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# Cyclosporin A inhibits programmed cell death and cytochrome *c* release induced by fusicoccin in sycamore cells

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**Summary.** Programmed cell death plays a vital role in normal plant development, response to environmental stresses, and defense against pathogen attack. Different types of programmed cell death occur in plants and the involvement of mitochondria is still under investigation. In sycamore (*Acer pseudoplatanus* L.) cultured cells, the phytotoxin fusicoccin induces cell death that shows apoptotic features, including chromatin condensation, DNA fragmentation, and release of cytochrome *c* from mitochondria. In this work, we show that cyclosporin A, an inhibitor of the permeability transition pore of animal mitochondria, inhibits the cell death, DNA fragmentation, and cytochrome *c* release induced by fusicoccin. In addition, we show that fusicoccin induces a change in the shape of mitochondria which is not prevented by cyclosporin A. These results suggest that the release of cytochrome *c* induced by fusicoccin occurs through a cyclosporin A-sensitive system that is similar to the permeability transition pore of animal mitochondria and they make it tempting to speculate that this release may be involved in the phytotoxin-induced programmed cell death of sycamore cells.

**Keywords:** *Acer pseudoplatanus*; Cyclosporin A; Cytochrome *c*; Fusicoccin; Permeability transition pore; Programmed cell death.

**Abbreviations:** BiP binding protein; CsA cyclosporin A; ER endoplasmic reticulum; FC fusicoccin; NO nitric oxide; PCD programmed cell death; PTP permeability transition pore; TUNEL terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

## Introduction

Programmed cell death (PCD) is a genetically controlled process present in all living organisms, aimed at eliminating unwanted or detrimental cells.

Biochemical, genetic, and molecular studies highlight the importance of mitochondria in the regulation of PCD in animals (Desagher and Martinou 2000). The role of mitochondria seems to include interpretation as well as amplification of cell stress signals originating from other cell structures and compartments. These signals can modify the permeability of the outer mitochondrial membrane, leading to the release of a number of PCD activators. Cytochrome *c* is the best studied of these activators. It binds the apoptotic ATPase APAF1-CED4 in the cytosol and activates caspase-9, the first member of a specific family of cysteine proteases called caspases. Caspase-9 in turn recruits and proteolytically activates other caspases, already present as zymogens, and the last caspases then cleave specific target proteins (Green 2000).

PCD in plants plays a pivotal role in many developmental processes and it is involved in defense mechanisms against biotic and abiotic stresses (Lam 2004). A role for the mitochondrion as integrator of cell stress and regulator of PCD has also been proposed for plants (Jones 2000). Although the translocation of cytochrome *c* to the cytoplasm has been reported and correlated with the induction of cell death in some plant systems (Lam et al. 2001), its possible function as a cell death signal, rather than a mere consequence of mitochondrial destruction, remains to be shown (Lam 2004).

There are at least two mechanisms for the release of cytochrome *c* in animal mitochondria. The first one involves the formation of a transient pore, the permeability transition pore (PTP), constituted by the transient complex of the voltage-dependent anion channel on the outer membrane, the adenine nucleotide transporter from the inner membrane, and cyclophilin D in the matrix. It is thought that the rapid flux of water and solutes through this nonselective

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channel causes the decay of membrane potential, the uncoupling of oxidative phosphorylation from electron flux, and the swelling of the matrix, leading to the rupture of the outer membrane and release of cytochrome *c* (Green and Reed 1998). The second mechanism occurs directly via the voltage-dependent anion channel when specific members of the family of Bcl-2 proteins bind to the channel and modify its permeability (Shimizu et al. 1999). This mechanism, at least in some cases, does not involve the swelling of mitochondria. The immunosuppressive drug cyclosporin A (CsA) has been used to discriminate between the two mechanisms. CsA specifically binds cyclophilin D in the matrix, preventing the formation of the PTP and, thus, the release of cytochrome *c* (Halestrap and Davidson 1990).

