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REVIEW

Plant mitochondrial pathway leading to programmed cell death

Angelo Vianello*, Marco Zancani, Carlo Peresson, Elisa Petrussa, Valentino Casolo, Jana Krajňáková, Sonia Patui, Enrico Braidot and Francesco Macrì

Section of Plant Biology, Department of Biology and Agro-Industrial Economics, University of Udine, Via Cotonificio, 108, I-33100 Udine, Italy

Correspondence
*Corresponding author,
e-mail: biolveg@uniud.it

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Programmed cell death (PCD) is a finely tuned process of multicellular organisms. In higher plants, PCD regulates many developmental processes and the response of host plants to incompatible pathogens (hypersensitive response). Four types of PCD have been described in plants, mainly associated to vacuole rupture, that is followed by the appearance of the typical PCD hallmarks (i.e. nuclear DNA fragmentation and cell shrinkage). However, in some cases vacuole collapse is preceded by an early alteration of other subcellular organelles, such as mitochondria. In particular, the central role played by mitochondria in PCD has been largely recognised in animal cells. This review deals with the involvement of mitochondria in the manifestation of plant PCD, in comparison to that described in animal PCD. The main hallmark, connecting animal and plant PCD via mitochondria, is represented by the release of cytochrome *c* and possibly other chemicals such as nucleases, which may be accomplished by different mechanisms, involving both swelling and non-swelling of the organelles.

Introduction

Programmed cell death (PCD) may be defined as a genetically regulated process of cell suicide, which accomplishes a central role in development, homeostasis and integrity of multicellular (eukaryotic) organisms. The origin, evolution and nature of PCD seem to be as old as the very first cell, because the mechanisms controlling homeostasis and preventing self-destruction may involve the PCD machinery (Ameisen 2002). Therefore, this apparatus has always been present in all cells from the origin. It has, indeed, been recognised in several prokaryotes and unicellular eukaryotes and related to numerous phenomena. Only later, during evolution of multicellular organisms, PCD has been 'fine tuned' for purposes such as the social control of cell members (Gray 2004).

Generally, three types of programmed (physiological) cell death were described in developing vertebrate embryos, heterophagy (apoptosis), autophagy and non-lysosomal death, on the basis of the location and role of lysosomes. The most widespread and studied form is apoptosis, that can be described considering both morphological and biochemical hallmarks (Van Doorn and Woltering 2005). The peculiar morphological changes include cell shrinkage, chromatin condensation, followed by nuclear fragmentation, formation of apoptotic bodies and, finally, their engulfment by neighbouring

Abbreviations – AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; Apaf-1, apoptotic protease activating factor-1; AOX, alternative oxidase; CARD, caspase recruitment domain; CsA, cyclosporin A; ETC, electron transport chain; HR, hypersensitive response; HSP, heat-shock protein; IAP, inhibitor of apoptosis protein; IMM, inner mitochondrial membrane; IMS, intermembrane mitochondrial space; $m\Delta\Psi$, mitochondrial electrical potential; mK^+_{ATP}, mitochondrial K^+_{ATP}; OMM, outer mitochondrial membrane; PCD, programmed cell death; PTP, permeability transition pore; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel; VPE, vacuolar-processing enzymes.

cells or macrophages. The main biochemical markers concern breakdown of DNA into large (50–300 kbp) and then small (200 bp) nucleosomal fragments (DNA laddering), and release of apoptogenic factors (see below). The apoptotic process is active, requires adenosine triphosphate (ATP), and is finely regulated by a set of pro- and antiapoptotic proteins, belonging to the Bcl-2 family (Droin and Green 2004). Autophagy plays a major role as a degradation and recycling system, because it contributes to the turnover of cells by delivering parts of the cytoplasm to lysosomes where they are digested. Non-lysosomal PCD, also named necrosis-like PCD, has been recognised in a few cases. It does not involve lysosomes and cells kill themselves by inhibiting the main biosynthetic pathways, disrupting their membranes, or by other unknown mechanisms (Van Doorn and Woltering 2005).

In contrast, necrosis (necrotic death) is considered an uncontrolled form of cell death. Necrotic death results from acute metabolic disruption with ATP depletion, ion disregulation, mitochondrial and cellular swelling and activation of degradative enzymes, all culminating in plasma membrane rupture. However, necrosis is not opposed to apoptosis. Rather, they appear to be the extremes of the same phenomenon, named 'necroapoptosis' (Lemasters 1999).

In higher plants, PCD is also a common feature involved in many developmental processes. The most common examples are stamen or ovary abortion during male and female flower formation, megaspore abortion, tapetal layer and suspensor degeneration, endosperm and aleurone degradation (in monocotyledons), tracheary element differentiation, leaf senescence, aerenchyma formation, death of root cell cap, etc. (Gray 2004). In addition, PCD is well documented in relation to the manifestation of the hypersensitive response (HR) caused by the interaction between a host plant and an incompatible pathogen (Hatsugai et al. 2004).

