

# Having a swell time – mitochondrial morphology and plant cell death programmes

D. C. LOGAN

School of Biology, Sir Harold Mitchell Building, University of St Andrews, St Andrews, Fife, KY16 9TH, Scotland, United Kingdom

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## Summary

Cell death is a vital process in multi-cellular eukaryotes. Rather than being a contradiction in terms, this statement highlights the importance of limited and localized cell killing to the health and normal development of complex organisms. The main focus of this article is the role of mitochondrial morphological changes during cell death programmes, and the conserved role of mitochondrial permeability transition (increased permeability of either the outer or inner membrane) as an early mechanistic event preceding cell death in both plant and non-plant eukaryotes. A second focus of this article is a review of the terminology and fundamental paradigms underpinning cell death research. Because of the importance of the process of cell death, there has been an enormous quantity of research performed to try to understand the underlying biological mechanisms. One result of such a large and varied research effort, and a result that is perhaps particularly evident to investigators coming into the field anew, is that some of the basic tenets of cell death research appear to have become confused. In this short article, I make an attempt to clarify the subject, focussing on the role of mitochondria, and the difficulties in comprehensibility arising from the sometimes-erroneous, or at least unnecessarily confusing use of specific terminology; there are several key terms in the cell death literature that appear interchangeable when they are not, or are interchanged when they should not be.

## Introduction

In recent years, the most frequently documented changes to mitochondrial morphology and distribution are those occurring during the initiation and progression of events leading to cell death. Studies in the 1970s and 1980s identified changes in mitochondrial morphology during

programmed cell death (PCD) in animal cells. However, there was considerable variability in the documented intracellular morphological changes that took place (Ellis *et al.*, 1991); in some cells, developmental PCD was associated with mitochondrial swelling, whereas in other cells, no major changes in mitochondria morphology occurred (Pilar & Landmesser, 1976). In fact, when the term *apoptosis* was coined the manner of cell death as described did not involve gross changes to mitochondrial morphology (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Indeed, it was postulated that an alternative form of death termed 'necrosis', rather than apoptosis, was characterized by the swelling of mitochondria and other organelles (see Kroemer *et al.*, 1998). Recently, however, researchers are collectively realizing that cell death is often not amenable to simple pigeonholing, as programmed, or non-programmed, on the basis of a few morphological criteria.

## Necrosis and apoptosis and confusion

Before a discussion of the role of mitochondria in cell death in more detail, it is necessary to define a few terms because the use of these terms has, in the plant science literature at least, become confused, if not fatuous. Apoptosis, previously termed *shrinkage necrosis* (Kerr, 1965; Kerr, 1971) was defined by Kerr *et al.* (1972) as a specific, morphologically distinct type of PCD (i.e. apoptosis is not synonymous with PCD) typically affecting scattered cells, and occurring during embryonic development, normal cell turnover and atrophy associated with hormone withdrawal. The morphological hallmarks are nuclear and cytoplasmic condensation followed by 'the formation of small, roughly spherical or ovoid cytoplasmic fragments, some of which contain pyknotic remnants of nuclei', termed *apoptotic bodies* (Kerr *et al.*, 1972). A further key feature of apoptosis is the phagocytosis and degradation of the apoptotic bodies by other cells (Kerr *et al.*, 1972). Necrosis is classically used as the name for non-apoptotic death; however, the problem with the

Correspondence to: Tel: 00 44 1334 463367; fax: 00 44 1334 463366; e-mail: david.logan@st-andrews.ac.uk

name necrosis is that it is a term used in pathology to define the presence of dead cells or tissue irrespective of the biochemical means by which death occurred, that is the features associated with necrosis are features associated with the cell's cadaver and not the mechanism of death (Majno & Joris, 1995). To make a discussion of necrosis clear, when the term is used (as by others) as a name for some means of death, it will be encased in inverted commas: 'necrosis'. 'Necrosis' is postulated to occur following accidental cell death, as a result of a severe, often violent shock to numbers of cells at once, this form of death has been characterized as involving dilation of all cytoplasmic organelles, including mitochondria, followed by rupturing of the plasma membrane (Wyllie *et al.*, 1980; Kroemer *et al.*, 1998), which causes inflammation of the neighbouring viable tissue, although this inflammation has since been proposed to result more from the fact than numerous cells are dying at once than from a key mechanistic difference between apoptosis and 'necrosis' (Majno & Joris, 1995). As argued by Fink and Cookson use of the term 'necrosis' to describe non-apoptotic death likely arose from attempts to increase clarity in scientific communication and aid the formulation of testable hypotheses (Fink & Cookson, 2005). Thus, a simple dichotomy between apoptotic and 'necrotic' cell death was easy to deal with, and became somewhat fixed in the literature and in many peoples minds; as researchers discovered more about the intricate molecular and biochemical processes involved in death, so there appeared a need to simplify the meaning of it all (Fink & Cookson, 2005).

