Chapter 2
Mitochondrion and Chloroplast Regulation of Plant Programmed Cell Death

Theresa J. Reape, Niall P. Brogan, and Paul F. McCabe

2.1 Introduction

Programmed cell death (PCD) is a sequence of events that lead to the controlled and organised destruction of the cell [1]. PCD is a fundamental process in plants, controlling the elimination of cells during development, defence (the hypersensitive response) and stress responses (see Kacprzyk et al. [2] for comprehensive review). The decision as to whether a plant cell activates PCD, or not, is determined by information it receives from a number of sources, including its environment, for example, cell survival signals, developmental cues, pathogen recognition, stress signals or internal information such as developmental history, cellular damage and metabolic state [3]. Different modes of PCD occur in plant cells; one type is strongly characterised by condensation of the protoplast away from the cell wall. This distinctive morphology has been visualised in cells which have died following stress, during developmental programming or during the hypersensitive response [4]. In terms of a stress response, cells can often survive a mild stress, while higher levels of stress induce the cells to initiate a PCD programme resulting in corpse cells with a condensed protoplast [5]. Increasing the stress to an even higher level leads, of course, to death, but cells do not display this distinctive morphology and are deemed to have died via necrosis [5] (see Fig. 2.1). By monitoring the level of stress applied, an abiotic stress such as heat shock can cause plant cells to die via PCD, and following heat shock, the classic condensed protoplast morphology has been observed in many species such as Arabidopsis, carrot, tobacco, soybean, Zea mays, Quercus robur, Medicago truncatula and lace plant [6–11]. Following biotic stresses such as the hypersensitive response (HR), cells at the site of pathogen invasion undergo PCD, isolating the pathogen and preventing further spread [12, 13].
Fig. 2.1 Cell types present following a 10-min 53 °C heat treatment of *Arabidopsis thaliana* suspension cells. (a) Cells that are alive have the ability to cleave FDA and fluoresce under light at a wavelength of 490 nm. (b) Necrotic cells cannot cleave FDA and so do not fluoresce and show no evidence of protoplast condensation. (c) In cells that undergo PCD, the protoplast retracts from the cell wall. These cells cannot cleave FDA and so do not fluoresce. As this is a moderate stress, the majority of the cells die via PCD. Adapted from Diamond, M.; Reape, T. J.; Rocha, O.; Doyle, S. M.; Kacprzyk, J.; Doohan, F. M.; and P. F. McCabe (2013). The *Fusarium* mycotoxin deoxynivalenol can inhibit plant apoptosis-like programmed cell death. *PLoS ONE* 8: e69542. DOI:10.1371/journal.pone.0069542, with permission from the authors.
When HR cell death is induced in oats with the host-selective toxin victorin, cell shrinkage is found to be associated with death [14], and during and following this shrinkage, the plasma membrane remains intact. HR elicitors also induce this condensed morphology in soybean and tobacco [12, 15]. This mode of PCD has also been observed in many examples of normal plant tissue development such as leaf morphogenesis [16], senescence [17], embryogenesis [6], tapetum development [18] and premature death of anther tissues during cytoplasmic male sterility [19].

The plant cell is constantly sensing signals from its internal and external environment and must act on those signals. How does the plant cell coordinate these signals and make this ultimate decision to undergo PCD? We know that the mitochondrion plays a central role in control of plant PCD (reviewed recently by Diamond and McCabe [20]), but our understanding of its role as executioner is far from complete. We can manipulate in vitro models and study the contributions of individual organelles, proteins and genes to PCD; however, in the context of the whole plant, it is unlikely that any organelle or molecule will act alone during PCD or indeed behave in a uniform fashion in different parts of the plant. The orchestration of PCD throughout a plant is without doubt extremely complex and reliant on numerous factors. However, we can progress our understanding by studying these less complicated in vitro models, and recently, further insight into the role of the chloroplast in plant PCD has been explored in the *Arabidopsis* suspension cell heat shock model [21, 22]. In this review, we discuss how the mitochondrion can act on environmental cues and orchestrate plant PCD and examine what we know to date of the involvement of the chloroplast in this process.

### 2.2 The Mitochondrion and PCD

Mitochondria were first shown to be components of PCD regulation in mammalian cells [23, 24]. Since then mitochondria have proved to have a central regulatory role in integrating environmental signals which determine whether a cell will live or die. The mitochondrion does this by coordinating death signals that lead to the initiation of cell death and by releasing molecules that drive the destruction of the cell (see reviews by Diamond and McCabe [20], Green and Reed [25], Jones [26], Bras et al. [27]).