Mammalian and plant PCD share similar morphological and biochemical hallmarks (Table 1). However, there are some differences in the formation of apoptotic bodies, which are rarely observed in plants, because engulfment by neighbouring cells is hindered by the presence of the cell wall. In addition, surveys of the complete *Arabidopsis thaliana* and *Oryza sativa* genomes have failed to uncover obvious orthologs of animal genes that regulate animal apoptosis (e.g. genes for caspases or Bcl-2 proteins) (Danon et al. 2000, Dickman and Reed 2004). Nevertheless, the capability of mammalian Bcl-2 proteins, introduced into plant cells by heterologous expression, of modifying cell death supports the view that plants and animals share common PCD pathways (Dickman et al. 2001, Lacomme and Santa Cruz 1999).

Table 1. Comparison between morphological and biochemical hallmarks linked to animal and plant programmed cell death. m$\Delta\Psi$, mitochondrial electrical potential.

	Animals	Plants
Cell shrinkage	+	+
Apoptotic bodies	+	−
Chromatin condensation	+	+
Nuclear fragmentation	+	+/−
Mitochondrial swelling	+/−	+/−
m$\Delta\Psi$ dissipation	+	+
High ATP level	+	+/−
Pro-apoptotic proteins	Bcl-2 family	BAX-like?
Cytochrome *c* release	+	+
Endonuclease release	+	+
Caspases	+	Caspase-like proteins (metacaspases)
Vacuolar-processing enzymes	−	+
Apoptosome	+	?

The cytological events that accompany cell death in plants may be categorized into four types, three of which are related to autophagy (autolysis) by vacuole and designated micro-, macro- and mega-autophagy (Van Doorn and Woltering 2005). Micro-autophagy implies the sequestration of small portion of the cytoplasm by vacuole, whereas macro-autophagy is associated to the engulfment of larger parts. Mega-autophagy is a neologism, introduced by Van Doorn and Woltering (2005), to describe an autophagic process accomplished by the permeabilization of the tonoplast. In all cases, vacuole permeabilization (rupture) to large molecules immediately precedes nuclear DNA fragmentation and organelle disruption. Mega-autophagy appears to be the most common type during plant development (developmental PCD), such as transdifferentiation of mesophyll cells into mature xylem cells, aerenchyma formation and senescence (Lam 2004). The fourth form, occurring during HR, implies chromatin condensation and DNA cleavage into 50-kbp fragments that happen before an apparent vacuole rupture. Blebbing of the vacuole and plasma membrane, and degradation of organelles are observed only at a late stage. This observation opens the possibility for a role of organelles (mitochondria, but also chloroplasts) in the chain of events leading to plant PCD.

The involvement of mitochondria in plant PCD has been suggested by Jones (2000) and then, in particular, related to the manifestation of HR (Lam et al. 2001). However, the role of mitochondria in PCD (apoptosis) has been largely recognised in animal cells, where they accomplish, in many cases, the true role of execution central (Desagher and Martinou 2000, Green and Kroemer 2004, Kroemer and Reed 2000).

This article focuses on the role of plant mitochondria in the manifestation of PCD. In doing this, at least two assumptions have to be made. Firstly, from the above it appears clear that the acronym PCD covers several phenomena; it is difficult to believe that only one mechanism underlies such different processes. Secondly, as already seen, PCD occurs also in prokaryotes; therefore, considering the endosymbiotic origin of mitochondria and chloroplasts, it is rational to suppose that they have a role in PCD and that the machinery involved utilizes genes derived from such organelles (Ameisen 2002).

Intrinsic (mitochondrial) pathway in animal cells

In animal cells, apoptotic death may be reached by two main routes, known as extrinsic and intrinsic pathways. The extrinsic pathway, which is unique to vertebrates, is described in detail in a recent publication (MacFarlane and Williams 2004). In this pathway, specific 'death receptors' may, after activation, directly recruit caspase-activating multimeric protein complexes.

In contrast, the intrinsic pathway is evolutionarily conserved in multicellular organisms and is characterised by signal sensing and amplification by mitochondria, mediated by the release of the mitochondrial apoptogenic factors (Desagher and Martinou 2000, Green and Kroemer 2004, Kroemer and Reed 2000). A key role in the activation process is played by proteins belonging to the Bcl-2 family that reside immediately upstream of mitochondrial apoptosis pathway (Kaufmann and Hengartner 2001). The Bcl-2 family consists of three main groups including pro- and antiapoptotic members, on the basis of their ability to promote or inhibit the apoptotic process. Group I contains antiapoptotic members (e.g. Bcl-2, Bcl-X_L) that are present on the outer surface of the outer mitochondrial membrane (OMM) and that would act by preventing the translocation of proapoptotic proteins (Bax/Bak, see below) to the mitochondria (Kaufmann and Hengartner 2001). Group II (e.g. Bax, Bak) and III (e.g. Bid, Bad) members are all proapoptotic proteins, which respond to a wide variety of stimuli, suggesting that they could act as sensors for cellular integrity and functionality (Kaufmann and Hengartner 2001).

