Review

Plant programmed cell death: can't live with it; can't live without it

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The decision of whether a cell should live or die is fundamental for the wellbeing of all organisms. Despite intense investigation into cell growth and proliferation, only recently has the essential and equally important idea that cells control/programme their own demise for proper maintenance of cellular homeostasis gained recognition. Furthermore, even though research into programmed cell death (PCD) has been an extremely active area of research there are significant gaps in our understanding of the process in plants. In this review, we discuss PCD during plant development and pathogenesis, and compare/contrast this with mammalian apoptosis.

INTRODUCTION

Nearly all cells in all multicellular organisms possess an intrinsic program for cell suicide (Kimchi, 2007). This altruistic programme instructs the cell to eliminate itself for the 'greater good' or overall survival of the organism. Current theories suggest that cell suicide responses arose during evolution as a response to viruses, effectively providing a mechanism for limiting viral replication and spread by triggering cell suicide prior to virion genome replication (Iriti and Faoro, 2007). Although this theory is debatable, examples of cell suicide can even be found in unicellular bacteria and yeast (Ameisen *et al.*, 1995). Presumably, this was selected for by viruses or other evolutionary pressures where it proved advantageous to sacrifice a minority of the 'mother cell's' progeny for propagation of the genome in future 'daughter cell' generations (Ameisen, 2002).

Over evolutionary time, the cell suicide programme became exploited for processes associated with fetal development, such as creation of body cavities, removal of redundant cells, and sculpting of body appendages and other structures. Many of these developmental cell deaths are genetically 'programmed',

often requiring the induction of specific genes to activate the cell death machinery, thus termed 'programmed cell death' (PCD). Additionally, dysregulation of this natural cell death pathway significantly contributes to diseases characterized by either excessive cell accumulation (e.g. cancer, restenosis, autoimmunity), or inappropriate cell death (e.g. stroke, myocardial infarction, inflammation, AIDS, Alzheimer's disease, necrotrophic fungal disease) (Ameisen, 1994; Edwards, 1998; Kondo, 1988; Leijon *et al.*, 1994; Takaoka *et al.*, 1997; Vachon *et al.*, 1997; Wellington *et al.*, 1998). Importantly, many viruses and intracellular bacteria control the cell death pathway in the host cells they infect, thus linking PCD to infectious diseases (Limonta *et al.*, 2007; Roulston *et al.*, 1999).

As in animals, a programmed type of cell death occurs in plants to aid in normal growth and development, including reproduction, seed germination, aerenchyma formation, tracheary element differentiation, sieve element differentiation and senescence (Azeez *et al.*, 2007; Bassham *et al.*, 2006; Groover *et al.*, 1997; Turner *et al.*, 2007; Visser and Bogemann, 2006; Yu *et al.*, 2002). Regulation of cell death pathways also occurs in response to abiotic stimuli such as drought or temperature stress (Harrak *et al.*, 2001; Koukalova *et al.*, 1997). Finally, cell suicide programmes are activated, at least in some cases, during pathogen challenge, intriguingly, in both resistant and susceptible interactions (Abramovitch *et al.*, 2003; Desaki *et al.*, 2006; Kim *et al.*, in press; Mittler and Lam, 1996; Veneault-Fourrey *et al.*, 2006).

CONSERVATION ACROSS VAST EVOLUTIONARY DISTANCES

The genes that control PCD are conserved across wide evolutionary distances, defining a core set of biochemical reactions that are regulated by inputs from diverse upstream pathways (Boyce *et al.*, 2004; Chae *et al.*, 2003). These genes encode either anti-apoptotic or pro-apoptotic proteins, which direct the cell to make life-death decisions. Ectopic over expression of certain types of anti-apoptotic genes can render animal cells markedly resistant to a wide range of cell death stimuli, including nutrient deprivation, irradiation, cytotoxic chemicals and hypoxia (Gazitt *et al.*, 1998; Li *et al.*, 2001; Marcelli *et al.*, 2000). Functional conservation of

