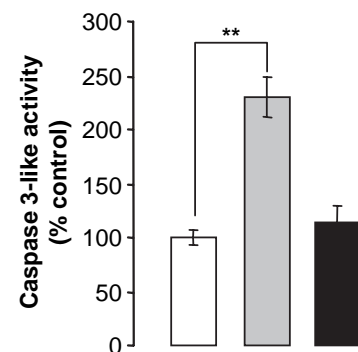
Detection of CPA-induced chromatin condensation and morphological changes

To further explore the nature of CPA-induced cell death, we evaluated the cell morphological changes by light microscopy and double staining of the nuclei with the fluorophores HO and PI. Untreated cells show a HO fluorescence homogenously distributed in the cell nuclei without chromatin condensation (Fig. 8B) and a negative PI staining (Fig. 8C). This staining pattern occurs in about 95% of the control cells analyzed. The nuclei of CPA-treated (24 hours) cells exhibit condensed chromatin with regions of nonhomogeneous HO fluorescence (Fig. 8E,H), a staining pattern consistent with apoptosis in animal cells (Maruyama et al., 2000). The presence of both early (HO⁺ and PI⁻ nuclei) and late (HO⁺ and PI⁺ nuclei) PCD stages is observable in the same treated cell population (Fig. 8E,F,H,I) probably because of its asinchronicity. Fig. 8G shows the detachment of the plasma membrane from the cell wall, which is considered as a hallmark of PCD in plant cells (Beers and McDowell, 2001). In CPA-treated cells this alteration
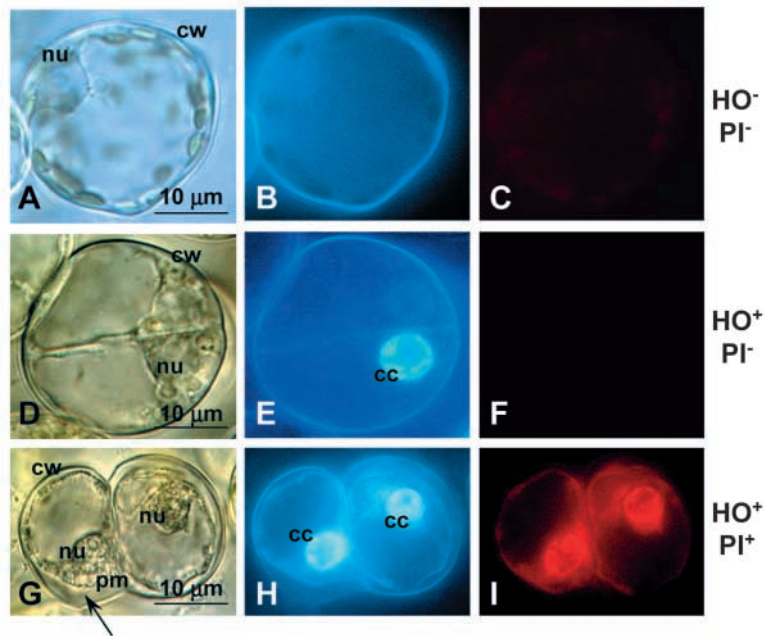
Fig. 8. Changes in nuclear morphology in response to CPA treatment. Nuclei of 24-hour CPA-treated cells were stained with Hoechst 33342 and propidium iodide and processed for fluorescent microscopy. (A-C) Untreated cells. (D-I) 50 µM CPA-treated (24 hours) cells. (E,F) Early PCD nucleus. (H,I) Late PCD nuclei. Pictures represent typical examples. cc, chromatin condensation; cw, cell wall; nu, nucleus; pm, plasma membrane. The arrow indicates the detachment of the plasma membrane from the cell wall in CPA-treated cells.

occurs progressively as the PCD program advances (Fig. 8D,G). This morphology is not present in untreated cells (Fig. 8A). The analysis of genomic DNA extracted from CPA-treated soybean cells by agarose gel did not show the presence of DNA fragmentation within the 24 hours of treatment (data not shown).