### A new paradigm

As mentioned at the end of the penultimate paragraph, there has been an increasing understanding that the simple paradigm of apoptotic versus necrotic cell death of animal cells does not fit the growing number of empirical observations (Majno & Joris, 1995; Chipuk & Green, 2005; Fink & Cookson, 2005; Green, 2005). What is also clear is that in most cell death programmes investigated, the role of the mitochondrion, which was not thought to undergo any particular changes during apoptosis by Kerr and colleagues (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; see below), is key. For example, although attempts were made to clarify the use of the term *apoptosis*, by redefining it in terms of the activation of cytosolic caspases (a family of cysteine proteases), it is now known that apoptosis can occur in the absence of caspase activity (see Chipuk & Green, 2005). In addition, based on the results of two studies (Baines *et al.*, 2005; Nakagawa *et al.*, 2005) using mice lacking cyclophilin D, a protein proposed to be involved in the inner mitochondrial membrane permeability transition (mPT, see below), it is now accepted that changes to mitochondrial morphology can be necessary features of otherwise non-apoptotic (classically necrotic, now often termed programmed 'necrosis') cell death, reaffirming the inappropriate use of the terms 'necrotic cell death' to define any symptoms.

So what does all this mean for studies of cell death in plants? One fact is clear, based on the definition of apoptosis (e.g. formation of apoptotic bodies and their eventual phagocytosis), this is a process that does not occur in plants; the description of plant cell death phenomena, as being apoptotic, is therefore clearly incorrect. Even use of the term 'apoptosis-like' death is confusing since the features of the cell death process being described as apoptosis-like are frequently shared by non-apoptotic death programmes (van Doorn & Woltering, 2005). Plant researchers also have to be careful when describing any cell death as necrotic if the intention is to infer anything useful based on the classical definition of 'necrosis' from the animal literature. Since plant tissues do not become inflamed, one specific and defining hallmark of necrotic death is missing. Of course if we simply use the definition of necrosis as used by pathologists to describe a cell, or collection of cells that is/are dead and are in equilibrium with their environment, then necrosis, as a state, does occur in plants, just as necrosis occurs in animals, but as in animals, necrosis is best left as a description of the presence of dead cells, and not used as terminology for a means to achieve death. In plants, like in animals, and no doubt in other organisms, including yeast and protists, there is a spectrum of PCD, and changes in mitochondrial morphology are often central. In an insightful and well-argued paper, Jones (2000) made many sensible points specifically related to plant cell death, in part echoing many of the points specific to animal cell death made by Manjo & Joris (1995). Jones (2000) suggested that many plant cell deaths appear to be oncotic, meaning death with swelling, as opposed to apoptotic death which is death with shrinkage. In his article Jones asks the question, can oncosis be programmed in plants? The argument I hope to convey here is that apart from a violent, often accidental, cell death, which the cell has no possible chance to control, all cell death in plants, and probably animals too, is likely to be programmed, in so far as there is a sequence of controlled events, and that the mitochondrion is a key player in the programme.

### The mitochondrial morphology changes in more detail

Based on the results of transmission electron microscopy of dead cells and apoptotic bodies, mitochondria were not thought to undergo major morphological change during apoptosis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). However, various reports have since described changes in the ultra-structure of mitochondria, termed *ultra-condensation*, as a key feature of apoptosis (Zhuang *et al.*, 1998; Martinou *et al.*, 1999); condensation results from a reduction in the volume of the matrix with a concomitant increase in the intra-cristal space (Hackenbrock, 1968; Logan, 2006). It was not until researchers used mitochondrial-targeted GFP and light microscopy to visualize the chondriome in three dimensions that it became clear that the induction of apoptosis results in a rapid transition of the animal chondriome

from a reticulo-tubular to a punctiform phenotype (Frank *et al.*, 2001), a process that has been termed the *thread-grain transition* (Skulachev *et al.*, 2004). The higher plant chondriome typically exists, in steady state, as a population of physically discrete organelles, a discontinuous whole (Logan, 2006), and is therefore akin to the grain state of mammalian chondriomes. However, treatment of leaves of the *network* mutant of *Arabidopsis*, where the chondriome exists as inter-connected threads, with strong oxidants (e.g. hydrogen peroxide or methyl viologen) causes fragmentation of the threads suggesting that the components required to execute the thread-grain transition are also present in plants (Scott & Logan, 2007). Following the thread-grain transition, mitochondria cluster around the nucleus (De Vos *et al.*, 1998; Suen *et al.*, 2000; Skulachev *et al.*, 2004), a phenotype that, at least when induced by tumour necrosis factor, has been shown to result from a perturbation to the microtubule motor protein, kinesin (De Vos *et al.*, 1998). Mitochondria have even been seen within the nucleus following the induction of apoptosis (Skulachev *et al.*, 2004). The role of the perinuclear and occasional intra-nuclear location of mitochondria is unknown but has been suggested to enable the direct movement of death proteins from mitochondria to the nucleus (Skulachev *et al.*, 2004). The events downstream of the thread-grain transition and discussion of the role, if any, of this event in the release of pro-apoptotic cytochrome *c* and other inter-membrane space proteins are out with the scope of this article and readers are instead directed to several recent reviews (Youle & Karbowski, 2005; Cereghetti & Scorrano, 2006; Cheung *et al.*, 2007).