#### 2.2.1 The Mitochondrion and Apoptosis

Many studies investigating the role of mitochondria in plant PCD have focused on possible similarities with mammalian apoptosis, as the mitochondrion is a central component during this form of PCD. Mitochondria coordinate death signals in the induction phase, which involves the perception of the death-inducing stimulus by the cell and initiation of the death programme and then triggers the cell death
programme through the release of pro-apoptotic molecules during the effector phase, where the cell commits irrevocably to death [28]. The release of these pro-apoptotic molecules or mitochondrial factors is of critical importance to the progression of apoptosis. However, the mechanism by which the pro-apoptotic molecules are released from mitochondria is still under investigation. When mitochondria perceive stimuli, for example, death signals within the cell trigger alterations in the inner mitochondrial membrane (IMM), resulting in a loss of the mitochondrial transmembrane potential ($\Delta \Psi_m$) and opening of the mitochondrial permeability transition pore (PTP). This loss of the mitochondrial transmembrane potential allows for the release of two main groups of pro-apoptotic proteins from the intermembrane space (IMS) into the cytosol [29]. The first group of proteins consists of cytochrome c (cyt c), SMAC/DIABLO and the serine protease Omi/HtrA2 [30–34]. Cyt c is a soluble protein found in the intermembrane space loosely associated with the MM [35]. Under normal conditions, cyt c is a necessary component of the electron transport chain in mitochondrial respiration [24, 36]. However upon its release, cyt c forms a complex with procaspase-9, dATP and the apoptosis protease-activating factor (Apaf-1), which leads to the formation of the apoptosome. This apoptosome can then activate downstream effector caspases which regulate apoptosis. Caspases are amongst the most specific of proteases, with an absolute requirement for cleavage of aspartic acid, consistent with the observation that apoptosis is not accompanied by indiscriminate protein digestion. The binding of cyt c and Apaf-1 increases the affinity of Apaf-1 for dATP and induces a conformational change that exposes the caspase recruitment domain (CARD) of Apaf-1 [27]. This CARD interacts with procaspase-9 creating a holoenzyme with the ability to activate caspase-3 and caspase-7, leading to the cleavage of procaspase-2, procaspase-6, procaspase-8 and procaspase-10 resulting in a proteolytic cascade eventually causing cellular destruction [37, 38]. However, regulatory mechanisms do exist both to prevent the unnecessary activation of apoptosis and also to prevent the inhibition of apoptosis where cell death is necessary. The activation of caspases can be negatively regulated by inhibitor of apoptosis proteins (IAPs). These IAPs bind and inhibit caspase-3, caspase-7 and/or caspase-9; however, upon apoptosis induction, these IAPs can also be inhibited through the antagonising effects of the mitochondrial proteins SMAC/DIABLO and Omi/HtrA2 [39].

Further regulation checks are also in place for the release of pro-apoptotic molecules, in the form of B cell lymphoma 2 (Bcl-2) family of proteins, which contain both pro- and anti-apoptotic members [40]. The anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL contain four Bcl-2 homology regions (BH1-4). The pro-apoptotic members consist of multidomain proteins containing three Bcl-2 homology regions (BH1-3) such as Bax and Bak and single-domain BH3-only proteins including Bid and Bad. Under normal homeostatic conditions, Bak is localised in the outer mitochondrial membrane (OMM), while Bax is localised to the cytosol. However, once apoptosis is induced, the activation of Bax by the cleaved Bid results in homooligomerization within the OMM forming a molecular opening that facilitates the release of pro-apoptotic molecules from mitochondria and induction of cell death. On the other hand, it is believed that the anti-apoptotic molecules are normally
localised in the OMM, so they can interact, bind and inhibit the pro-apoptotic Bcl-2 members to prevent mitochondrial membrane permeabilisation (MMP) and the release of pro-apoptotic molecules from the mitochondria into the cytosol. The pro-apoptotic function of BH3-only molecules is completed in two distinct ways, (1) activation of the BH123 proteins to induce MMP, that is achieved by translocation of Bax to the OMM, or (2) binding to BH1234 molecules so they cannot interact with pro-apoptotic Bcl-2 family proteins [41], thus indirectly facilitating apoptosis.