Different mechanisms have been suggested to explain the release of apoptogenic factors from mitochondria, induced by proapoptotic proteins (Bernardi et al. 2001, Kroemer and Reed 2000, and references therein). The first involves Bax that could simply oligomerise in OMM to form a channel. Alternatively, Bax, in association with either the voltage-dependent anion channel (VDAC) or truncated Bid (tBid), could promote the formation of pores allowing the passage of soluble proteins.

Alternative models have been suggested in which, during early stages of apoptosis, the inner mitochondrial membrane (IMM) plays a key role. The first one implies that water and solutes enter the mitochondrial matrix, inducing swelling of mitochondria (Bernardi et al. 2001, Kroemer and Reed 2000). This process is mediated by either VDAC or the opening of a permeability transition pore (PTP) (Desagher and Martinou 2000). The PTP may be defined as a voltage-dependent, cyclosporin A (CsA)–sensitive, high-conductance inner membrane channel. The pore open–closed transitions are highly regulated by multiple effectors at discrete sites. Factors affecting PTP can be subdivided into matrix and membrane effectors. The former include both openers (Ca^{2+}, phosphate, oxidizing agents, ^{-}OH and atractylate) and inhibitors (CsA, ADP, H^+, bongkrekate and reducing agents). Among the latter, a high (inside-negative) membrane potential tends to stabilize the PTP in a closed conformation, whereas depolarisation by different uncouplers determines its aperture. PTP is also regulated by quinones, which prevent Ca^{2+}-dependent pore opening. The molecular structure of PTP is still unknown, although evidence suggests that it may be formed of several components, including matrix cyclophilin D, the outer membrane VDAC, the inner membrane adenine nucleotide translocase (ANT), peripheral benzodiazepine receptor and Bcl-2, hexokinase bound to VDAC, and intermembrane creatine kinase (Bernardi 1999, Zoratti and Szàbo 1995). In addition, the proapoptotic protein Bax induces PTP opening through binding to ANT, suggesting the involvement of the Bcl-2 family proteins in this process (Bernardi et al. 2001). The permeabilization of the IMM to solutes with molecular mass up to 1.5 kDa, caused by the aperture of the PTP results in the complete dissipation of mitochondrial electrical potential ($m\Delta\Psi$). Consequently, the high concentration of solutes present in the matrix induces an osmotic swelling that could ultimately lead to OMM rupture and the consequent release of proteins from the intermembrane mitochondrial space (IMS) (Kroemer and Reed 2000).

In addition to the above-mentioned molecular mechanisms, a new mitochondrial system, which could be potentially involved in apoptosis of animal cells, has been described (Eliseev et al. 2002). In human H1-60 cells, treated with etoposide, a K^+ accumulation into mitochondria precedes the permeability transition and the manifestation of apoptotic symptoms. This K^+ transport, mediated by a mitochondrial K^+_{ATP} (mK^+_{ATP}) channel (Garlid and Paucek 2003), is accompanied by matrix osmotic swelling and is sensitive to Bcl-2 family proteins. This slight swelling determines the release of cytochrome *c* and is accompanied by a moderate membrane depolarisation (Eliseev et al. 2003). Although the molecular nature of this mK^+_{ATP} channel is still unknown, it is suggested

that this mechanism plays a potential role in the prevention of ischemic heart disease and mammalian cell death (Ardehali and O'Rourke 2005). Furthermore, Szabò et al. (2005) provided evidence for a new margatoxin-sensitive K^+ channel (Kv1.3), located in the IMM of T lymphocytes, which is involved in an apoptotic event.

The proteins released from the IMS into the cytosol during the early events of apoptosis are subdivided into two classes: those that directly or indirectly activate caspases and those that function independently from caspases (van Gurp et al. 2003). The latter group includes the apoptosis-inducing factor (AIF, a flavoprotein that shows similarities with bacterial, plant and fungal oxidoreductases) and endonuclease G (a highly conserved protein in the eukaryotic kingdom) (Ameisen 2002, van Gurp et al. 2003) that, once released from the IMS, are transferred to the nucleus where they could digest nuclear DNA.

In contrast, second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI (Smac/DIABLO), a mitochondrial serine protease (Omi/HtrA2) and cytochrome *c* can trigger apoptosis through a caspase-dependent process. These proteins, once released into the cytosol, contribute to caspase activation by sequestering the inhibitor of apoptosis proteins (IAP) through their IAP-binding motif (van Gurp et al. 2003).