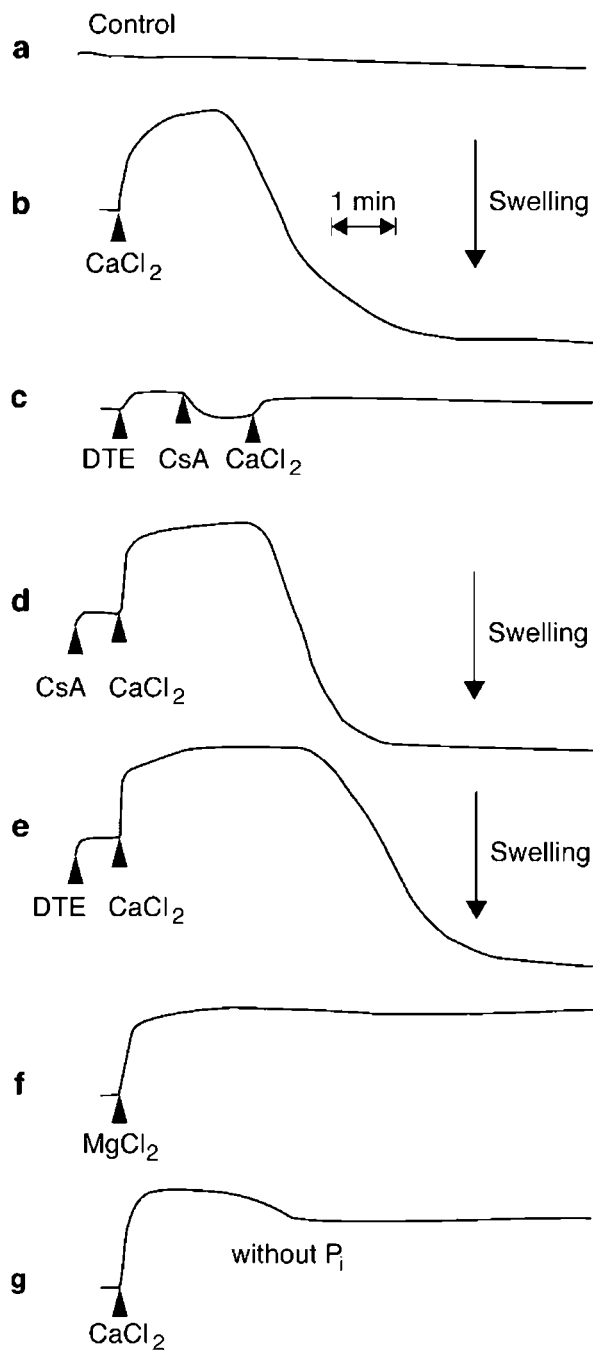
#### *The mitochondrial permeability transition (mPT)*

*In animals.* Induction of the death of some animal cells, resulting from some perturbations, leads to a second type of mitochondrial morphology change: swelling of the matrix, which results from a change in the permeability of the inner mitochondrial membrane. The mitochondrial permeability transition (mPT) was, until recently, considered to be solely a feature of certain types of apoptotic cell death. However, it is now considered also to be involved in a mitochondrial-dependent pathway of non-apoptotic cell death (Baines *et al.*, 2005; Nakagawa *et al.*, 2005; see above). The mPT was first described by Hunter and colleagues following the addition of low concentrations of calcium to isolated bovine heart mitochondria (Hunter *et al.*, 1976). In addition to an increase in permeability, such treatment caused a conformational change of the matrix from condensed to orthodox, induction of ATPase activity, uncoupling of oxidative phosphorylation and loss of respiratory control. Interestingly, it was reported that the swelling itself was not correlated with the state of coupling; even grossly swollen mitochondria being capable of coupled respiration (Hunter *et al.*, 1976). A turning point in the study of this mPT was the discovery that the immunosuppressant, cyclosporin A (CsA), inhibited the transition (measured as

swelling of isolated rat liver mitochondria; Broekemeier *et al.*, 1989). Subsequent electrophysiological experiments identified a non-selective 'megachannel' conductance of the inner mitochondrial membrane (Kinnally *et al.*, 1989; Petronilli *et al.*, 1989) that was, based on its inhibition by CsA (Szabo & Zoratti, 1991) and modulation by calcium (Szabo *et al.*, 1992), responsible for the calcium-dependent permeability transition first detected in isolated mitochondria by Hunter and colleagues (Hunter *et al.*, 1976).