The extrinsic pathway is mediated by molecules known as “death receptor” molecules that are located on the cell surface; this “death receptor” pathway is activated from outside the cell by ligation of transmembrane death receptors such as Fas, TNF, TRAIL and DR3–6 receptors with their corresponding ligands. Upon activation, the adaptor molecules bind to the cytosolic ends of the death receptors to generate a signal, known as the death-inducing signalling complex (DISC), by recruiting the adaptor Fas-associated death domain (FADD) and procaspase-8 and procaspase-10, resulting in caspase-8 and caspase-10 activation, leading to cleavage of effectors caspase-3 and caspase-7. Through this pathway, the death receptors can directly activate caspases and initiate apoptosis without mitochondrial involvement, as active caspase-8 can activate the downstream effector caspases [42]. However, if the death receptors do not generate a signal sufficient enough to induce cell death, mitochondrion-dependent apoptotic pathways can amplify this weakened signal. In this scenario, caspase-8 can cleave Bid and initiate the release of cyt c from IMS, which leads to the initiation of apoptosis in a manner that does not seem to cause typical mitochondrial swelling [43].

2.2.2 The Mitochondrion and Plant PCD

2.2.2.1 Cytochrome c

Changes in mitochondrial morphology, loss of $\Delta \Psi_m$ and cyt c release from the mitochondria are the earliest markers of plant PCD following stress [14, 44–47]. Cyt c release from the mitochondria has been documented in numerous in vitro stress models of plant PCD [44–46, 48–50], during developmental models [19, 51, 52] and following activation of the HR [14, 53]. Unlike apoptosis, plant cells do not contain caspases [54], and therefore, cyt c release from plant mitochondria cannot directly result in an apoptotic-like proteolytic cascade of events leading to the demise of the cell [45, 49]. However, although plants lack canonical caspases, there is well-documented evidence for caspase-like activity during plant PCD [2, 4], and some of this activity has been shown to be associated with plant subtilisin-like proteases, saspases and phytaspases which hydrolyse a range of caspase substrates following the aspartate residue [55]. The addition of broken plant mitochondria to Arabidopsis nuclei in a cell-free system results in chromatin condensation, high molecular weight DNA cleavage and DNA laddering, whereas addition of purified cyt c has no effect [45]. Pharmacological studies and submitochondrial localisation
suggested that a Mg\textsuperscript{2+}-dependent nuclease which resides in the mitochondrion IMS is responsible for the high molecular weight DNA cleavage and chromatin condensation, but DNA laddering required addition of a cytosol extract in addition to the mitochondria [45]. Therefore, evidence points towards there being molecules, in addition to cyt c, which are released from plant mitochondria during PCD and are involved in some way in plant death-associated protease activity. However, as of yet, these molecules have not been identified.

2.2.2.2 Mechanisms of Release of Mitochondrial Proteins

How are cyt c and potential death-inducing molecules released from the mitochondria during stress response? One way in which MMP is achieved in mammalian cells is via a specific Bax/Bcl-2 controlled pore. As mentioned earlier, the Bcl-2 family of proteins is composed of pro- (Bid, Bad, Bak and Bax) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) [56]. Under normal conditions, mitochondrial outer membrane integrity is maintained through a balance between these pro- and anti-apoptotic proteins, and proteins such as cyt c are prevented from being released from the mitochondria. There is an endoplasmic reticulum-located Bax inhibitor-1 (BI-1) conserved in animals and plants, and overexpression of Arabidopsis AtBI-1 can downregulate mammalian Bax-induced PCD [57]. Murine Bax (BI-1) can induce cell death and initiate a hypersensitive-like response in tobacco when expressed from a viral vector [58], and the anti-apoptotic Bcl-xL, when expressed in tobacco, can confer resistance to death induced by known activators of cellular ROS [59]. However, no plant homologs of the Bcl-2 family have been identified to date, and although these animal proteins can operate in plants, there is no direct evidence for a Bcl-2 family-formed pore occurring during plant PCD.