Cytochrome *c*, the most investigated protein involved in caspase activation, binds the scaffolding protein, named apoptotic protease activating factor-1 (Apaf-1), leading to an ATP- or dATP-dependent conformational change that induces Apaf-1 oligomerisation (van Gurp et al. 2003). This high molecular mass complex, called the apoptosome, is assembled by binding cytochrome *c* and Apaf-1 with procaspase-9 through the interaction between their *ca*spase *r*ecruitment *d*omains (CARDs). Procaspase-9 activity is greatly enhanced in the apoptosome that, in turn, proteolytically activates caspase-3, finally resulting in the morphological and biochemical changes associated with apoptosis (Kaufmann and Hengartner 2001).

PCD-associated mitochondrial signalling pathway in plant cells

The above-described models, involving mitochondria in trigger of apoptosis, have still not been completely described in plants, where experimental evidence is limited and, in some cases, contradictory. Therefore, the discussion of a putative role of the mitochondrial signalling pathway needs an overview of the known mitochondrial hallmarks in plant PCD.

Release of cytochrome *c*

The most common hallmark used to identify the involvement of plant mitochondria in PCD is the release of cytochrome *c*. The first evidence is related to the observation that the addition of cytochrome *c* to carrot cytoplasmic extracts induces an apoptotic degradation of added mouse nuclei, which is prevented by caspase inhibitors (Zhao et al. 1999). Then, the release of cytochrome *c* from mitochondria has been detected in different plant systems, in which PCD was induced. In particular, the release of cytochrome *c* precedes the appearance of PCD symptoms and has been recognised in *A. thaliana* cells treated with mannose, where the effect is also associated to endonuclease activation (Stein and Hansen 1999), and in maize cells infected by *Agrobacterium* sp. (Hansen 2000). In addition, harpin (a bacterial proteinaceous elicitor)–induced HR in tobacco cells is associated with an alteration of mitochondrial functions (Xie and Chen 2000). The initial steps of cell death are accompanied by an oxidative burst, depletion of ATP, collapse of the $m\Delta\Psi$ and release of cytochrome *c*. A strong stimulation of the expression of the alternative oxidase (AOX) and small heat-shock proteins (HSPs) has also been described (Krause and Durner 2004). Consistent with this, induction of PCD in *A. thaliana* cell cultures by ceramide, protoporphyrin IX and an elicitor of HR (AvrRpt2) leads to the dissipation of $m\Delta\Psi$, followed by morphological changes and cytochrome *c* release (Yao et al. 2004).

Balk and Leaver (2001) studied the *Hehianthus petiolarus* sub sp. *petiolarus*–cytoplasmic male-sterility (PET1-CMS) mitochondrial mutation in sunflower. This mutation causes a premature PCD of the tapetal cells, associated with a release of cytochrome *c*, that precedes the appearance of the gross morphological changes related to cell death. However, the release of cytochrome *c* is only partial and, surprisingly, precedes the loss of other mitochondrial functions. This anomalous result could be because of Percoll purification of organelles, which leads to a separation of different mitochondrial populations (de Virville et al. 1994), determining a selection of the best coupled ones, not involved in cytochrome *c* release.

In contrast to the above findings, no cytochrome *c* release has been observed during petal senescence even if there are no differences in the experimental system used to detect the appearance of such a protein in the cytosolic fraction (Xu and Hanson 2000). Furthermore, other authors show that cytochrome *c* release happens only after mitochondrial disorganization (Yu et al. 2002).

Endonuclease release

Several DNase activities and nuclease genes have been recognised to be upregulated in different models of plant PCD (Sugiyama et al. 2000). Most of the experimental

evidence shows that cytochrome *c* release is associated with DNA fragmentation, although the induction of endonuclease activities, during PCD, has been described only in a few cases (Balk et al. 2003, Stein and Hansen 1999). In particular, using a cell-free system from *A. thaliana*, it has been shown that the addition of mitochondria facilitates nuclear DNA degradation through a putative Mg^{2+}-dependent nuclease activity that has been associated to the IMS (Balk et al. 2003). More recently, a cell-free system to analyse nucleus degeneration in nucellar cells from wheat grains has been developed (Domínguez and Cejudo 2006). In this system, nuclear extracts from such cells have been shown to be capable of triggering DNA fragmentation in both plant and human nuclei, demonstrating that similar features of nucleus degradation could be shared between plant and animal cells.

Mechanisms of cytochrome *c* (and endonuclease) release

By analogy with animal mitochondria, several authors have correlated the detected release of cytochrome *c* to the activity of PTP, on the basis of the inhibitory effect exhibited by CsA (Balk and Leaver 2001, Lin et al. 2005, Tiwari et al. 2002). This contention seems to be confirmed by the observation that NO-induced programmed death in *Citrus sinensis* cell cultures is also prevented by CsA (Saviani et al. 2002).