The composition of the mPT channel has been the subject of a great deal of research, and is still a matter of debate (see Brenner & Grimm, 2006). Some studies point to a multipartite, organized structure that localizes at contact points between the outer and inner mitochondrial membranes and is composed of the adenine nucleotide transporter (ANT) (Brustovetsky & Klingenberg, 1996; Faustin *et al.*, 2004), the outer membrane voltage-dependent anion channel (also called Porin; Crompton *et al.*, 1998) and cyclophilin D (Woodfield *et al.*, 1998), whereas others have suggested that the permeability pore/channel forms by aggregation of misfolded integral membrane proteins damaged by oxidative and other stresses (He & Lemasters, 2002). There is certainly empirical evidence that questions the central roles of both ANT and cyclophilin D as components of the channel (Kokoszka *et al.*, 2004; Baines *et al.*, 2005; Nakagawa *et al.*, 2005).

*In plants.* Evidence for activation of an mPT in plants was first obtained from *in vitro* experiments on oat (Curtis & Wolpert, 2002), wheat (Virolainen *et al.*, 2002), potato (Fortes *et al.*, 2001; Arpagaus *et al.*, 2002) and *Arabidopsis* mitochondria (Tiwari *et al.*, 2002). Swelling of isolated potato mitochondria was induced by Ca<sup>2+</sup>, but not Mg<sup>2+</sup>, and Ca<sup>2+</sup>-induced swelling was inhibited by CsA, but only in the presence of the reducing agent dithioerythritol (Arpagaus *et al.*, 2002; Fig. 1 cf. (c–e)); swelling, induced by oxidative stress, of isolated *Arabidopsis* mitochondria was also sensitive to CsA (Tiwari *et al.*, 2002). Swelling was reported to be insensitive to CsA in experiments with either oat, or wheat (Curtis & Wolpert, 2002; Virolainen *et al.*, 2002), or in the potato study by Fortes *et al.* (2001), although this may be due to the reported requirement for reduced conditions prior to the addition of CsA (Arpagaus *et al.*, 2002), a situation that was not tested in the other studies. Swelling led to disruption of the outer, but not inner, mitochondrial membrane and release of cytochrome *c* (Arpagaus *et al.*, 2002). The studies by Arpagaus *et al.* (2002) and Virolainen *et al.* (2002) are two of many that provide evidence for the release of cytochrome *c* during events implicated in the induction of plant cell death. Cytochrome *c* release has a clear role in apoptosis through its role in the formation of the caspase-activating apoptosome. However, it is not clear if cytochrome *c* release has a specific role to play in the execution of a PCD pathway in plant cells, nor is it known if there is a link between mechanisms underlying the mPT and cytochrome *c* release in plants; no links have been established *in vivo* between these two events. For a review of cytochrome