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these mammalian PCD pathways has been witnessed in the plant kingdom and demonstrated in other eukaryotes (e.g. *Caenorhabditis elegans*, *Drosophila*). For example, *CED-9* mutants of *C. elegans* are partially complemented by human *Bcl-2* even though the two genes have limited sequence similarity. The transgenic over-expression of animal anti-apoptosis genes such as *CED-9* and *Bcl-2* in plants also leads to enhanced tolerance to many abiotic stresses, including, salt, drought, cold and heat, and, importantly, disease resistance (Chen and Dickman, 2004; Dickman *et al.*, 2001; Li and Dickman, 2004; Lincoln *et al.*, 2002; Xu *et al.*, 2004). Recently, structural and functional homologues of the mammalian BAG (*Bcl-2* athanogene) family were identified in Arabidopsis via bioinformatics to illustrate further the conservation of key PCD regulators (Doukhanina *et al.*, 2006). While displaying similarities [DNA cleavage (laddering), DNA fragmentation and the activation of caspase-like proteases] plant cells also exhibit distinctive features of PCD. The presence of chloroplasts, a prominent vacuole and the cell wall are all unique aspects of plant cells and effect PCD responses accordingly (Hatsugai *et al.*, 2006; Samuilov *et al.*, 2003). Current theories speculate that chloroplasts, through the regulation of reactive oxygen species (ROS), may serve as a global messaging system in many plant PCD responses (Samuilov *et al.*, 2002; Zapata *et al.*, 2005). One clear difference between mammals and plants is the involvement of macrophages and phagocytosis by mammals to remove apoptotic cells cleanly in a non-inflammatory manner. A combination of the plant vacuole and autophagy may represent a 'plant' alternative to this mobile phagocytosis system (Hatsugai *et al.*, 2006). The observation of characteristic apoptotic-like hallmarks in certain necrotrophic pathogen infections suggests that, at least in these cases, PCD is induced by the pathogen and not the plant (Coffeen and Wolpert, 2004; Tada *et al.*, 2005). Therefore, PCD can be beneficial or harmful to the plant in a context-dependent manner, representing a culmination of factors including developmental, environmental stimuli and the source of the signal. In this review, we summarize the current knowledge of plant-based PCD processes. As the underlying mechanisms driving PCD in plants are somewhat obscure, plant-PCD pathways will initially be discussed in relation to the well-established mammalian systems.

MAMMALIAN PCD

Currently, several forms of cell death are recognized in mammals, including necrosis, apoptosis and autophagy (Assuncao Guimaraes and Linden, 2004). Despite this categorization, the lines distinguishing each form are not distinct, with considerable overlap often observed. Therefore, cell death may be viewed as a continuum, ranging from the 'accidental' cell death (necrosis) at one end to the genetically controlled, highly orchestrated apoptosis at the other, with autophagy (sequestration and delivery of unwanted

cytoplasm and organelles to lysosomes or the vacuole for degradation) in between but on the genetically programmed side of the 'line'. In the next few sections, we described the various forms of mammalian cell death starting with apoptosis.

Apoptosis—'leaves falling from a tree-senescence'

Apoptosis (*apo*—from, *ptosis*—falling), the morphological equivalent of PCD, refers to a constellation of characteristic morphological changes that cells generally undergo when dying by activation of the endogenous cell suicide programme (Kerr *et al.*, 1972). Typically, animal apoptosis is associated with hallmark characteristics such as chromatin condensation, cell shrinkage, plasma membrane blebbing, inter-nucleosomal DNA cleavage, externalization of the inner membrane lipid, phosphatidylserine ('eat me' signal), the formation/packaging of apoptotic bodies, and finally engulfment and removal of these cells by ordered phagocytosis (Kerr *et al.*, 1972). Most of these morphological and ultrastructural changes can be traced to the actions of the caspases, a family of intracellular cysteine proteases that become activated during the cell suicide response (Li *et al.*, 1997; Villa *et al.*, 1997). The genes that constitute the core components of the apoptosis machinery of animal cells are known and relatively well characterized (Fig. 1).

Caenorhabditis elegans; a classic model for the study of mammalian apoptosis systems

The paradigm for the cell death pathway of animal cells was derived largely from pioneering studies of Horvitz and colleagues with the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Ellis *et al.*, 1991). In this organism, exactly 131 of the 1090 cells that form the mature adult undergo PCD during development (Ellis *et al.*, 1991). Using the translucent digestive tract and differential interference contrast microscopy (DIC) as a guide, genetic mutants with defects in the cell death machinery or so-called 'cell death defective' (CED) mutants were generated, identified and tracked at the single cell level. The results of these studies established a genetic basis for cell suicide (Fig. 1) (Ellis *et al.*, 1991).

In the worm, all developmental cell deaths are dependent upon a caspase-family cell death protease, CED-3 (Yuan *et al.*, 1993). The activation of CED-3 requires CED-4, an ATPase that directly binds the CED-3 zymogen and triggers proteolytic processing of pro-CED-3, thereby generating autonomously active CED-3 protease. To prevent untimely cell death, the actions of CED-4 are suppressed by CED-9, an anti-apoptotic protein that binds CED-4 and prevents activation of the pro-CED-3 zymogen (Chen *et al.*, 2000). In some cells of the worm, CED-3 activity is further regulated by EGL-1, a negative repressor of CED-9, to allow CED-4 interaction/activation of CED-3 and ultimately PCD (Conradt and Horvitz, 1998).

Fig. 1 Schematic representation of the core biochemical pathways leading to apoptosis in (a) *Caenorhabditis elegans*, (b) mammals and (c) plants.

Although much simpler, PCD in *C. elegans* has clear parallels in humans and other animal species, with homologues of CED-3, CED-4, CED-9 and EGL-1 all present/identified in humans and mammals (Fig. 1b) (Earnshaw *et al.*, 1999). The greater diversity of tissues and biological processes in mammals, however, is accompanied by increased numbers and complexity in the homologues of the cell death pathway genes, i.e. *C. elegans* has one unique caspase family member (CED-3), while mammals have at least 14 (Earnshaw *et al.*, 1999; Rupinder *et al.*, 2007).