Discussion

In this paper, we demonstrate that in soybean cells CPA determines an ER stress, which in turn induces a PCD program involving a biphasic increase in $[Ca^{2+}]_{cyt}$, generation of H_2O_2, release of mitochondrial cytochrome c, activation of caspase-like proteases and chromatin condensation.

Accumulating evidence shows that besides its crucial role as an important Ca^{2+} store and in protein maturation, folding and transport, the ER is emerging as a central player in apoptosis (Rizzuto et al., 1998; Berridge, 2002). The data so far available comes mainly from mammalian cells, although the precise involvement of the ER in the apoptotic response has not yet been completely unraveled. In animal cells disturbance of ER functions have been shown to trigger apoptotic pathways involving a cross-talk between ER and mitochondria, which is regulated by Bcl-2 protein, and the activation of ER-resident caspase 12 as mediator of death signaling (Hacki et al., 2000; Nakagawa and Yuan, 2000; Nakagawa et al., 2000). In particular, caspase 12 knockout mice are resistant to ER stress-induced apoptosis while their cells undergo apoptosis in response to stimuli (i.e. staurosporine) that do not involve ER stress (Nakagawa and Yuan, 2000). Compared with animal systems, relatively scarce knowledge is available on the detailed mechanism of PCD in plants. However, some aspects of the molecular machinery of PCD seem to be conserved between plants and animals. So far, the role of the ER in plant PCD has been suggested in sycamore cell cultures treated with tunicamycin and brefeldin A (Crosti et al., 2001). The authors argue that chemicals that interfere with ER functions induce PCD with apoptotic features.

In a wide variety of animal cell types, inhibiting ER Ca^{2+}-ATPase activity with TG or structurally unrelated Ca^{2+}-ATPase antagonists, such as CPA, induces apoptosis, which is triggered by ER Ca^{2+} depletion (Zhou et al., 1998; Nguyen et al., 2002; Zuppini et al., 2002). We found that in soybean cell suspension cultures CPA induces cell death when applied at 50 µM for 24 hours. Our data suggest that lowering ER Ca^{2+} concentration by CPA treatment is a sufficient signal to initiate a specific PCD program. Incubation of cells with CPA causes an ER stress response, highlighted by an enhanced transcription of CRT and BiP. Both proteins have been shown to increase their expression in response to stimuli that disrupt ER homeostasis.

In animal systems apoptosis is mediated by the key enzymatic activity of a class of specific proteases: the caspases (Nicholson, 1999). Although no direct homologues of caspase genes have been identified in the plant genome so far, caspase-like activities have been reported in PCD induced by different treatments and during HR cell death (del Pozo and Lam, 1998; Sun et al., 1999). Recently, based on homology searches, a family of caspase-related proteases (the metacaspases) has been identified in *Arabidopsis* (Uren et al., 2000). In the investigations reported here, western blot analysis on protein extracts from soybean cells with an antibody raised against murine caspase 12 showed the presence of a cross-reacting protein band, which seems to increase in its expression after the CPA treatment and to be processed into proteolytic fragments of a molecular weight comparable to mammalian active caspase 12. Although a molecular approach is needed to firmly establish the nature of this protein, our data suggest the involvement of a protein structurally related to animal caspase 12 (one of the most important mediator of ER stress-induced apoptosis) in plant ER-mediated PCD.

The role of cytosolic Ca^{2+} as pro-apoptotic messenger involved in triggering apoptosis and in regulating death-specific enzymes has been ascertained in animal and plant systems (Levine et al., 1996; Nicotera and Orrenius, 1998). In this study we show that inhibition of ER Ca^{2+}-ATPase activity after the application of CPA causes, in soybean cells, a biphasic

increase in $[Ca^{2+}]_{cyt}$ as a result of the ion release mainly from intracellular stores (ER and vacuole). The participation of the vacuolar compartment in the CPA-induced Ca^{2+} signaling is suggested by the annulment of the second rise in $[Ca^{2+}]_{cyt}$ by bafilomycin A_1. PCD induction seems to be signaled in response to ER Ca^{2+} lowering rather than $[Ca^{2+}]_{cyt}$ elevation. Indeed, inhibiting the increase of cytosolic Ca^{2+} with BAPTA-AM did not prevent CPA-induced cell death.