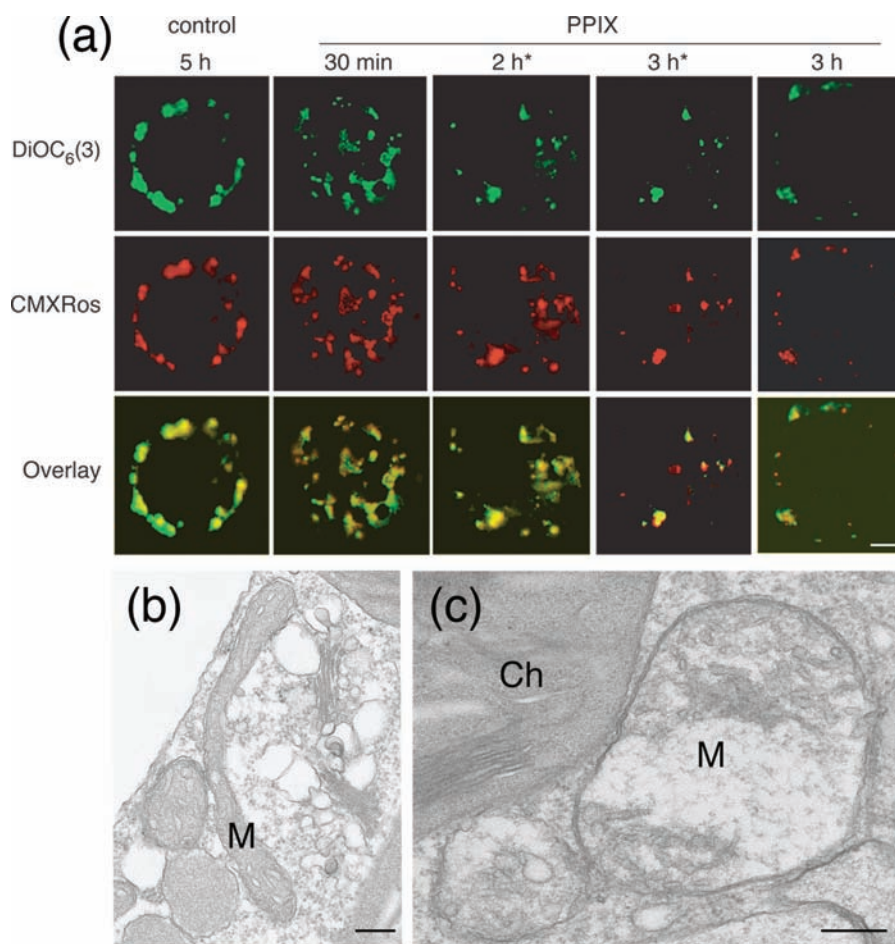


**Fig. 1.** Phosphate-dependent  $\text{Ca}^{2+}$ -induced swelling of isolated potato mitochondria measured by light scattering. There is an initial contraction of the mitochondria (rise in amplitude of the trace) followed by a pronounced swelling, marked by the downward-pointing arrow. (a) Control, no additions; (b) plus 5 mM  $\text{CaCl}_2$ ; (c) pre-treatment with dithioerythritol (DTE, 1 mM) and cyclosporin A (CsA, 1.6  $\mu\text{M}$ ) prior to addition of 5 mM  $\text{CaCl}_2$ ; (d) pre-treatment with CsA alone prior to addition of 5 mM  $\text{CaCl}_2$ ; (e) pre-treatment with DTE alone prior to addition of 5 mM  $\text{CaCl}_2$ ; (f) addition of 5 mM  $\text{MgCl}_2$  instead of  $\text{CaCl}_2$ ; (g) as (b) but phosphate was omitted from the medium. Reproduced from Arpagaus *et al.* (2002) with permission. © 2002 The American Society for Biochemistry and Molecular Biology, Inc.

*c* release in plant PCD and its significance, see Diamond & McCabe (2007).

The role of an mPT in plant PCD has been demonstrated in a number of studies using a variety of death inducers. Yu and colleagues showed that CsA could inhibit PCD of tracheary elements in *Zinnia elegans* and that there were subtle changes in mitochondrial ultra-structure prior to loss of tonoplast integrity and cellular autolysis (Yu *et al.*, 2002). It was reported that the changes to mitochondrial ultra-structure were different from those that occur during apoptosis of animal cells, but in fact an increased density of the matrix and swelling of the intra-cristal spaces, as reported, are the same as has been reported to occur during apoptosis (Zhuang *et al.*, 1998; Martinou *et al.*, 1999). What was not observed was any significant swelling of the mitochondria (Yu *et al.*, 2002). Using a hypersensitive response elicitor, ceramide or protoporphyrin IX (PPIX), a molecule similar to the proposed substrate of ACCELERATED DEATH 2, which is a red chlorophyll catabolite reductase, it was reported that death of *Arabidopsis* leaf cells was accompanied by a loss of mitochondrial membrane potential (Fig. 2(a)) and nuclear DNA cleavage and that these features could be partially abrogated by CsA, as could death itself (Yao *et al.*, 2004). This is one of the few plant cell death studies in which death was measured rather than inferred. Subsequent work by Yao & Greenberg confirmed the effect of PPIX treatment on mitochondrial morphology in wild-type protoplasts: the mitochondria became swollen, and more round in shape (Figs 2(b) & (c); Yao & Greenberg, 2006).

We have recently published the results of an investigation into the changes in mitochondrial morphology in *Arabidopsis* protoplasts expressing mitochondrial-targeted GFP (Logan & Leaver, 2000) during the induction of cell death (Scott & Logan, in press). Chemical treatment with strong oxidants, or a mild heat shock, caused a rapid and consistent change in mitochondrial morphology, characterized as an increase in the size of individual organelles (and termed the mitochondrial morphology transition, Fig. 3), that preceded measured cell death by many hours. Treatment of protoplasts with a cell permeable superoxide dismutase analogue, TEMPOL, blocked this morphology change. Furthermore, incubation of protoplasts in micromolar concentrations of the calcium channel-blocker lanthanum chloride, or the permeability transition pore inhibitor CsA, prevented both the mitochondrial morphology transition and subsequent cell death. The mitochondrial morphology transition can be observed much earlier than similar changes in morphology previously reported *in vivo* (Yoshinaga *et al.*, 2005; Zottini *et al.*, 2006). We concluded that the mitochondrial morphology transition that we measured directly in intact protoplasts, or leaf cells, is synonymous with the mPT, as measured *in vitro* by others, and that perturbation of the permeability of the inner mitochondrial membrane is at least one important mechanism promoting cell death in plants (Scott & Logan,