The presence of PTP, whose aperture is induced by Ca^{2+} and inhibited by CsA, has been documented in potato mitochondria (Arpagaus et al. 2002). However, its activity and role(s) are not easily and unequivocally detectable, because the Ca^{2+} sensitivity can be found only in the presence of dithioerythritol. Differently, a reductant-independent and CsA-insensitive Ca^{2+}-induced permeability transition has been described in potato tuber (Fortes et al. 2001) and wheat (Virolainen et al. 2002) mitochondria. Nevertheless, plant mitochondria have the main components probably involved at the contact sites of OMM and IMM, e.g. ANT, VDAC (Godbole et al. 2003) and cyclophilin (Yokota et al. 2004). In any case, the opening of this channel would determine the entry into mitochondria of osmotically active solutes and water. This would cause a mitochondrial swelling with the consequent rupture of the OMM and release of cytochrome *c* (and endonuclease).

Like its animal counterpart, an ATP-sensitive K^+ channel has been identified in the inner membrane of wheat (Pastore et al. 1999) and pea stem (Petrussa et al. 2001) mitochondria. The aperture of the channel of pea mitochondria is stimulated by CsA and NO, but inhibited by H_2O_2 (Petrussa et al. 2001). This mK^+_{ATP} channel may function as an outwardly or inwardly rectifying channel, regulating the matrix volume together with a dibucaine-sensitive K^+/H^+ antiporter. This channel is responsible for a low amplitude permeability transition, associated with an increase of mitochondrial volume (Petrussa et al. 2004), which is followed by a release of cytochrome *c*, with a mechanism that is modulated by NO (stimulation) and H_2O_2 (inhibition) (Chiandussi et al. 2002). The mitochondria remain structurally and functionally intact, as recently reported for mammals (Gogvadze et al. 2004). The latter observation suggests that cytochrome *c* release could occur in a PTP-independent manner and that a simple modulation of the volume suffices to induce a cytochrome *c* loss. Indeed, these effects are linked only to a slight decrease of $m\Delta\Psi$. Therefore, this mK^+_{ATP} channel may turn out to be the mechanism to mediate the release of apoptogenic factors during plant PCD. In agreement with this hypothesis, the NO- or H_2O_2-induced PCD of soybean suspension cell cultures implies a mitochondrial phase, with a release of cytochrome *c* dependent on the activity of this mK^+_{ATP} channel (Casolo et al. 2005).

A further model refers to the non-swelling mechanism involving the OMM. In this mechanism a crucial role is performed by VDAC, which interacts with Bax, forming a pore through which cytochrome *c* is released (Lam et al. 2001). The first evidence derives from a study in which the overexpression of mammalian Bax gene in tobacco plants causes hypersensitive-like lesions and induces defence genes (Lacomme and Santa Cruz 1999). Recent experimental findings seem to corroborate this mechanism, suggesting that VDAC can play a crucial role in PCD pathway being a conserved element in both plants and animals (Godbole et al. 2003, Swidzinski et al. 2004). In agreement, VDAC expression increases during HR, senescence and heat-induced PCD in *A. thaliana* cells (Lacomme and Roby 1999, Swidzinski et al. 2004). This evidence indicates a putative dual role for VDAC, as a component of PTP or as a channel that interacts with Bax.

Energetic state of cells undergoing necrotic or programmed death

ATP is a ubiquitous energy source that can also act as a signalling molecule in cellular metabolism. In animals, it is now emerging as the connection between bioenergetics and apoptosis (Hammerman et al. 2004). After the mitochondria have been triggered, it has been shown that the level of ATP may drive cells towards apoptosis (high ATP) or necrosis (low ATP), determining the type of death occurring after the signal reception or toxic stress (Lemasters 1999).

Necroapoptosis in plant mitochondria has been now elucidated in soybean cell cultures treated with different H_2O_2 concentrations (Casolo et al. 2005). Hydrogen

peroxide (5 m*M*) induces PCD (apoptotic-like symptoms), which is accompanied only by a slight decrease in ATP and glucose-6-P levels; at a higher H_2O_2 (20 m*M*) concentration, cells become necrotic and the level of both energetic molecules drops. In addition, ATP depletion after PCD induction in *A. thaliana* (Krause and Durner 2004, Tiwari et al. 2002) and tobacco BY-2 (Mlejnek et al. 2003) suspension cell cultures has been reported. However, it is not clear if this depletion is because of the collapse of $m\Delta\Psi$, which is linked to the release of cytochrome *c* and inhibition of respiration, or to ATP consumption by PCD processes. This discrepancy may be explained, however, considering that animal apoptosis, at its early stage, is accompanied by increase of $m\Delta\Psi$ and ATP concentration, whereas at the end it results in $m\Delta\Psi$ collapse and ATP decrease (Skulachev 2006).