Another mode of release of apoptotic factors from the mitochondria, mentioned above, is via the PTP, a polypeptide complex formed at contact sites between the IMM and OMM and thought to act through the interaction between the voltage-dependent anion channel (VDAC) on the outer membrane, the adenine nucleotide transporter (ANT) from the inner membrane and cyclophilin D (CypD) in the matrix and other proteins [25, 26]. The PTP can be formed following cellular stress (build up of Ca\textsuperscript{2+}, changes in phosphate and/or ATP levels, ROS production), resulting in loss of the inner $\Delta\Psi_m$, osmotic swelling of the mitochondria, disruption of the OMM and subsequent release of IMS proteins. There is strong evidence for the PTP playing a role in plant MMP. Homologs exist in plants for the major constituents of the PTP complex, VDAC, ANT and CypD [46]. In plants, an early loss of $\Delta\Psi_m$, preceding cell shrinkage and DNA degradation, has been detected after induction of PCD in oat seedlings following treatment with victorin, a host-selective toxin produced by Cochliobolus victoriae [14]. Similar findings have also been reported for Arabidopsis protoplasts treated with ceramide, methyl jasmonate or ultraviolet-C overexposure ([46, 60, 61], respectively) and in Arabidopsis suspension cells treated with harpin and acetylsalicylic acid [48, 62]. PTP opening can be inhibited by cyclosporin A (CsA) which acts by displacing the binding of CypD to ANT [63],
and this has been used as a tool to investigate the physiological role of the PTP in animal cells. Similarly, CsA has been used in plant PCD models to pharmacologically provide evidence for the existence of the PTP. CsA has been shown to inhibit Ca²⁺-induced swelling of potato mitochondria [64], betulinic acid-induced PCD in tracheary element cells of Zinnia elegans [51], nitric oxide-induced PCD in Citrus sinensis [65], ROS-induced PCD in Arabidopsis cells [66] and caspase-1-like activity and PCD and actin breakdown during leaf perforation formation in the lace plant [67, 68]. In addition, CsA has been shown to protect against loss of ΔΨₘ and cyt c release after protoporphyrin IX treatment of Arabidopsis protoplasts [46], prevent mitochondrial swelling by ROS following methyl jasmonate treatment of Arabidopsis protoplasts [60] and reduce the rate of fusaric acid-induced cell death in tobacco cells [69].

2.2.2.3 Control of MMP During Plant PCD

Hexokinase, the enzyme which catalyses the initial step in intracellular glucose metabolism, may control MMP in plants. Studies have shown that mitochondria-associated hexokinases play an important role in the regulation of PCD in Nicotiana benthamiana [70]. Hexokinase can bind with high affinity to mitochondria at sites in the OMM through its interaction with VDAC [71]. This interaction between hexokinase and the mitochondria is maintained by the serine/threonine kinase Akt and has been shown to play an important role in the control of mammalian apoptosis in the presence or absence of Bax and Bak [72]. Kim et al. [70] used tobacco rattle virus-based virus-induced gene silencing (VIGS) to investigate the function of signalling genes in N. benthamiana, and this screen revealed that VIGS of a mitochondria-associated hexokinase gene Hxk1 caused the formation of necrotic lesions in leaves similar to those formed during the HR. When cells in the affected areas were examined, they displayed hallmark features of PCD—nuclear condensation and DNA fragmentation—and death was associated with loss of ΔΨₘ, cyt c release, activation of caspase-like activities and expression of genes known to be induced during HR. Overexpression of the mitochondria-associated Arabidopsis hexokinases HXK1 and HXK2 can protect against oxidative stress-induced PCD [70]. Similar findings have been reported in potato tubers, where mitochondrial-bound hexokinase is thought to be involved in antioxidant function [73]. Cell death induced by heterologous expression of rice VDAC (OsVDAC4) in the tobacco bright yellow cell-2 line (BY2) and in leaves of N. benthamiana is reduced by co-expression of N. benthamiana hexokinase (NtHxK3), implying that VDAC/hexokinase interactions can modulate PCD in plants [74]. As in mammalian apoptosis, it looks like this association of hexokinase with the mitochondria is important in maintaining mitochondrial integrity during plant PCD. Of considerable interest is the fact that dissociation of hexokinase from animal mitochondria in the presence of apoptotic stimuli but in the absence of Bax and Bak still results in cyt c release (though not as effective) which is not suppressed by Bcl-2 [72]. It is possible that while both animal and plant cells employ hexokinases to control MMP, animals may
have evolved a further level of control involving the Bcl-2 family of proteins. Plants on the other hand may have found this mechanism sufficient for PCD or indeed may have evolved further plant-specific controls that have not been identified yet.