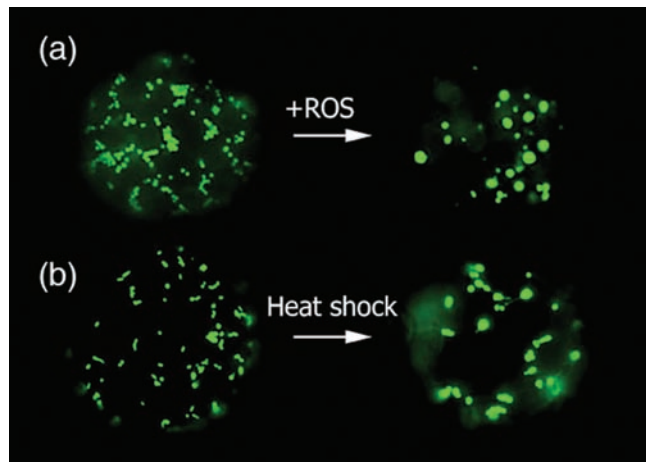
**Fig. 2.** Mitochondrial morphology and depolarization during protoporphyrin IX (PPIX) treatment of wild-type *Arabidopsis* protoplasts. (a) Effect of PPIX on mitochondrial membrane potential in wild-type (WT) protoplasts. Cells were double stained with 5 nM DiOC<sub>6</sub> and 50 nM CMXRos for 5 min. The cell in the middle (2 h\*) was the same as the cell to its right (3 h\*). Note that fluorescence intensity of DiOC<sub>6</sub> or CMXRos was reduced at 3 h compared with 2 h. Scale bar = 10 μm. Reproduced with permission from Yao *et al.* (2004), © 2004 Blackwell Publishing Ltd. (b) and (c) Protoplasts from wild-type leaves treated with (c), or without (b) 10 μM PPIX for 5 h in the light and then fixed and prepared for electron microscopy. Note that mitochondria are more swollen and rounded after PPIX treatment (c) than in control cells (b). Ch, chloroplast; M, mitochondria. Scale bar = 200 nm. Reproduced from Yao & Greenberg (2006) with permission, © 2005 American Society of Plant Biologists.

in press). Swelling of mitochondria has also been reported *in vivo* during the self-incompatibility response in poppy pollen (Geitmann *et al.*, 2004) and during abortion of microspores in CMS sunflower (Laveau *et al.*, 1989). However, although the changes to mitochondrial morphology can be postulated (and indeed are likely) to be linked to the PCD that occurs in these systems, further studies are required to show direct links.

#### *Outer versus inner membrane permeabilization*

Induction of the mPT causes matrix swelling leading to increased permeability and possibly the rupturing of the mitochondrial outer membrane, and thereby the loss to the cytosol of inter-membrane space localized proteins known to be potent inducers of apoptosis. However, since apoptosis frequently does not result in swelling of individual

mitochondria following the thread-grain transition, and the absence of cyclophilin D does not reduce the effectiveness of various pro-apoptotic proteins such as Bax, Bak or Bid in inducing cell death (Baines *et al.*, 2005; Nakagawa *et al.*, 2005) the role of the mPT in apoptosis has been questioned. Instead, an alternative sequence of events has been proposed whereby it is an increased permeability of the outer mitochondrial membrane (mitochondrial outer-membrane permeability [MOMP]), without changes to the permeability of the inner membrane and therefore no matrix swelling, that is a common feature of mitochondria-dependent apoptosis (for review, see Danial & Korsmeyer, 2004; Green, 2005). The MOMP is suggested to occur via complicated interactions of BH3-only (Bad, Bid) proteins with anti-apoptotic Bcl-2 family proteins and multi-domain (Bak, Bax) pro-apoptotic proteins, with Bax forming the pore in the outer



**Fig. 3.** Mitochondrial morphology transition in *Arabidopsis* mesophyll protoplasts expressing mito-GFP. Typical epifluorescence micrograph of protoplasts before and after: (a) incubation in 5  $\mu\text{M}$  methyl viologen for 24 h or (b) 5 min after a 10-min heat shock at 45°C. See Scott & Logan (2008) for more details.

mitochondrial membrane (Antonsson *et al.*, 1997; Schlesinger *et al.*, 1997). It is unclear how this role for Bax as a channel-forming protein fits with the evidence that, during the early stages of apoptosis, both Bax and Bak coalesce into large foci on the surface of the OMM (Nechushtan *et al.*, 2001) that co-localize with DRP1 and MFN2 at mitochondrial fission sites (Karbowski *et al.*, 2002). There have been no reports, to my knowledge, of the specific involvement of MOMP in plant cell death.