Mitochondria function as major checkpoint in dictating cell survival or cell death by integrating Ca^{2+} and apoptotic stimuli in animal cells. Mitochondrial Ca^{2+} accumulation seems to trigger the opening of permeability transition pore with the consequent release of apoptogenic proteins (Bernardi et al., 1999). Previous data on endothelial cells have shown an accumulation of Ca^{2+} within mitochondria as a result of CPA treatment (Jornot et al., 1999). Several reports also suggest the key role of mitochondria in plant PCD induced by various stimuli (Lam et al., 1999; Jones, 2000). Particularly, the requirement of Ca^{2+} localization in the mitochondrial matrix seems to be important for the permeability transition pore opening and cytochrome c release (Virolainen et al., 2002). Cytochrome c leakage and activation of caspase-like proteases were detected in our study during CPA-induced PCD, suggesting the involvement of mitochondria in the death pathway. Our results indicate an increase in caspase 9-like activity in cytosolic extracts of soybean cells treated with CPA, which is blocked by the specific inhibitor Ac-LEHD-CHO of animal caspase 9. Furthermore, the presence of caspase 3-like protease activity was detected from the specific cleavage of the caspase 3 substrate in cells treated with CPA. Also in this case, the elevation in the protease activity was successfully prevented by the use of the caspase 3 inhibitor Ac-DEVD-CHO. The existence of a caspase-like proteolytic activity and its essential role in plant PCD have been strongly suggested in several plant systems (Sun et al., 1999; Mlejnek and Prochàzka, 2002). Caspase-like activity has been reported in tobacco tissue undergoing HR (del Pozo and Lam, 1998) and specific inhibitors of animal caspase 1 and 3 prevent menadione-induced PCD in tobacco protoplasts (Sun et al., 1999).

A number of recent studies have tried to prove whether plant PCD induced by various elicitors occurs with morphological and physiological features similar to those observed during apoptotic cell death in animal cells (Mlejnek and Prochàzka, 2002; Hoeberichts and Woltering, 2003). One of such hallmarks is the condensation of chromatin followed by DNA fragmentation into 180 bp fragments or multiples of these (ladders). Damage of DNA with ladder formation has been observed in CPA-induced cell death in animal cells (Zhou et al., 1998). In plants, DNA fragmentation has been reported in several PCD processes (Sun et al., 1999; Mlejnek and Prochàzka, 2002), although plant PCD occurring without laddering has also been observed (Dangl et al., 1996; Fukuda, 2000). We provide evidence that CPA induces chromatin condensation in soybean cells, but no DNA fragmentation into ladders was found within 24 hours of treatment (data not shown). Similar results have been obtained for PCD induced by other stimuli. Nitric oxide for example induces apoptosis in mammalian cells occurring with DNA laddering (Pinsky et al., 1999), whereas in *Arabidopsis* suspension cultures a condensation of nuclear chromatin without DNA laddering has been observed during nitric oxide-induced PCD (Clarke et al.,

2000). Thus, despite the recognized evolutionary conservation of some mechanisms underlying PCD in plants and animals, some features seem to rely on the considered cell type and PCD-inducing stimulus.

In summary, our data show that inhibition of the ER Ca^{2+}-ATPase by CPA causes stress in the ER of soybean cells. In response to the induced stress, cells transiently increase $[Ca^{2+}]_{cyt}$ and undergo a PCD program involving mitochondria and caspase-like activities. We propose that the PCD pathway activated by CPA might involve a cross-talk between ER and mitochondria to fulfil the cell death program.

References

Aridor, M. and Balch, W. E. (1999). Integration of endoplasmic reticulum signaling in health and disease. *Nat. Med.* **5**, 745-751.

Arnaudeau, S., Frieden, M., Nakamura, K., Castelbou, C., Michalak, M. and Demaurex, N. (2002). Calreticulin differentially modulates calcium uptake and release in the endoplasmic reticulum and mitochondria. *J. Biol. Chem.* **277**, 46696-46705.

Beers, E. P. and McDowell, J. M. (2001). Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. *Curr. Opin. Plant Biol.* **4**, 561-567.

Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V. and di Lisa, F. (1999). Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur. J. Biochem.* **264**, 687-701.

Berridge, M. J., Lipp, P. and Bootman, M. D. (2000). The versatility and universality of calcium signaling. *Nat. Rev. Mol. Cell. Biol.* **1**, 11-21.

Berridge, M. J. (2002). The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* **32**, 235-249.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Brini, M., Marsault, R., Bastianutto, C., Alvarez, J., Pozzan, T. and Rizzuto, R. (1995). Transfected aequorin in the measurements of cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$). *J. Biol. Chem.* **270**, 9896-9903.

Brostrom, C. O. and Brostrom, M. A. (1998). Regulation of translational initiation during cellular responses to stress. *Prog. Nucleic Acid Res. Mol. Biol.* **58**, 79-125.

Cain, K., Bratton, S. B. and Cohen, G. M. (2002). The Apaf-1 apoptosome: a large caspase-activating complex. *Biochimie* **84**, 203-214.

Cessna, S. G. and Low, P. S. (2001). An apoplastic Ca^{2+} sensor regulates internal Ca^{2+} release in aequorin-transformed tobacco cells. *J. Biol. Chem.* **276**, 10655-10662.

Clarke, A., Desikan, R., Hurst, R. D., Hancock, J. T. and Neill, S. J. (2000). NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. *Plant J.* **24**, 667-677.

Crosti, P., Malerba, M. and Bianchetti, R. (2001). Tunicamycin and Brefeldin A induce in plant cells a programmed cell death showing apoptotic features. *Protoplasma* **216**, 31-38.

Dangl, J. L., Dietrich, R. A. and Richberg, M. H. (1996). Death don't have no mercy: cell death programs in plant-microbe interactions. *Plant Cell* **8**, 1793-1807.

Danon, A., Delorme, V., Mailhac, N. and Gallois, P. (2000). Plant programmed cell death: a common way to dye. *Plant Physiol. Biochem.* **38**, 647-655.

del Pozo, O. and Lam, E. (1998). Caspases and programmed cell death in the hypersensitive response of plants to pathogens. *Curr. Biol.* **8**, 1129-1132.

Desagher, S. and Martinou, J. C. (2000). Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* **10**, 369-377.

Fukuda, H. (2000). Programmed cell death of tracheary elements as a paradigm in plants. *Plant Mol. Biol.* **44**, 245-253.

Grebenová, D., Kuzelová, K., Smetana, K., Pluskalová, M., Cajthamlová, H., Marinov, I., Fuchs, O., Soucek, J., Jarolim, P. and Hrkal, Z. (2003). Mitochondrial and endoplasmic reticulum stress-induced apoptotic pathways are activated by 5-aminolevulinic acid-based photodynamic therapy in HL60 leukemia cells. *J. Photochem. Photobiol.* **69**, 71-85.

Gunawardena, A. H., Pearce, D. M., Jackson, M. B., Hawes, C. R. and Evans, D. E. (2001). Characterisation of programmed cell death during aerenchyma formation induced by ethylene or hypoxia in roots of maize (*Zea mays* L.). *Planta* **212**, 205-214.

Hacki, J., Egger, L., Monney, L., Conus, S., Rosse, T., Fellay, I. and Borner, C. (2000). Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene* **19**, 2286-2295.

Harper, J. F., Hong, B., Hwang, I., Guo, H. Q., Stoddard, R., Huang, J. F., Palmgren, M. G. and Sze, H. (1998). A novel calmodulin-regulated Ca^{2+}-ATPase (ACA2) from *Arabidopsis* with an N-terminal autoinhibitory domain. *J. Biol. Chem.* **273**, 1099-1106.

Heath, M. C. (2000). Nonhost resistance and nonspecific plant defences. *Curr. Opin. Plant Biol.* **3**, 315-319.

Hetz, C. A., Hunn, M., Rojas, P., Torres, V., Leyton, L. and Quest, A. F. G. (2002). Caspase-dependent initiation of apoptosis and necrosis by the Fas receptor in lymphoid cells: onset of necrosis is associated with delayed ceramide increase. *J. Cell Sci.* **115**, 4671-4683.