The mechanism of this release in plant mitochondria is poorly understood. Although CsA-sensitive changes in the permeability of the inner mitochondrial membrane were reported years ago in isolated pea mitochondria (Vianello et al. 1995), convincing evidence for the presence of a CsA-sensitive system similar to the PTP has only recently been provided both in intact cells and isolated mitochondria. Arpagaus and co-workers (2002) have shown that $Ca^{2+}$ induces a phosphate-dependent swelling in isolated potato mitochondria that is fully inhibited by CsA. This swelling is favored by anoxic conditions and involves the release of cytochrome *c*, which appears to be inhibited by CsA. In addition, nitric oxide (NO) donors cause a PCD in *Citrus sinensis* cultured cells that shows chromatin condensation and fragmentation and a loss of the mitochondrial membrane potential, all of which are fully prevented by CsA (Saviani et al. 2002). Finally, CsA reduces the loss of mitochondrial membrane potential and the concomitant PCD induced in *Arabidopsis thaliana* protoplasts by different stimuli (Yao et al. 2004). However, a CsA-insensitive change in membrane permeability induced by $Ca^{2+}$ accumulation and a CsA-insensitive release of cytochrome *c* induced by anoxia together with $Ca^{2+}$ accumulation have been reported for isolated potato and wheat mitochondria (Fortes et al. 2001, Virolainen et al. 2002). The inner membrane of wheat and pea mitochondria possesses a $K^+$ channel whose opening is inhibited by ATP and stimulated by CsA (Pastore et al. 1999, Petrussa et al. 2001). The opening of this channel in medium containing $K^+$ is associated with swelling and consequent release of cytochrome *c* by a mechanism modulated by $H_2O_2$ and NO. Its involvement in a low-amplitude permeability transition has recently been demonstrated in isolated pea stem mitochondria (Petrussa et al. 2004). Thus, it appears that higher-plant mitochondria can release cytochrome *c* by at least two different pores or channels, namely, the PTP and the $K^+$ channel, whose activities are regulated, in some cases, in opposing manners by CsA.

Sycamore (*Acer pseudoplatanus* L.) cultured cells can be induced to undergo a PCD with apoptotic features, including chromatin condensation, DNA fragmentation, and release of cytochrome *c* from the mitochondria, by treatments with the animal PCD inducers tunicamycin and brefeldin A and with the phytotoxin fusicoccin (FC) (Crosti et al. 2001; Malerba et al. 2003a, 2004a). In addition to the well-known effects of the toxin, proton extrusion and $H^+$-ATPase activation (Marrè 1979, Palmgren 2001), FC stimulates several stress-related responses of sycamore cells: production of ethylene, $H_2O_2$, and NO, stimulation of alternative respiration through the activation of the alternative oxidase protein, and accumulation of 14-3-3 proteins in the cytosol and of the endoplasmic reticulum (ER)-resident molecular chaperone binding protein (BiP) with concomitant modification of the ER architecture (Malerba et al. 1995, 2003b, 2004a, 2005). In this work, we show the effect of CsA on FC-induced cell death, DNA fragmentation, and cytochrome *c* release. In addition, we investigate the effect of CsA on the FC-induced $H_2O_2$ production and the effect of FC and CsA on the morphology of the mitochondria of sycamore cells.

## Material and methods

### *Cell culture growth*

Sycamore (*Acer pseudoplatanus* L.) cell cultures (a kind gift of Prof. R. Bligny, Laboratoires de Physiologie Cellulaire Végétale, CNRS-INRA, Grenoble, France) were maintained in White's modified medium supplemented with 20 g of sucrose and 1 mg of thiamine-HCl per liter, and $4 \times 10^{-6}$ M 2,4-dichlorophenoxyacetic acid, as previously described (Crosti et al. 2001).

### *Experimental conditions*

Cells in the exponential phase of growth (7-day cultures) were utilized in the experiments. The cells were gathered by gentle centrifugation (2 min at 300 **g**) and resuspended in fresh culture medium at a final density of $10^6$ cells per ml. During the experimental period the cells were maintained in a growth chamber at 25 °C under light on a gyratory shaker (120 rpm).

### *$H_2O_2$ assay and NO imaging*

$H_2O_2$ accumulation in the culture medium was measured using the xylenol orange colorimetric assay, as previously described (Jiang et al. 1990, Malerba et al. 2005). Briefly, 500 µl aliquots of culture medium were added at the indicated times to 500 µl of reaction mixture containing 500 µM ammonium ferrous sulfate, 50 mM $H_2SO_4$, 200 µM xylenol orange, and 200 mM sorbitol. After 45 min at room temperature, the absorbance value was determined at 560 nm with a Jasco V-530 spectrophotometer (Jasco Corporation, Tokyo, Japan). The amount of $H_2O_2$ was calculated from a standard curve obtained by adding known amounts

of $H_2O_2$ to 500 μl of the culture medium. The experiments were run in triplicate and repeated at least three times. NO was visualized with the NO-reactive cell-permeant fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate (Alexis Biochemicals, Lausen, Switzerland), as previously described (Malerba et al. 2005).

*pH measurements*

The pH of the external medium was measured with a flat-tip combined electrode (GK 732511 model; Radiometer Analytical, Villeurbanne, France). The experiments were run in triplicate and repeated at least three times.

*Cell viability assay*

The cells were collected and stained for 5 min with the Evans Blue dye at a final concentration of 150 μg/ml. Dead cells stained blue, while living cells remained unstained. The cells were examined with a light microscope and the percentage of dead (Evans Blue-stained) cells was calculated from the observation of at least 1000 cells.