2.3 Reactive Oxygen Species and Antioxidant Control of Plant PCD

Continual production of reactive oxygen species (ROS) in mitochondria and chloroplasts throughout the life cycle of the plant is a by-product of metabolic processes such as respiration and photosynthesis, in peroxisomes during photorespiration and by enzymes such as plasma membrane NADPH oxidases, cell wall peroxidases and apoplastic amine oxidases [66, 75]. ROS present in plant cells include singlet oxygen (\(^{1}O_2\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals formed via the transfer of one, two or three electrons to oxygen, respectively [75]. ROS are highly toxic, with the ability to oxidise and damage cell components such as membrane lipids, proteins, enzymes and nucleic acids, and to balance this in plant cells, antioxidant scavengers of ROS have evolved to alleviate the toxic effects of ROS within the cell [75, 76]. Antioxidants and antioxidant enzymes are capable of quenching ROS without themselves undergoing conversion to destructive radicals. The interaction between ROS and antioxidants provides an interface for metabolic and environmental signals that can modulate induction of the cell’s acclimation to stress or, alternatively, activation of PCD [77]. Therefore, despite their potential toxicity, ROS are important signalling molecules in plant cells [78]. The ROS signalling network during PCD is complex given the presence of different kinds of ROS within the cell, different sites of production and their interaction with other molecules involved in PCD [77, 79]. Levels of ROS, antioxidants and antioxidant enzymes act in balance during cell signalling [80]. Antioxidants are crucial to a plant cell’s defence against oxidative stress, and there is evidence for their involvement in the control of plant PCD [21, 81–83]. Although release of cyt c from the mitochondrial IMS may not directly activate cytoplasmic components of plant PCD [45, 49], its release results in disruption of electron transport leading to generation of toxic levels of ROS. Activation or suppression of PCD in Arabidopsis cells by antioxidants has been shown to be dependent on the type and cellular localisation of ROS [82].

Disruption of mitochondrial electron transport has previously been suggested as the primordial mechanism by which the protomitochondrion killed its host [84]. Employing antimycin A to inhibit mitochondrial electron transport, in tobacco cells, results in induction of genes thought to be involved in senescence and defence responses. Interestingly, these same genes are induced by H\(_2\)O\(_2\) and salicylic acid treatment which cause a rise in ROS and, when inhibited by antioxidant treatment, prevent the gene induction [85]. This work demonstrates that disruption of electron transport can certainly result in mitochondrial signals being transported to the nucleus,
possibly as a result of PTP opening, and results in alteration of gene expression. It may be that a ROS has at least two roles in plant PCD; it acts as a signalling molecule which leads to the opening of the PTP, which would lead to release of and the generation of more ROS, causing a feedback loop which amplifies the original PCD-inducing stress signal [86]. Cyt c release does not only lead to an increase in ROS, but it can interact with \( \text{H}_2\text{O}_2 \) and superoxide radicals to form hydroxyl radicals, which are amongst the most reactive and mutagenic molecules known [87], thereby escalating the damage caused by ROS. A study using rice protoplasts showed that overexpression of mitochondrial heat shock protein 70 inhibited MMP and cyt c release, preventing amplification of ROS and inhibiting PCD [88].

Blackstone and Kirkwood [87] hypothesise that the protomitochondrial electron transport chain, and ROS, may have played an important role in signalling between protomitochondria and host cell. These redox signalling mechanisms may have subsequently been adapted into primordial PCD mechanisms which were driven by ROS production and release of cyt c (causing increased ROS production). These authors further suggest that in animal cells, this release of cyt c could have been refined over time leading to the recruitment of caspases as the terminal effectors of apoptosis. As plants and animals share a common unicellular ancestor, it would not be surprising if plant cells also use ROS production and release of cyt c in cell death pathways.

### 2.4 The Chloroplast and PCD

In nature, plants need to continuously adapt to fluctuating environmental conditions including light. Light is a major factor in the control of growth, development and survival and therefore has a significant effect on a plant’s response to biotic or abiotic stress, for example, progression of the hypersensitive response [89, 90] or the wound response [91].

As with the mitochondria, the chloroplast is also a major source of ROS in plant cells, and excess excitation energy (any light that the chloroplasts receive in excess of the amount required for photosynthesis in photosystem II (PSII) of chloroplasts) results in a decrease in photosynthetic efficiency and inhibition of plant growth [80]. Excess excitation energy increases with higher light intensity and also rises during stress responses, when photosynthesis is less efficient, causing more ROS production. Thus, the chloroplast, like the mitochondrion, is an organelle capable of sensing stress and initiating ROS signalling [92, 93]. It is not surprising, therefore, that the chloroplast plays a role in PCD. Indeed, illumination was found to be required for UV-induced PCD in *Arabidopsis* protoplasts and seedlings [94], cell death induced by fumonisin B1 in *Arabidopsis* protoplasts is light dependent [95], and cyanide-induced PCD in guard cells of pea epidermal cells is enhanced by light [96]. Cell death induced by avirulent pathogen inoculation was found to occur in light-grown, but not dark-grown, *Arabidopsis* leaves [89, 97], and likewise a dark
treatment immediately following avirulent pathogen inoculation suppressed the HR in Arabidopsis leaves [90]. Seo et al. [98] found that the HR was accelerated by the loss of chloroplast function in tobacco.