An alternative scenario leading to MOMP is the formation of ceramide channels in the OMM and their regulation by shingosine-mediated channel disassembly (Siskind *et al.*, 2002; Anishkin *et al.*, 2006). These results are particularly interesting given the complex effects of the fungal toxin fumonisins B1, an inhibitor of ceramide synthase (Merrill *et al.*, 1993), on plant and animal PCD (Dombrinkkurtzman *et al.*, 1994; Stone *et al.*, 2000; Chivasa *et al.*, 2005; Kitatani *et al.*, 2006).

#### *Pro-apoptotic proteins and mitochondrial morphology and their absence from plants*

No functional homologues of any of the animal Bcl-2 family have been identified in plant genomes to date. This result is not surprising given the recent evidence that in healthy mammalian cells Bax, or Bak, is required for normal fusion of mitochondria into elongated tubules (Karbowski *et al.*, 2006), a mitochondrial morphology that does not generally occur in plant cells (Logan, 2006). However, over-expression of mammalian Bax in tobacco (Lacomme & Santa Cruz, 1999) and in *Arabidopsis* (Kawai-Yamada *et al.*, 2001) causes cell death. In the *Arabidopsis* study, the mouse Bax gene was

expressed in plant cells under an inducible promoter system, and upon induction, the plants exhibited cell death at the whole-plant level (Kawai-Yamada *et al.*, 2001). It is not known if heterologous Bax localizes to plant mitochondria, or if Bax can cause MOMP in plants. Despite the absence of obvious Bax homologues, the *Arabidopsis* and rice genomes contain homologues of the negative regulator of apoptosis, Bax inhibitor-1 (BI-1). Expression of the plant BI-1 homologue has been reported to suppress the Bax-induced PCD in yeast and *Arabidopsis* (Kawai *et al.*, 1999; Kawai-Yamada *et al.*, 2001, 2004). AtBI-1 has been used as bait to fish for *Arabidopsis* cell death-inducing genes (Kawai-Yamada *et al.*, 2005). The screen involved identification of *Arabidopsis* cDNAs that, upon expression in yeast, induced death in an AtBI-1-dependent manner. Screening 20000 cDNA clones enabled the identification of a family of genes named *Cdfs* (cell growth defect factor) encoding polypeptides of circa 30 kDa (Kawai-Yamada *et al.*, 2005). *Cdf1* has no significant homology to proteins of known function nor does it contain any recognizable protein motifs (Kawai-Yamada *et al.*, 2005). The two *Arabidopsis* homologues, At3g51140 and At2g20929 share 32.9 and 22.4% identity with *Cdf1* (Kawai-Yamada *et al.*, 2005). There are similarities in the mode of action of *Cdf1* and Bax in inducing yeast cell death: (1) both proteins induce similar changes to the internal structure of yeast cells, (2) both proteins are ineffective against the Bax-resistant yeast mutant, BRM1 and (3) both localize to yeast mitochondria (Kawai-Yamada *et al.*, 2005). It is not known whether or not *Cdf1* has death-inducing activity in *Arabidopsis*, whether *Cdf1* localizes to *Arabidopsis* mitochondria, or whether it has any effect on mitochondrial morphology. However, taken together the data discussed in the above section suggest that certain features of PCD are conserved between plant and non-plant eukaryotes.

#### **Mitochondria and calcium**

Finely tuned regulation of intra-cellular and intra-organellar  $\text{Ca}^{2+}$  activities is fundamental for the maintenance of intra- and inter-cellular signalling and the survival or death of the cell or organism. For many years, it was considered that mitochondrial  $\text{Ca}^{2+}$  uptake simply reflected changes in the cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{cyt}}$ ) with the mitochondria acting as  $\text{Ca}^{2+}$  buffers. However, thanks to experiments using the  $\text{Ca}^{2+}$  indicator, aequorin, targeted to mitochondria, it is now clear that fluxes in the mitochondrial calcium concentration ( $[\text{Ca}^{2+}]_{\text{mit}}$ ) are integrated components of intra-cellular signalling (Rizzuto *et al.*, 2000). In plants  $[\text{Ca}^{2+}]_{\text{mit}}$  has also been shown to be regulated (Logan & Knight, 2003). Intra-cellular  $\text{Ca}^{2+}$  over-load can initiate a series of catastrophic events leading to cell death. For example, efflux of  $\text{Ca}^{2+}$  from ER microdomains, where the ER and mitochondria are in close physical proximity, leads to  $\text{Ca}^{2+}$  influx into mitochondria and an mPT. Bcl-2 family proteins