Reactive oxygen species and NO involvement in mitochondrial pathway leading to PCD

Reactive oxygen species (ROS) and NO can function as important effectors/regulators of plant PCD. ROS are generated in different compartments of plant cells, including cell wall (peroxidases and polyamine oxidases), cytoplasm (xanthine oxidase), mitochondria, chloroplasts and peroxisomes. NO is also produced by different mechanisms in some plant cell sites (Hancock et al. 2002), including also mitochondria (Guo and Crawford 2005). In particular, H_2O_2 and NO may be the link that connects the cell death trigger to the cellular responses, acting as secondary signals in the activation of plant PCD (Hoeberichts and Woltering 2002).

In this context, plant mitochondria can play a major role, because superoxide anion, which then dismutates to H_2O_2, is generated at the level of complexes I and III of the electron transport chain (ETC) (Møller 2001). Indeed ROS, generated by plant mitochondria, increase during the early stages of PCD (Vacca et al. 2004). The mitochondrial oxidative burst could play a crucial role in $m\Delta\Psi$ dissipation, preceding cell death response (Malerba et al. 2003, Tiwari et al. 2002, Yao et al. 2002, Yoshinaga et al. 2005). Dissipation of $m\Delta\Psi$ and accumulation of ROS also precede ultrastructural changes related to the manifestation of a plant PCD (ovule abortion) (Hauser et al. 2006). The same authors showed that mitochondrial physiological changes occur without reproducible modifications in their structure. Accordingly, the antitumour agent camptothecin induces an initial DNA damage in sugar beet protoplasts, eventually proceeding to extensive late cell death, which results in a higher ROS level generated by mitochondria (Weir et al. 2003). Simultaneous measurement of $m\Delta\Psi$ by flow cytometry shows a concomitant hyperpolarisation of the IMM, followed by a depolarisation at the final stages of apoptosis, similar to what was described in camptothecin-treated mammalian cells by the same authors.

NO is another important signalling molecule potentially involved in the manifestation of plant PCD. This evidence arises from experiments with *C. sinensis* suspension cell cultures, in which NO (sodium nitroprusside) triggers a cell death that is accompanied by the typical symptoms of PCD (apoptosis) and dissipation of $m\Delta\Psi$ (Saviani et al. 2002). In agreement, NO-treated soybean cells undergo PCD, which is associated to a moderate dissipation of $m\Delta\Psi$ and uncoupling (Casolo et al. 2005).

Regulation of mitochondrial pathways leading to PCD

Regulation of mammalian PCD (apoptosis) is under control of pro- and antiapoptotic members of Bcl-2 family, some of which are located in mitochondria (Kaufmann and Hengartner 2001). However, as discussed above, it is still problematic to extend such a mechanism to plant PCD. The only experimental evidence is linked to the observation that the heterologous expression of mammalian proapoptotic proteins (Bax) causes PCD in *A. thaliana* and tobacco with a mechanism, which is downregulated by the overexpression of Bax inhibitor-1 (BI-1) (Bolduc and Brisson 2002, Kawai-Yamada et al. 2004).

Nevertheless, there are numerous plant-specific regulators of PCD, including the main plant hormones (Hoeberichts and Woltering 2002). ROS signals directly or indirectly interact with other signalling pathways based on NO or hormones, such as salicylic acid, jasmonic acid and ethylene. The interaction and the balance among these pathways determine whether a cell lives or dies (Overmyer et al. 2003). Therefore, the regulation of the mitochondrial pathway leading to PCD has to involve mechanisms controlling ROS formation and scavenging.

One of the most studied systems is represented by AOX, which has been compared to an antiapoptotic protein (Maréchal and Baldan 2002). Indeed, the non-phosphorylating nature of this pathway and the ability to prevent ROS formation (Møller 2001) could play a major role in determining the fate of plant cells towards PCD.

Few studies have, however, shown a protective effect of AOX on plant PCD. In all cases, the increased expression of AOX is associated with the onset of HR (Vanlerberghe et al. 2002), suggesting that this pathway could influence PCD progression. Similarly, Robson and Vanlerberghe (2002) demonstrated that the lack of AOX in transgenic tobacco cells causes a higher susceptibility of these cells to different death-inducing treatments. In particular, death induced by H_2O_2 or salicylic acid occurs by

a mitochondria-dependent pathway, which is associated with cytochrome *c* loss prior to the appearance of the typical PCD hallmarks. Conversely, cantharidin-induced PCD was shown to occur in a mitochondria-independent way, leading to the suggestion that plants, like animals and yeasts, possess different ways to die. In addition, AOX could cooperate with cellular redox state-modulating enzymes, such as catalase and ascorbate peroxidase, in a mutually compensating way regulating PCD, by acting as a safety valve in lowering ROS accumulation and oxidative damage (Mizuno et al. 2005).