Some insight into the involvement of chloroplasts in plant PCD emerged from a series of studies using Arabidopsis suspension cultures (ASC) as models for PCD [21]. Light-grown ASC contain functional chloroplasts [21]. However, dark-grown ASC do not, providing an excellent experimental system to study the role of the chloroplast in plant PCD. While total levels of cell death (i.e. PCD + necrosis) are similar in light- and dark-grown ASC after heat stress, dark-grown cells undergo significantly higher levels of PCD than light-grown cells, the latter undergoing more necrosis. Light-grown ASC lacking functional chloroplasts, due to norflurazon treatment, also responded to heat stress with higher levels of PCD compared to untreated light-grown cultures, suggesting chloroplast involvement. \(^{1}\)O\(_2\) is the major ROS produced in photosystem II of chloroplasts, and in another study using light and dark ASC, mild photooxidation damage to cells using rose bengal (RB), a potent artificial \(^{1}\)O\(_2\) sensitizer that accumulates inside chloroplasts, results in \(^{102}\)-mediated PCD only in cells containing functional chloroplasts [22]. Similarly, studies on the leaf-variegated mutant variegated2 show that \(^{1}\)O\(_2\)-mediated PCD is only activated in green leaf sectors of the plant containing fully developed chloroplasts but not in white sectors containing undifferentiated plastids [99]. The data in these papers suggest that \(^{1}\)O\(_2\) can be a potent signal that initiates PCD but only when it is produced in the chloroplast. This is in agreement with the findings of Doyle and McCabe [82] that showed that antioxidants could suppress PCD but did not always do so, suggesting again that ROS per se does not induce PCD but rather the contexts—the location and type of ROS—are the important determinants.

Are chloroplasts a source of pro- or anti-PCD proteins? Another study linking the chloroplast and PCD came from the finding that palmitoleic acid induces cell death in eggplant cells and protoplasts, in a process involving cytochrome \(f\) (cyt \(f\)) [100]. Cyt \(f\) is a subunit of the cyt \(b_{6}f\) complex, an essential component of the major redox complex of the thylakoid membrane catalysing the transfer of electrons from photosystem II to photosystem I. Cyt \(f\) has also been shown to be released from the chloroplast into the cytosol following heat shock treatment of the unicellular green alga Chlorella saccharophila, accompanied by thylakoid membrane structure alterations, culminating in PCD [101]. Release of cyt \(f\) from chloroplasts has also been shown in senescent rice leaves prior to PCD [102], and these authors also demonstrate that cyt \(f\) can activate caspase-3-like activity in a cell-free system. Release of trace amounts of cyt \(f\) from the thylakoid membrane has also been observed in the flu mutant; however, in this case cyt \(f\) release was only observed after onset of cell death leading the authors to conclude that cyt \(f\) is not a trigger for \(^{1}\)O\(_2\)-mediated PCD but rather a marker for cellular collapse [99]. However, as in the case of cyt \(c\) release from the mitochondria, disruption of the cyt \(b_{6}f\) complex and release of cyt \(f\) will have a major impact on ROS production which will subsequently govern the type of death a cell undergoes. So, despite these interesting findings, it is not clear whether this loss of function of cyt \(f\) relates it to plant PCD or a more direct regulatory role exists as in the case of cyt \(c\) in apoptosis [101, 102].
While our understanding of the role of the chloroplast in PCD is increasing, it is far from complete. What is certain though is that the presence of chloroplasts, while not necessary for plant PCD, plays a significant role in the regulation of PCD and increases the complexity of ROS-mediated PCD pathways in cells containing functional chloroplasts (see Fig. 2.2 for schematic representation of mitochondrial and chloroplast involvement in PCD).
2.5 Mitochondrial and Chloroplast Crosstalk During PCD

2.5.1 Proximity of Organelles During PCD

Mitochondria and chloroplasts have an intimate relationship within the plant cell, and the environment of the mitochondria is dynamically influenced through metabolic interactions and redox exchange with the chloroplasts. Under normal conditions, plant mitochondria are localised around chloroplasts in an even distribution [46, 60, 103], and this is thought to be due to oxygen and carbon dioxide gradients which establish metabolite interchange between the two organelles [103]. During PCD, a different picture emerges and has been observed by several different laboratories employing different inducers of PCD in vitro: mitochondria begin to aggregate and later in the process show a more clumped/clustered morphology surrounding chloroplasts or aggregated within other areas of the cytoplasm [60, 104, 105]. Chloroplasts also show changes in their morphology and position from close to the plasma membrane to being evenly distributed throughout the cytoplasm [104]. In the case of cadmium-induced PCD, which increases ROS in *Arabidopsis* protoplasts, pretreatment with ascorbic acid or catalase prevents subsequent organelle changes and death [105]. Mitochondrial aggregation is also observed in developmentally regulated PCD in the lace plant [67]. Early stages of PCD in the lace plant, before loss of $\Delta \Psi_m$, are associated with an increase in observable transvacuolar strands, and mitochondria and chloroplasts can be seen moving along these transvacuolar strands in a seemingly orderly fashion, and later, chloroplasts could also be seen forming a ringlike structure around the nucleus [106] (see Fig. 2.3, kindly provided by A. Gunawardena; for more information and figures on this topic, see Chap. 1). It will be interesting to learn what the significance of these organelle distribution patterns is, but the close proximity of the chloroplast and mitochondria and their changes in morphology during PCD strongly support the involvement of the chloroplast in mitochondrial control of PCD.