Hoeberichts, F. A. and Woltering, E. J. (2003). Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. *BioEssays* **25**, 47-57.

Jabs, T. (1999). Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem. Pharmacol.* **57**, 231-245.

Jimbo, A., Fujita, E., Kouroku, Y., Ohnishi, J., Inohara, N., Kuida, K., Sakamaki, K., Yonehara, S. and Momoi, T. (2003). ER stress induces caspase-8 activation, stimulating cytochrome c release and caspase-9 activation. *Exp. Cell Res.* **283**, 156-166.

Jones, A. (2000). Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? *Trends Plant Sci.* **5**, 225-230.

Jornot, L., Maechler, P., Wollheim, C. B. and Junod, A. F. (1999). Reactive oxygen metabolites increase mitochondrial calcium in endothelial cells: implication of the Ca^{2+}/Na$^+$ exchanger. *J. Cell Sci.* **112**, 1013-1022.

Katoch, B., Sebastian, S., Sahdev, S., Padh, H., Hasnain, S. E. and Begum, R. (2002). Programmed cell death and its clinical implications. *Indian J. Exp. Biol.* **40**, 513-524.

Kuriyama, H. and Fukuda, H. (2002). Developmental programmed cell death in plants. *Curr. Opin. Plant Biol.* **5**, 568-573.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lam, E., Pontier, D. and del Pozo, O. (1999). Die and let live – programmed cell death in plants. *Curr. Opin. Plant Biol.* **2**, 502-507.

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

Llewellyn, D. H., Kendall, J. M., Sheikh, F. N. and Campbell, A. K. (1996). Induction of calreticulin expression in HeLa cells by depletion of the endoplasmic reticulum Ca^{2+} store and inhibition of N-linked glycosylation. *Biochem. J.* **318**, 555-560.

Maruyama, W., Sango, K., Iwasa, K., Minami, C., Dorstert, P., Kawai, M., Moriyasu, M. and Naoi, M. (2000). Dopaminergic neurotoxins, 6,7-dihydroxy-1-(3′,4′-dihydroxybenzyl)-isoquinolines, cause different types of cell death in SH-SY5Y cells: apoptosis was induced by oxidized papaverolines and necrosis by reduced tetrahydropapaverolines. *Neurosci. Lett.* **291**, 89-92.

Mason, M. J., Garcia-Rodriguez, C. and Grinstein, S. (1991). Coupling between intracellular Ca^{2+} stores and the Ca^{2+} permeability of the plasma membrane. Comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. *J. Biol. Chem.* **266**, 20856-20862.

McCabe, P. F. and Leaver, C. J. (2000). Programmed cell death in cell cultures. *Plant Mol. Biol.* **44**, 359-368.

Meldolesi, J. and Pozzan, T. (1998). The endoplasmic reticulum Ca^{2+} store: a view from the lumen. *Trends Biochem. Sci.* **23**, 10-14.

Mithöfer, A., Ebel, J., Bhagwat, A. A., Boller, T. and Neuhaus-Url, G. (1999). Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with β-glucan or chitin elicitors. *Planta* **207**, 566-574.

Mlejnek, P. and Prochàzka, S. (2002). Activation of caspase-like proteases and induction of apoptosis by isopentenyladenosine in tobacco BY-2 cells. *Planta* **215**, 158-166.

Nakagawa, T. and Yuan, J. (2000). Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* **150**, 887-894.

Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A. and Yuan, J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* **403**, 98-103.

Navazio, L., Baldan, B., Dainese, P., James, P., Damiani, E., Margreth, A. and Mariani, P. (1995). Evidence that spinach leaves express calreticulin but not calsequestrin. *Plant Physiol.* **109**, 983-990.

Navazio, L., Bewell, M. A., Siddiqua, A., Dickinson, G. D., Galione, A. and Sanders, D. (2000). Calcium release from the endoplasmic reticulum of higher plants elicited by the NADP metabolite nicotinic acid adenine dinucleotide phosphate. *Proc. Natl. Acad. Sci. USA* **97**, 8693-8698.

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.

Neill, S., Desikan, R. and Hancock, J. (2002). Hydrogen peroxide signaling. *Curr. Opin. Plant Biol.* **5**, 388-395.