*TUNEL procedure*

To detect nuclear DNA cleavage, cells were treated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling procedure (TUNEL) using the fluorescein-dUTP-based cell death detection kit (Roche Diagnostics S.p.A., Monza, Italy) as previously described (Crosti et al. 2001). At the end of the TUNEL procedure, the cells were counterstained with Hoechst 3342 (5 μg/ml) in phosphate-buffered saline, pH 7.4, and analyzed with a microscope under UV-visible light. The percentage of Hoechst-labeled nuclei that were positive for the TUNEL reaction was calculated from the observation of at least 1000 cells.

*DNA extraction and analysis*

Extraction of whole genomic DNA was performed using the method of Dellaporta et al. (1983), slightly modified as described previously (Crosti et al. 2001) to obtain the maximum recovery of oligonucleosome-related size fragments. The DNA content of the different samples was spectrophotometrically determined and equal amounts (10 μg) were separated on a 1.5% agarose gel by electrophoresis (2 h at constant 90 V). DNA was visualized and evaluated under UV light using a GEL DOC 2000 densitometer (Bio-Rad Instruments, Hercules, Calif., U.S.A.) equipped with the Multianalyst software (Bio-Rad) after staining with 0.1 μg of ethidium bromide per ml.

*Cell fraction preparation*

Cells were collected by gentle centrifugation, frozen in liquid nitrogen and homogenized for 5 min at maximum speed with a Ultra-Turrax T25 device (International PBI, Milan, Italy) at a density of 1 g of fresh weight per 2 ml of homogenizing buffer (25 mM 2-(N-morpholino)ethanesulfonic acid-2-(bis(2-hydroxymethyl)amino)-2-(hydroxymethyl)-1,3-propanediol, pH 7.8, 250 mM sucrose, 5 mM EDTA, 0.2% bovine serum albumin, and 0.2% casein) freshly supplied with 2 mM dithiothreitol and 10 μl of plant protease inhibitor cocktail (Sigma cat. nr. P 9599) per ml of cell homogenate. The homogenate was centrifuged at 1000 **g** for 10 min and the supernatant was centrifuged again at 10,000 **g** for 15 min. The supernatant was centrifuged at 48,000 **g** for 60 min and the resultant pellet, representing the microsomal fraction, was resuspended in 10 mM Tris-HCl, pH 6.5, 1 mM EDTA, 1 mM dithiothreitol and 20% (v/v) glycerol supplemented with the protease inhibitor cocktail, and stored at −80 °C until use. The remaining supernatant was centrifuged at 200,000 **g** for 3 h and the resultant supernatant, representing the cytosolic (soluble) fraction, was stored at −80 °C until use. All the above reported procedures were performed at 4 °C.

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot experiments*

Equal amounts of proteins, determined with the Bio-Rad microassay using bovine serum albumin as standard, were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4% stacking, 10% resolving gel), performed as described previously (Laemmli 1970) in a Mini Protean II apparatus (Bio-Rad). The proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, Mass., U.S.A.) using a Bio-Rad Mini Gel Trans Blot cell and immunodecorated. Immunodecoration of cytochrome *c* was performed on the cytosolic fraction with a polyclonal antibody against full-length cytochrome *c* from horse heart (Santa Cruz Biotechnology, Santa Cruz, Calif., U.S.A.). Immunodecoration of cytosolic 14-3-3 proteins was performed with an antibody against the BMH1 isoform of *Saccharomyces cerevisiae*, a generous gift from Prof. Paul van Heusden, Leiden University, the Netherlands. Immunodecoration of BiP was performed on the microsomal fraction with an antibody against tobacco BiP, a generous gift from Dr. A. Vitale, Istituto di Biologia e Biotecnologia Agraria, CNR, Milano, Italy. Relative abundance of immunodecorated protein was quantified with a Bio-Rad GEL DOC 2000 densitometer.

*Confocal microscopy*

The mitochondrial morphology was analyzed by confocal microscopy utilizing the vital dye rhodamine 123 (Wu 1987, Zhang et al. 1999). About 100,000 cells were washed, resuspended in fresh growth medium, and stained with 4 μg of the dye per ml. After 15 min of incubation at room temperature, the cells were observed with a Leica DMIRE2 confocal laser scanning microscope equipped with Leica TCS-NT software and fluorescein isothiocyanate filter sets. The data acquisition settings (laser power, pinhole size, scan conditions, detector settings, and so on) were identical for all experimental conditions. All images were edited by Photoshop 6.0 (Adobe, San Josè, Calif., U.S.A.). To evaluate the effect of the chemicals on mitochondrial shape, at least 200 cells were observed for each treatment and the percentage of round mitochondria (the number of round mitochondria divided by the total number of mitochondria and multiplied by 100) was determined in each cell.