![Fig. 2.3](Image)

Chloroplast distribution during PCD of the lace plant *A. madagascariensis*. (a) Regular chloroplast distribution in the cytoplasm during early PCD. (b) Perinuclear distribution of chloroplasts during late PCD (images are taken using differential interference contrast (DIC) optics and kindly provided by A. Gunawardena, Dalhousie University, Canada)
2.5.2 Dynamic Location of Mitochondrial and Chloroplast Proteins Involved in PCD During Oxidative Stress

2.5.2.1 Accelerated Cell Death 2 Protein

Some evidence for crosstalk between mitochondria and chloroplasts during PCD comes from a series of studies involving the chloroplast-localised protein, accelerated cell death 2 (ACD2). Overexpression of Arabidopsis ACD2, which encodes red chlorophyll catabolite reductase, can protect against disease symptoms and PCD in leaves caused by infection with virulent Pseudomonas syringae [107]. The acd2 mutant undergoes excessive PCD during infection and displays spontaneous spreading cell death [108]. ACD2 localises to chloroplasts in mature leaves but in young seedlings localises to both chloroplasts (or plastids) and mitochondria [107]. During infection of leaves with P. syringae or protoporphyrin IX treatment, ACD2 also localises to both chloroplasts and mitochondria in leaves [46] and is thought to protect cells from pro-death mobile substrate molecules, some of which may originate in the chloroplast but have major effects on mitochondria by causing increases in ROS levels and ultimately cell death [109].

2.5.2.2 Antioxidative Enzymes

As we have discussed, the chloroplast, like the mitochondrion, is a major regulator of cellular redox homeostasis within the cell. While import of proteins into the mitochondria and chloroplasts is generally considered to be organelle specific, several key antioxidative enzymes are dual targeted to chloroplasts and mitochondria [110]. Dual targeting involves localisation of a single protein in more than one cellular compartment, and that protein is encoded by a single gene in the nucleus and translated in the cytosol as a single translation product but targeted to both mitochondria and chloroplasts [111–113]. It is now thought that dual targeting is not just an evolutionary solution to increase the number of cellular functions without increasing the number of genes, as in some cases both dual-targeted and specific protein isoforms exist in the same organelle, suggesting a regulatory mechanism for some processes [113]. Dual targeting could have important control implications for plant PCD in the context of mitochondria/chloroplast crosstalk. Of the approximately 100 proteins found to be dual targeted to date, most are involved in nucleotide metabolism [111, 114], but three of the four enzymes involved in the ascorbate-glutathione cycle, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and glutathione reductase (GR), are dual targeted. The fourth enzyme involved in the cycle, glutathione-dependent dehydroascorbate reductase (DHAR), is not [110]. Interestingly, MDAR is a homolog of AIF [115]. AIF is a flavoprotein with NADH oxidase activity that (like cyt c) is normally found in the mitochondrial IMS but can translocate to the nucleus upon induction of apoptosis where it can bring about chromatin condensation and cleavage of DNA into large (~50 kb) fragments in a caspase-independent manner [116]. Although AIF has both apoptotic and anti-apoptotic activities [117, 118], indications in plant cells are that MDAR, being
involved in the ascorbate-glutathione cycle and crucial for ascorbate regeneration, has a protective role against oxidative stress in plants. In examining amino acid sequences of *Arabidopsis* MDAR and human AIF, Yamada et al. [119] found close homology between the oxidoreductase domains in both proteins, but not between the C terminal AIFM1 domains (DNA binding) which have the apoptotic function, leading these authors to conclude that higher plant MDARs do not have a similar pro-apoptotic function in PCD. In animal cells, DNA binding-defective AIF mutants remain capable of translocating to the nucleus but cannot induce cell death [120]. MDAR1 and DHAR overexpression in transgenic tobacco confers enhanced resistance to ozone, salt and polyethylene glycol stress, all known to increase levels of ROS ([121, 122], respectively). In a study looking at ascorbate metabolism in broccoli florets during postharvest senescence, where PCD occurs, gene expression of both the chloroplastic genes MDAR 1 and DHAR was found to be downregulated [123]. However, Locato et al. [81] found that during heat shock-induced PCD in tobacco BY-2 cells, mitochondrial MDAR activity decreases and becomes undetectable in cells undergoing PCD, while mitochondrial DHAR activity does not diminish, and the authors discuss the possibility of DHAR activity increase being involved in a regulatory feedback mechanism improving ascorbate regeneration from dehydroascorbate when ascorbate is depleted at the site of production.