Nguyen, H. N., Wang, C. and Perry, D. C. (2002). Depletion of intracellular calcium stores is toxic to SH-SY5Y neuronal cells. *Brain Res.* **924**, 159-166.

Nicholson, D. W. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* **6**, 1028-1042.

Nicotera, P. and Orrenius, S. (1998). The role of calcium in apoptosis. *Cell Calcium* **23**, 173-180.

Pahl, H. L. (1999). Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol. Rev.* **79**, 683-701.

Paschen, W. and Doutheil, J. (1999). Disturbance of endoplasmic reticulum functions: a key mechanism underlying cell damage? *Acta Neurochir.* **Suppl. 73**, 1-5.

Pinsky, D. J., Aji, W., Szabolcs, M., Athan, E. S., Liu, Y., Yang, Y. M., Kline, R. P., Olson, K. E. and Cannon, P. J. (1999). Nitric oxide triggers programmed cell death (apoptosis) of adult rat ventricular myocytes in culture. *Am. J. Physiol.* **277**, H1189-H1199.

Pittman, J. K. and Hirschi, K. D. (2003). Don't shoot the (second) messenger: endomembrane transporters and binding proteins modulate cytosolic Ca^{2+} levels. *Curr. Opin. Plant Biol.* **6**, 257-262.

Rao, R. V., Hermel, E., Castro-Obregon, S., del Rio, G., Ellerby, L. M., Ellerby, H. M. and Bredesen, D. E. (2001). Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. *J. Biol. Chem.* **276**, 33869-33874.

Rizzuto, R., Pinton, P., Carrington, W., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. and Pozzan, T. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. *Science* **280**, 1763-1766.

Sun, Y. L., Zhao, Y., Hong, X. and Zhai, Z. H. (1999). Cytochrome c release and caspase activation during menadione-induced apoptosis in plants. *FEBS Lett.* **462**, 317-321.

Sze, H., Liang, F., Hwang, I., Curran, A. C. and Harper, J. F. (2000). Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 433-462.

Takahashi, K., Isobe, M., Knight, M. R., Trewavas, A. J. and Muto, S. (1997). Hypoosmotic shock induces increases in cytosolic Ca^{2+} in tobacco suspension-culture cells. *Plant Physiol.* **113**, 587-594.

Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA* **87**, 2466-2470.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Uren, A. G., O'Rourke, K., Aravind, L. A., Pisabarro, M. T., Seshagiri, S., Koonin, E. V. and Dixit, V. M. (2000). Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. *Mol. Cell* **6**, 961-967.

Vercammen, D., Beyaert, R., Denecker, G., Goossens, V., van Loo, G., Declercq, W., Grooten, J., Fiers, W. and Vandenabeele, P. (1998a). *J. Exp. Med.* **187**, 1477-1485.

Vercammen, D., Brouckaert, G., Denecker, G., van de Craen, M., Declercq, W., Fiers, W. and Vandenabeele, P. (1998b). Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J. Exp. Med.* **188**, 919-930.

Virolainen, E., Blokhina, O. and Fagerstedt, K. (2002). Ca^{2+}-induced high amplitude swelling and cytochrome c release from wheat (*Triticum aestivum* L.) mitochondria under anoxic stress. *Ann. Bot.* **90**, 509-516.

Wolff, S. P. (1994). Ferrous ion oxidation in presence of ferric ion indicator xylenol orange for measurement of hydroperoxides. *Methods Enzymol.* **233**, 182-189.

Zhou, Y. P., Teng, D., Dralyuk, F., Ostrega, D., Roe, M. W., Philipson, L. and Polonsky, K. S. (1998). Apoptosis in insulin-secreting cells. Evidence for the role of intracellular Ca^{2+} stores and arachidonic acid metabolism. *J. Clin. Invest.* **101**, 1623-1632.

Zuppini, A., Groenendyk, J., Cormack, L. A., Shore, G., Opas, M., Bleackley, R. C. and Michalak, M. (2002). Calnexin deficiency and endoplasmic reticulum stress-induced apoptosis. *Biochemistry* **41**, 2850-2858.