## Results

### *Effect of CsA on FC-induced cell death and nuclear DNA cleavage*

The effect of 10 μM CsA on cell death and nuclear DNA cleavage induced by 8 and 20 μM FC is shown in Fig. 1. Viability of the cells was determined by Evans Blue staining (Fig. 1A); the dead cells stained blue, while the living cells remained unstained. Nuclear DNA cleavage, an index of the percentage of cells undergoing programmed death, was detected in situ by the TUNEL reaction (Fig. 1B). The results show that CsA reduced the accumulation of dead (Evans Blue-stained) cells in the FC-treated cultures (Fig. 1A). The FC-induced appearance of cells with fragmented DNA (TUNEL-positive cells) was also reduced by CsA for up to 8 h of treatment and was com-

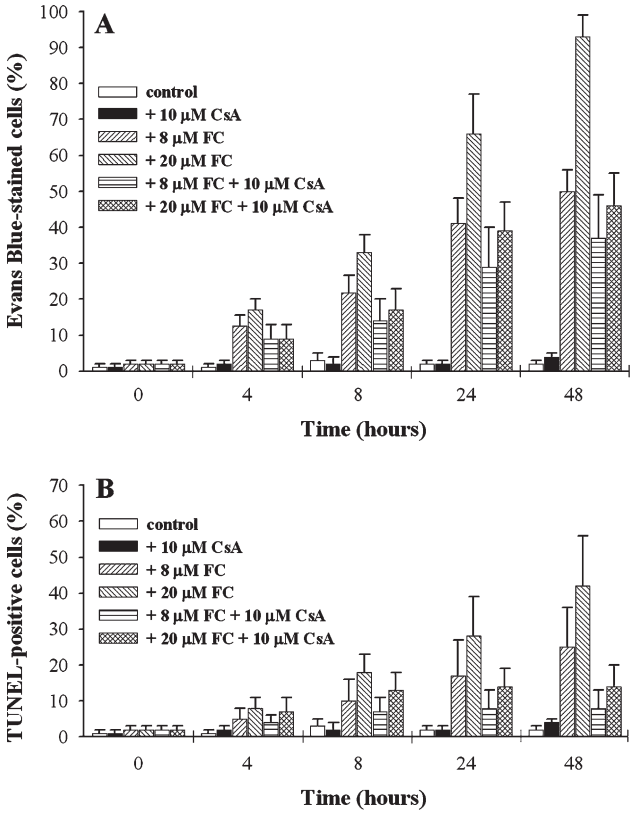


**Fig. 1.** Effect of CsA on FC-induced cell death (**A**) and nuclear DNA cleavage (**B**). At the indicated times, the cells were collected and stained with Evans Blue (**A**) or fixed and subjected to the TUNEL procedure (**B**). Means with standard deviation (bars) of at least 3 independent experiments run in duplicate are presented

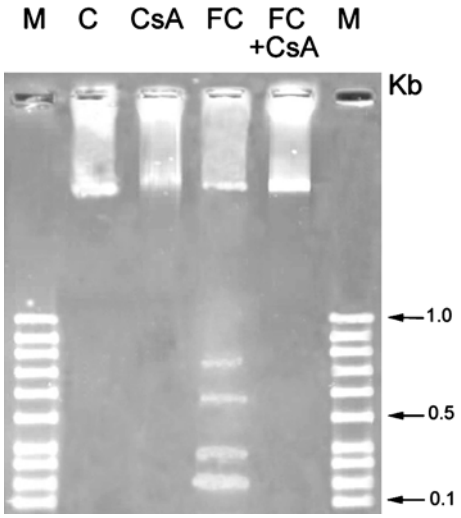


**Fig. 2.** Effect of CsA on FC-induced DNA specific fragmentation (laddering). *C* Control cells; *CsA* cells treated with 10 μM CsA; *FC* cells treated with 20 μM FC; *FC + CsA* cells treated with 20 μM FC and 10 μM CsA. *M* DNA markers (100–1000 bp ladder). The incubation period was 12 h and 10 μg of DNA were run in each lane. The results of a typical experiment (n = 3) are shown