However, besides preventing ROS formation, the protection mediated by AOX probably also relies on its ability to sustain mitochondrial respiration and functions during or after stress challenge, when the cytochrome pathway is restricted (Yip and Vanlerberghe 2001). Therefore, a coordinate regulation of the cytochrome and AOX pathways may be relevant to modulate the initiation of PCD. Indeed, biotic and abiotic stresses have been described to alter the ETC by inhibiting the cytochrome pathway and inducing AOX, thus suggesting that the ETC components may play an important role during PCD. In this context, it is relevant to note that transgenic tobacco cells, unable to express AOX, lose the respiratory capacity and undergo PCD, whereas in wild-type cells the induction of AOX expression allows the maintenance of high respiratory rates and cell survival (Vanlerberghe et al. 2002). Based on these results, the authors suggested a model where loss or dysfunction of the cytochrome pathway, downstream to ubiquinone, is critical for activating PCD pathway, whereas the activity or induction of AOX may prevent this activation.

Does cytochrome *c* release induce the execution of PCD in plant cells?

As described above, in animal cells the cytochrome *c* release is followed by the assembly of the apoptosome complex and the activation of the executioner caspases. Even though there is no evidence for the formation of apoptosome in plant cells, sequence alignments have revealed significant similarities among regions of *Caenorhabditis elegans* cell death gene that encodes a protease-activating factor (CED4), human Apaf-1 and several plant resistance genes. The products of such genes do not contain a CARD, but may function as controlling adaptors in plant protein complexes, which are activated during HR (Van Der Biezen and Jones 1998). In addition, HSPs can partially suppress apoptosis in animal cells, by preventing cytochrome *c* release and disrupting the apoptosome. It has been suggested that plant HSPs accomplish comparable effects (Hoeberichts and Woltering 2002) and a strong increase in HSP during harpin-stimulated HR in *A. thaliana* cells has been reported (Krause and Durner 2004).

The downstream executors of plant PCD have not been identified yet. Even though there are no caspase homologues in plants (Dickman and Reed 2004), there is indirect evidence for caspase-like proteins involved in plant PCD, related to the observation that (1) many plants extracts, obtained from cells undergoing PCD, are capable of cleaving synthetic caspase substrates; (2) natural caspase substrates [e.g. bovine and plant poly (ADP-ribose)polymerase] are cleaved by proteases; (3) caspase inhibitors suppress plant PCD and the associated morphological and biochemical features; (4) plant cell death may be blocked by heterologous expression of the baculovirus macromolecular caspase inhibitors IAP, Op-IAP and p35 (Hansen 2000, Woltering 2004). Although the nature of caspase-like proteases is a matter of ongoing debate, two types of cysteine proteases, with structural homology to caspases, have been described: metacaspases and legumains (vacuolar-processing enzymes, VPE); in addition, a class of serine proteases that exhibit aspartate-specific cleavage activity has been reported. All these proteases can potentially form a proteolytic network involved in plant PCD (Hatsugai et al. 2004).

Conclusions

The current view is that mitochondria may be involved in some forms of plant PCD, in particular in those that are not associated with an early rupture of the vacuole (i.e. the manifestation of HR) (Fig. 1). A complex array of stimuli/effectors alters the delicate equilibrium between ROS-producing and ROS-scavenging systems that determines the life-or-death of a plant cell. ROS, but also NO, can trigger mitochondrial dysfunction, which is associated with a decrease of $m\Delta\Psi$ and cellular ATP. This results in a release of cytochrome *c* from the IMS, which may be accomplished by at least three different mechanisms. The first does not imply mitochondrial swelling and happens at the level of OMM by involving pore-forming proteins (Bax? or Bax plus VDAC?). This possibility is corroborated by accumulating evidence, showing that Bax inhibitor-1 is an evolutionarily conserved protein that can regulate cell death pathways in both plants and animals (Watanabe and Lam 2004). The other two are related to a moderate mitochondrial swelling, as a consequence of an increase in the aperture of PTP or mK^{+}_{ATP} channel, both present in the IMM. Their opening would determine the entry into the matrix of osmotically active metabolites and water, causing swelling of the IMM and the partial rupture of OMM. These mechanisms may coexist and be utilized in different metabolic contexts. Endonucleases may also be released from mitochondria and delivered to