are also involved in the modulation of ER  $\text{Ca}^{2+}$  dynamics and hence  $[\text{Ca}^{2+}]_{\text{mit}}$ . Over-expression of Bcl-2 (Pinton *et al.*, 2001) or reduced expression of Bax or Bak (Scorrano *et al.*, 2003a, b) results in a reduced  $[\text{Ca}^{2+}]_{\text{er}}$ , reduced mitochondrial  $\text{Ca}^{2+}$  influx and prevention of apoptosis induction by  $\text{Ca}^{2+}$ -dependent stimuli.

Direct effects of  $\text{Ca}^{2+}$  on mitochondrial fission in a number of mammalian cell types have been reported. Treatment with A23187, a  $\text{Ca}^{2+}$  ionophore, promotes mitochondrial fragmentation in myoblasts and astrocytes (Duncan *et al.*, 1980) and in HeLa cells (Karbowski & Youle, 2003).  $\text{Ca}^{2+}$  has also been shown to have an important role in mitochondrial fragmentation during ceramide-induced apoptosis in HeLa cells (Pinton *et al.*, 2001; Rapizzi *et al.*, 2002). Ceramide induces an increase in  $[\text{Ca}^{2+}]_{\text{cyt}}$  that is associated with mitochondrial fragmentation; conditions that reduce  $[\text{Ca}^{2+}]_{\text{cyt}}$  decrease mitochondrial fragmentation and ceramide-induced cell death (Pinton *et al.*, 2001; Rapizzi *et al.*, 2002). Given the effect of  $\text{Ca}^{2+}$  on mitochondrial fragmentation and the fact that dynamin-regulated endocytosis is regulated by  $\text{Ca}^{2+}$ , it has been suggested that  $\text{Ca}^{2+}$  may regulate Drp1 directly and regulate other proteins involved in mitochondrial fission (Karbowski & Youle, 2003).

Little is known about the role of  $\text{Ca}^{2+}$  in mitochondrial morphology changes in plant cell death beyond its involvement in stimulation of an mPT (Arpagaus *et al.*, 2002; Curtis & Wolpert, 2002; Virolainen *et al.*, 2002; Curtis & Wolpert, 2004), the inhibitory effect of  $\text{LaCl}_3$  on the mPT measured as a mitochondrial morphology transition (Scott & Logan, in press), and death induction upon treatment of carrot cell suspension cultures with the  $\text{Ca}^{2+}$  ionophore, A23187 (McCabe *et al.*, 1997). Clearly, there is a role for intracellular  $\text{Ca}^{2+}$  homeostasis in shaping cell death programmes in plants even if those roles are currently un-defined. Also lacking is our understanding of  $\text{Ca}^{2+}$  and cell death in non-plant organisms. However, one possible role for  $\text{Ca}^{2+}$  is the regulation of oxidative stress and the cells response to oxidative stress.  $\text{Ca}^{2+}$  signalling and redox state are tightly linked (Moller & Rasmusson, 1998; Bowler & Fluhr, 2000; Pei *et al.*, 2000; Rentel & Knight, 2004; Kobayashi *et al.*, 2007) and are likely instrumental in regulating PCD in eukaryotes.

## Conclusions

Cell death is a means to shape organism structure and to defend against disease. In all cases where the cell is able to respond to death-inducing stimuli, be it developmentally programmed or the result of environmental stress (biotic or abiotic), the response is likely to be programmed, in so far as death occurs via a series of organized and genetically controlled events. In other cases, where cell death occurs owing to a severe, violent, often accidental perturbation, the response is uncontrollable cell death. Uncontrollable cell

death, by its violent nature, can therefore be defined as a physical phenomenon, rather than a biological one: it is the result of a severe physical perturbation that the cell cannot mitigate against. Despite the apparent complexity of death programmes and some confusion likely resulting from attempts to simplify this complexity, one common thread in many types of PCD is the involvement of mitochondria. Changes in mitochondrial permeability and morphology have been demonstrated to be involved very early in the series of events leading to death resulting from the execution of various types of PCD in both animals and in plants. However, the exact role(s) of mitochondria in the process of plant PCD, and in some animal PCD remains unclear; the precise mechanisms leading to morphological and permeability changes are unknown, as is, in plants, the identity of any genetic component. The conserved central role of mitochondria and mitochondrial morphological changes in PCD in multi-cellular eukaryotes, together with the clear differences, between animals and plants, in downstream events following the induction of PCD demonstrates that the study of PCD in plants has the potential to teach us a great deal about the evolution of PCD and highlight some of the key events at the mitochondria level.

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