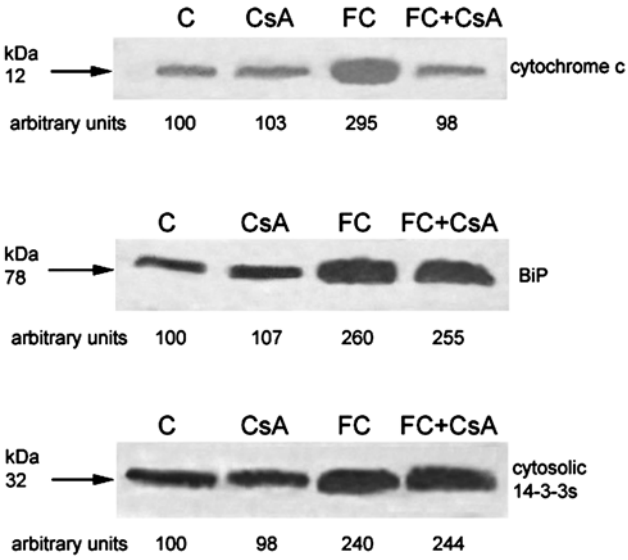


**Fig. 3.** Western blot analysis of the effect of CsA on the FC-induced release of cytochrome *c* and accumulation of cytosolic 14-3-3 proteins and BiP. *C* Control cells; *CsA* cells treated with 10 μM CsA; *FC* cells treated with 20 μM FC; *FC + CsA* cells treated with 20 μM FC and 10 μM CsA. The incubation period was 4 h and 50 μg of proteins were run in each lane. The results of a typical experiment (n ≥ 3) are shown. An arbitrary value of 100 was assigned to the amount of immunodecorated protein in the control cells

pletely suppressed thereafter (Fig. 1B). The FC-induced DNA fragmentation was also detectable by gel electrophoresis and resulted in the appearance of DNA fragments with a molecular size of 180 bp or multiples (laddering). Figure 2 shows that the FC-induced DNA laddering was also prevented by CsA.

### *Effect of CsA on the FC-induced release of cytochrome c and mitochondrial morphology*

The release of cytochrome *c* from mitochondria is a common feature of plant PCD triggered by different stimuli (Lam et al. 2001) and it is induced by FC in sycamore cells (Malerba et al. 2003b). The data in Fig. 3 show that CsA fully prevented the FC-induced release of cytochrome *c*, measured as appearance of the protein in the soluble fraction after 4 h of treatment. A corresponding decrease in the crude mitochondria could not be detected, probably because the released cytochrome *c* is a very small fraction of the total protein (data not shown) (see also Malerba et al. 2004a). The inhibitory effect of CsA was maintained even at the prolonged experimental times tested for this parameter (up to 12 h, data not shown). In order to rule out a general effect of CsA, we tested its effect on two other parameters affected by FC: the accumulation of cytosolic 14-3-3 proteins, a class of proteins playing a regu-

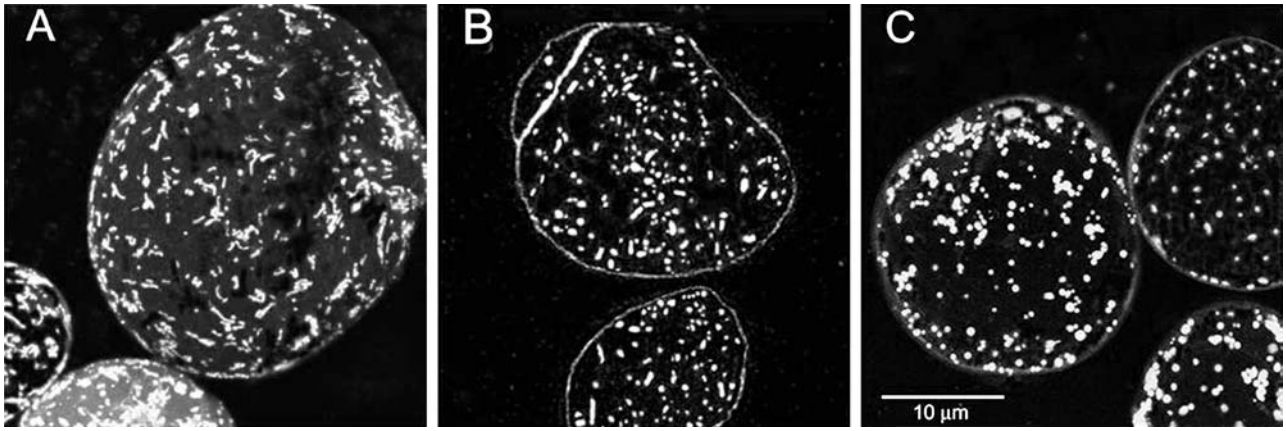


**Fig. 4 A–C.** Confocal microscopy analysis of the mitochondrial morphology. **A** Representative microphotograph of cells in which tubular mitochondria prevail; **B** representative microphotograph of cells in which tubular and round mitochondria are present; **C** representative microphotograph of cells in which round mitochondria prevail. For percentages of round mitochondria in control cells and cells treated with either CsA or FC or with FC and CsA, see text

latory role in many processes, including cytochrome *c* leakage and apoptosis (van Hemert et al. 2001), and the accumulation of BiP, a widely distributed and highly conserved ER-resident molecular chaperone whose synthesis is stimulated by a variety of abiotic and biotic stresses in plants (Alvim et al. 2001). Figure 3 shows that CsA did not affect the FC-induced accumulation of these proteins.