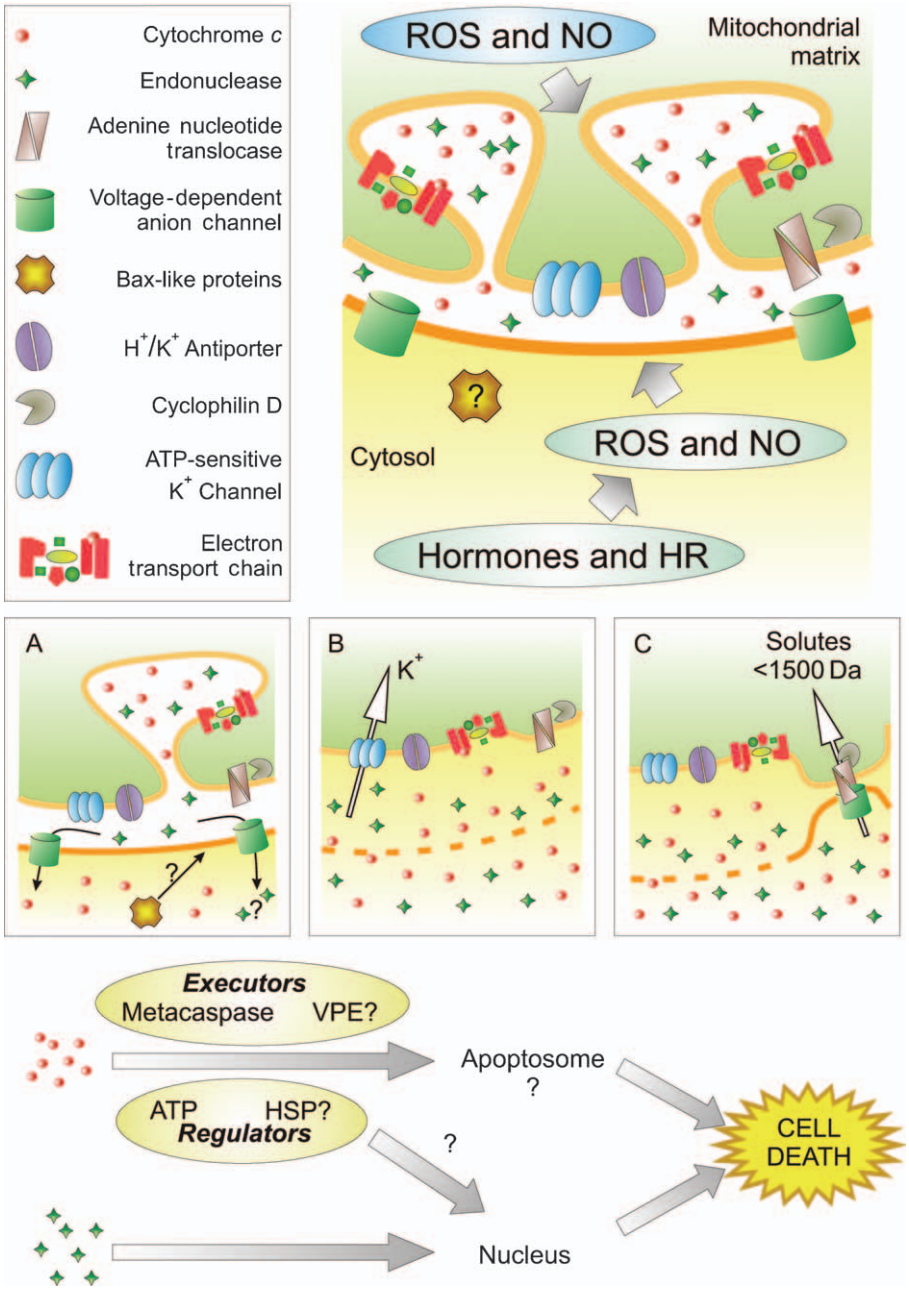

Fig. 1. Hypothetical mitochondrial signalling pathway leading to plant programmed cell death. In the response to numerous stimuli, plant cells increase reactive oxygen species and NO generation that can then trigger mitochondrial dysfunction, resulting in the release of cytochrome *c* and endonuclease from the intermembrane mitochondrial space (IMS). In panel A, pore-forming proteins are involved in such release without swelling and rupture of outer mitochondrial membrane (OMM). In panel B, the aperture of a mitochondrial K^+_{ATP} channel determines the entry into the matrix of K^+ and water, with a partial rupture of OMM. In panel C, permeability transition pore formation allows the entry into the matrix of osmotically active metabolites and water, with the rupture of the OMM. The subsequent fate of the IMS proteins is still largely unknown, as well as the steps that are involved in the execution of cell death (see text for further details).

nuclei, although there is evidence supporting the presence of endonucleases in nuclei, where they may be simply activated. More problematic is the subsequent fate of the cytochrome *c*, because the formation of a complex, like the apoptosome, is still largely speculative. If an apoptosome-like complex exists in plants, it may interact with caspase-like proteases (metacaspases, VPE) by analogy with that system in animal cells.

The model above presented, however, does not exclude other mechanisms, based on the role played by either caspase-dependent (Omi) or independent (AIF) pathways. Indeed, homologues of AIF have been identified in plants, but only on the basis of sequence alignments (Candé et al. 2002). On the other hand, by performing an National Center for Biotechnology Information-BLASTp search against *Viridiplantae* database, we found only two protein sequences from *A. thaliana* (NP 198118) and *O. sativa* (AAX96770), respectively, that are significantly similar to Omi (unpublished result). Therefore, at this stage, the involvement of such proteins in plant PCD is still hypothetical.

In conclusion, a body of evidence appears to support the involvement of plant mitochondria in PCD, even though the mechanism underlying this process is largely speculative and needs to be further corroborated.

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