### 2.5.2.3 Anti-apoptotic Genes

Earlier in our discussion on release of mitochondrial proteins during PCD, we discussed the Bcl-2 family of proteins and the fact that although there are no plant homologs, these animal proteins have been shown to operate in plants [58]. Chen and Dickman [124] developed transgenic tobacco plants containing anti-apoptotic genes, animal Bcl-2 and Bcl-xL and *Caenorhabditis elegans* CED 9, and subcellular fractionation revealed that the anti-apoptotic proteins associated not only with mitochondria and nuclear fractions, but also with chloroplast membranes. This finding was not surprising as all three proteins have transmembrane binding domains. However, what is of significance in the context of PCD is that light is required for herbicidal (whose primary site of action is the chloroplast) treatment of tobacco plants, resulting in production of lethal levels of ROS and subsequent death with apoptotic-like features, while transgenic plants expressing the anti-apoptotic genes survive.

### 2.6 An Evolutionary Perspective

It is clear that there are many shared features amongst plant PCD and other eukaryotic cell death programmes. However, the extent to which these shared features arise from an ancient unicellular death programme is uncertain and remains controversial. Have redox signalling mechanisms between the mitochondrial symbiont and host cell
become subsequently adapted into primordial PCD mechanisms [84, 87]? Although we don’t have the answer to this question, one point is clear and that is, as in animal apoptosis, the mitochondria appear to be key regulators of the cell death process. In the context of plant PCD, it is clear from the evidence discussed in this chapter that the chloroplast, the other endosymbiont-derived organelle, plays a role in PCD.

The theory that mitochondria and chloroplasts derive from bacteria taken into other cells as endosymbionts is particularly relevant to recent findings by Yang et al. [125] where plant orthologs to putative bacterial PCD proteins were found using a proteomic analysis of chloroplast envelope proteins. This plant Cid/Lrg ortholog AtLrgB may play a role in plant PCD in that a T-DNA insertion mutation of AtLrgB resulted in plants with interveinal chlorotic and premature necrotic leaves [125]. Interestingly, these Cid/Lrg bacterial proteins are thought to be evolutionarily linked with the Bcl-2 family of proteins [126]. The relevance of PCD in bacteria is a fairly recent consideration and connected to their ability to form biofilms [126]. The Staphylococcus aureus cid and lrg operons that control cell death and lysis are important in biofilm development [126], and these proteins have many similarities to the bacteriophage holin/antiholin family which regulate membrane permeability in lysis. The Bcl-2 proteins are able to functionally replace holins to promote lysis [127]. Thus, the finding of plant Cid/Lrg plant orthologs led the authors to hypothesise about the potential conservation of this membrane permeability control mechanism in animal, plants and bacteria and a similar evolutionary relationship between bacteria and chloroplasts [126], similar to that of bacteria and mitochondria and the control of PCD [84, 87].

2.7 Conclusions

The studies discussed in this review highlight the complexity involved when these two major plant organelles, the mitochondria and chloroplast, sense and respond to stress, which in turn determines whether the cell lives or dies via PCD or necrosis. Cell monitoring of fitness by major cellular organelles is probably a significant player in determining cell fate. When relating PCD research findings to the whole plant, one cannot consider results pertaining to mitochondria alone, as it is clear that when chloroplasts are present they are capable of mediating PCD under oxidative stress conditions.

References


82. Doyle SM, McCabe PF (2010) Type and cellular localisation of reactive oxygen species determine activation or suppression of programmed cell death in Arabidopsis suspension cultures. Plant Signal Behav 4:467–468
Mitochondrion and Chloroplast Regulation of Plant Programmed Cell Death


Plant Programmed Cell Death
Gunawardena, A.N.; McCabe, P. (Eds.)
2015, XI, 306 p. 60 illus., 47 illus. in color., Hardcover
ISBN: 978-3-319-21032-2