In animals, the induction of PCD programs by different stimuli, including oxidative stress, is tightly coupled to changes in mitochondrial morphology (for a review, see Perfettini et al. 2005). Mitochondrial dynamics play a role in the initiation and promulgation of cell death in plants, although there are specific differences in the genes and mechanisms involved relative to other higher eukaryotes (for a review, see Logan 2006). We, therefore, investigated the effects of FC and CsA on the morphology of the mitochondria of sycamore cells.

After staining with rhodamine 123, one of the most commonly employed dyes for delineating the mitochondrial morphology in live plant cells (Wu 1987, Zhang et al. 1999), mitochondria presented great morphological heterogeneity, with tubular and round mitochondria present even within a single cell. This is shown in Fig. 4, where three representative confocal extended-focus images of sycamore cells are presented. A 4 h treatment with FC induced changes in mitochondrial shape: while tubular mitochondria were prevalent (24.13% ± 15.36% [mean with standard deviation, n = 213] of mitochondria per cell were round) in control cells, a great number of round mitochondria (75.87% ± 16.41%, n = 208) were observed in cells treated with 20 µM FC. The mitochondrial morphology is not altered by CsA (10 µM) either in control (26.38% ± 16.67% [n = 200] of mitochondria were round) or in FC-treated cells (72.11% ± 15.22% [n = 200] of mitochondria were round).

### *Effect of CsA on FC-induced $H_2O_2$ and NO production*

Two other parameters affected by FC are $H_2O_2$ and NO accumulation in the culture medium of sycamore cells, resulting from changes in the rates of production (Malerba et al. 2003b, 2005). These two highly reactive molecules are often involved in plant PCD (Neill et al. 2002), although in our experimental material, PCD with apoptotic features can be induced by tunicamycin in the absence of a detectable accumulation of these molecules (Malerba et al. 2004a; M. Malerba et al. unpubl. data) and the FC-induced cytochrome *c* release is not strictly related to their production (Malerba et al. 2004b, 2005). Figure 5 shows

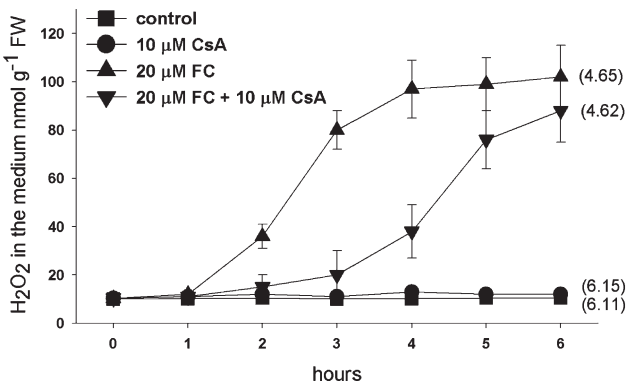


**Fig. 5.** Effect of CsA on FC-induced $H_2O_2$ accumulation in the culture medium. At the indicated times, aliquots of the culture medium were withdrawn and $H_2O_2$ was measured. Means with standard deviation (bars) are presented (n = 9). Figures in parentheses are the final pHs recorded in a typical experiment. The initial pH was 6.15 ± 0.1 for the different samples

that CsA delayed the $H_2O_2$ accumulation induced by FC in the culture medium. In fact, accumulation in the samples treated with FC and CsA was reduced by 75 and 14% after 3 and 6 h, respectively, compared with that in the samples treated with FC alone. FC-induced $H_2O_2$ accumulation is strongly dependent on the $H^+$-ATPase activity (Malerba et al. 2004b); however, the effect of CsA is not due to the inhibition of the $H^+$-ATPase. In fact, the final pHs (Fig. 5) and the kinetics of medium acidification (data not shown) were practically the same in the samples treated with FC or with FC and CsA. In contrast, CsA had no effect on the FC-induced NO accumulation, visualized by the specific fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (data not shown).

## Discussion

The reported results show that CsA inhibits the FC-induced cell death, DNA fragmentation, and cytochrome *c* release and delays the $H_2O_2$ production, but CsA does not inhibit the production of NO and the accumulation of cytosolic 14-3-3 proteins and BiP. In addition, FC induces changes in mitochondrial morphology that are not affected by CsA.

Although specific DNA cleavage and cytochrome *c* release from mitochondria, typical hallmarks of animal apoptosis, can be detected in plant cells induced to undergo PCD by different stimuli (Lam et al. 2001), including treatment with FC (Figs. 1B, 2, and 3) (see also Malerba et al. 2003a, 2004a), the use of the term apoptosis is controversial and other types of PCD occur in plants. In fact, besides the "nuclear" type of PCD showing apoptotic features and involving mitochondria, at least two other types of PCD have been reported in plants: the "chloroplastic" type, observed during leaf senescence, and the "vacuolar" type, typical of the formation of tracheary elements (Lam 2004). This can account for the difference between the percentages of Evans Blue-stained and TUNEL-positive cells observed in the presence of FC (Fig. 1A, B) and suggests that the toxin may activate different cell death programs. It is noteworthy that while CsA only partially reduces the number of Evans Blue-stained cells in the FC-treated cultures (Fig. 1A), it markedly inhibits the accumulation of TUNEL-positive cells (Fig. 1B) and totally prevents FC-induced DNA fragmentation, detectable by gel electrophoresis (Fig. 2). CsA also completely suppresses the release of cytochrome *c* induced by FC (Fig. 3) without preventing the changes in mitochondrial morphology (Fig. 4). Thus, CsA seems to affect the type of PCD that shows apoptotic features. As regards the effect of FC on mitochondrial morphology, it is interesting to note that similar modifications in the shape of mitochondria, from elongate to more spherical organelles, are observed during animal and plant PCD. In animals, these changes occur during the PCD induced by oxidative stress and by changes in cellular $Ca^{2+}$ levels (Scorrano and Korsmeyer 2003), two phenomena affected by FC in sycamore cells (Malerba et al. 2003b). In plants, expression of heterologous Bax in *A. thaliana* induces a change in the typical morphology of leaf mitochondria from elongate, bacilliform or sausage-shaped to more condensed spherical organelles, accompanied by a cessation of movement (Yoshinaga et al. 2005), and drought stress similarly affects the shape of mitochondria of spinach leaves (Zellnig et al. 2004).

The addition of CsA significantly delays the FC-induced accumulation of $H_2O_2$ in the culture medium (Fig. 5). The reason for this delay, which is similar to that reported for NaCl-stressed tobacco protoplasts by Lin et al. (2006), is unclear. Our previous work indicates that the production of $H_2O_2$ induced by FC is strictly related to the activation of the plasma membrane $H^+$-ATPase (Malerba et al. 2004b), which is not affected by CsA (Fig. 5). Potential sources of $H_2O_2$ production in the FC-treated cells appear to be the plasma membrane NADPH oxidase and the electron transport chain of the mitochondria (Malerba et al. 2003b). In particular, mitochondrial production of $H_2O_2$ could be the consequence of a reduced capacity of the cytochrome pathway possibly resulting from cytochrome *c* release (Malerba et al. 2003b). However, as shown in Fig. 5, the accumulation of $H_2O_2$ induced by FC is only delayed in the presence of CsA, and it almost completely recovers after 6 h, when the release of cytochrome *c* is still fully suppressed.

As regards the mechanism of FC-induced cytochrome *c* release, our results suggest the involvement of a system similar to the PTP of animal mitochondria rather than of the $K^+_{ATP}$ channel identified in isolated plant mitochondria (Pastore et al. 1999, Petrussa et al. 2001). In fact, under our experimental conditions, the PTP inhibitor CsA inhibits the release of cytochrome *c* and this occurs in the presence of an accumulation of NO and of a delayed production of $H_2O_2$. All these conditions (presence of CsA and NO, absence of $H_2O_2$) promote the opening of the $K^+_{ATP}$ channel and the release of cytochrome *c* (Petrussa et al. 2001, 2004).

The results as a whole suggest that the release of cytochrome *c* induced by FC in *A. pseudoplatanus* cultured cells occurs through a CsA-sensitive system, similar to the PTP of animal mitochondria, and they make it tempting to

speculate that this release may be involved in the activation of a cell death program showing apoptotic features by FC. In addition, FC and CsA may be useful tools to investigate the different types of plant PCD.

## References


Alvim FC, Carolino SMB, Cascardo JCM, Nunes CC, Martinez CA, Otoni WC, Fontes EPB (2001) Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. Plant Physiol 126: 1042–1054

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

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

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1: 19–21

Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol 10: 369–377

Fortes F, Castilho RF, Catisti R, Carnieri EG, Vercesi AE (2001) $Ca^{2+}$ induces a cyclosporin A-insensitive permeability transition pore in isolated potato tuber mitochondria mediated by reactive oxygen species. J Bioenerg Biomembr 33: 43–51

Green DR (2000) Apoptotic pathways: paper wraps stone blunts scissors. Cell 102: 1–4

Green DR, Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1312

Halestrap AP, Davidson AM (1990) Inhibition of $Ca^{2+}$-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem J 268: 153–160

Jiang ZJ, Woollard ACS, Wolff SP (1990) Hydrogen peroxide production during experimental protein glycation. FEBS Lett 268: 69–71

Jones AM (2000) Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? Trends Plant Sci 5: 223–230

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lam E (2004) Controlled cell death, plant survival and development. Nat Rev Mol Cell Biol 5: 305–315

Lam E, Kato N, Lawton M (2001) Programmed cell death, mitochondria and the plant hypersensitive response. Nature 411: 848–853

Lin J, Wang Y, Wang G (2006) Salt stress-induced programmed cell death in tobacco protoplasts is mediated by reactive oxygen species and mitochondrial permeability transition pore status. J Plant Physiol 163: 731–739

Logan DC (2006) Plant mitochondrial dynamics. Biochim Biophys Acta 1763: 430–441

Malerba M, Crosti P, Armocida D, Bianchetti R (1995) Activation of ethylene production in *Acer pseudoplatanus* L. cultured cells by fusicoccin. J Plant Physiol 145: 93–100

Malerba M, Cerana R, Crosti P (2003a) Fusicoccin induces in plant cells a programmed cell death showing apoptotic features. Protoplasma 222: 113–116

Malerba M, Crosti P, Cerana R, Bianchetti R (2003b) Fusicoccin stimulates the production of $H_2O_2$ in sycamore cell cultures and induces alternative respiration and cytochrome *c* leakage from mitochondria. Physiol Plant 119: 480–488

Malerba M, Cerana R, Crosti P (2004a) Comparison between the effects of fusicoccin, Tunicamycin, and Brefeldin A on programmed cell death of cultured sycamore (*Acer pseudoplatanus* L.) cells. Protoplasma 224: 61–70

Malerba M, Crosti P, Cerana R, Bianchetti R (2004b) Fusicoccin affects cytochrome *c* leakage and cytosolic 14-3-3 accumulation independent of $H^+$-ATPase activation. Physiol Plant 120: 386–394

Malerba M, Crosti P, Cerana R (2005) The fusicoccin-induced accumulation of nitric oxide in sycamore cultured cells is not required for the toxin-stimulated stress-related responses. Plant Sci 168: 381–387

Marrè E (1979) Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol 30: 273–288

Neill SJ, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and nitric oxide as signalling molecules in plants. J Exp Bot 53: 1237–1247

Palmgren MG (2001) Plant plasma membrane $H^+$-ATPases: powerhouses for nutrient uptake. Annu Rev Plant Physiol Plant Mol Biol 52: 817–845

Pastore D, Stoppelli MC, Di Fonzo N, Passarella S (1999) The existence of the $K^+$ channel in plant mitochondria. J Biol Chem 274: 26683–26690

Perfettini J-L, Roumier T, Kroemer G (2005) Mitochondrial fusion and fission in the control of apoptosis. Trends Cell Biol 15: 179–183

Petrussa E, Casolo V, Braidot E, Chiandussi E, Macrì F, Vianello A (2001) Cyclosporin A induces the opening of a potassium-selective channel in higher plant mitochondria. J Bioenerg Biomembr 33: 107–117

Petrussa E, Casolo V, Peresson C, Braidot E, Chiandussi E, Vianello A, Macrì F (2004) The $K^+_{ATP}$ channel is involved in a low-amplitude permeability transition in plant mitochondria. Mitochondrion 3: 297–307

Saviani EE, Orsi CH, Oliveira JFP, Pinto-Maglio CAF, Salgado I (2002) Participation of the mitochondrial permeability transition pore in nitric oxide-induced plant cell death. FEBS Lett 510: 136–140

Scorrano L, Korsmeyer SJ (2003) Mechanisms of cytochrome *c* release by proapoptotic BCL-2 family members. Biochem Biophys Res Commun 304: 437–444

Shimizu S, Narita M, Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. Nature 339: 483–487

van Hemert MJ, Steensma HJ, van Heusden GP (2001) 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. Bioessays 23: 936–946

Vianello A, Macrì F, Braidot E, Mokhova EN (1995) Effect of cyclosporin A on energy coupling in pea stem mitochondria. FEBS Lett 371: 258–260

Virolainen E, Blokhina O, 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

Wu FS (1987) Localization of mitochondria in plant cells by vital staining with rhodamine 123. Planta 171: 346–357

Yao N, Eisfelder BJ, Marvin J, Greenberg JT (2004) The mitochondrion – an organelle commonly involved in programmed cell death in *Arabidopsis thaliana*. Plant J 40: 596–610

Yoshinaga K, Arimura S, Hirata A, Niwa Y, Yun DJ, Tsutsumi N, Uchimiya H, Kawai-Yamada M (2005) Mammalian Bax initiates plant cell death through organelle destruction. Plant Cell Rep 24: 408–417

Zellnig G, Zechmann B, Perktold A (2004) Morphological and quantitative data of plastids and mitochondria within drought-stressed spinach leaves. Protoplasma 223: 221–227

Zhang Q, Wiskicha JT, Sooleb KL (1999) Respiratory activities in chloramphenicol-treated tobacco cells. Physiol Plant 105: 224–232