Caspases

CED-3 is a member of the cysteine protease family, which share homology with mammalian interleukin-1 β -converting enzyme (ICE) (Yuan *et al.*, 1993). These proteases were subsequently designated as caspases, a group of specific cysteine proteases requiring the recognition of an aspartate residue in the P1 position of their substrate (Alnemri *et al.*, 1996). Individual family members are defined by several categories, including substrate specificity, sequence/structural similarity and relative 'position' in the cell death pathway. Mutations in caspase-encoded genes from *C. elegans* to humans have established their importance in apoptosis as key executioners of the cell (Alnemri *et al.*, 1996).

Initially, caspases are synthesized as inactive pro-caspase zymogens and are regulated post-translationally, allowing rapid response to stimuli (Alnemri *et al.*, 1996). Generally, this activation requires the proteolytic removal of an N-terminal pro-domain

and assembly of two caspase subunits into the active heterotetramer protease. A plethora of substrates are targeted for caspase activity, including DNA repair enzymes, lamins, cytoskeletal proteins or other caspases. However, it remains unclear whether many of these targets are causal and required for execution or a mere by-product of the death process. The upstream caspases trigger cascades that amplify the 'death' signal to a threshold level required for an irreversible 'suicide' response once the life/death decision is made (Earnshaw *et al.*, 1999; Villa *et al.*, 1997).

Regulation of caspases and PCD—a series of checks and balances

Naturally, caspase activation must be carefully controlled to prevent gratuitous cell death while still allowing necessary PCD to occur. As such, there are a number of positive and negative regulators of caspase activation that 'battle' prior to the irreversible decision phase. Primary combatants of PCD are the *Bcl-2* family (see below) and the IAPs (inhibition of apoptosis protein).

Inhibition of apoptosis proteins

Originally identified in baculoviruses where they suppressed host-directed cell death during virus infection, a number of IAP family members have now been identified in a diverse range of organisms, including yeast, nematodes, *Drosophila* and humans (Yang and Li, 2000). Categorization of IAP family members is based on the presence of a ~70-amino-acid domain designated the baculoviral IAP repeat (BIR) (Crook *et al.*, 1993). Several IAPs also contain a RING (Really Interesting New Gene) domain that

confers E3 ubiquitin ligase activity and possibly a role in apoptosis regulation via the proteasome (Vaux and Silke, 2005). Effectively, RING domains direct the ubiquitination of target proteins for degradation by the proteasome (Morizane *et al.*, 2005; Ni *et al.*, 2005).

An inhibitor of an inhibitor

IAP activity is regulated by 'IAP-antagonists', another protein family whose members are characterized by the presence of a tetra-peptide motif that directly regulates IAP activity (Salvesen and Duckett, 2002; Yoo *et al.*, 2002). In contrast to *Drosophila*, in which many IAP-antagonists have been isolated (Reaper, Grim, HID, Sickie and Frac2), only two [second mitochondrial-derived activator of caspases (SMAC)/direct inhibitor of apoptosis protein binding-protein with low pI (Diablo) and Omi (HtrA2)] are known in humans (Holley *et al.*, 2002; Yoo *et al.*, 2002). These proteins (among others, including cytochrome C) are released during mitochondrial permeabilization (which occurs upon triggering of PCD) to inhibit IAPs, thus allowing PCD to proceed.

Bcl-2 family

Members of the *Bcl-2* gene family provide a critical role within mitochondrial-driven PCD pathways (see below) where they physically interact/dimerize to serve either pro- (*Bax*, *Bak*) or anti-apoptotic (*Bcl-2*, *Bcl-X_L*) functions (Reed, 1997; Reed *et al.*, 1998). The family is characterized by the presence of one or more BH (*Bcl-2* homology) regions designated 'BH1–4'. These BH regions are the only areas of sequence conservation between family members and strongly influence whether the family member is pro- or anti-apoptotic (Kelekar and Thompson, 1998). For example, *Bcl-2* proteins containing only BH-3 domains are generally pro-apoptotic, in contrast, multi-domain *Bcl-2* family proteins can be either pro- or anti-apoptotic (Kelekar and Thompson, 1998). One major model for PCD regulation posits that cell life and death comes down to the ratio of free *Bcl-2* family members vs. bound heterodimers, i.e. whichever class is in abundance dictates cell fate in the life/death decision (Borner, 2003). Several *Bcl-2* family proteins that target mitochondria (e.g. *Bax*) bear a striking resemblance to certain pore-forming proteins of bacteria, including diphtheria toxin and the colicins, suggesting a possible mechanism of action and may indicate an ancient pathway for cell death regulation (Minn *et al.*, 1997; Schlesinger *et al.*, 1997). Initially, colicins are synthesized and secreted in an inactive conformation that recognizes and binds to receptors on competing bacteria. Following binding, a voltage-dependent conformational change activates the colicin which then inserts into membranes to form channels, depolarize membranes, and kill the targeted bacteria. In an analogous manner, *Bax* normally resides in the cytosol in a latent state, but translocates to mitochondrial membranes upon perception of an apoptotic stimulus (or upon over-expression), and undergoes conformational changes associated

with its insertion into mitochondrial membranes (Antonsson *et al.*, 1997; Nouraini *et al.*, 2000). Considering that mitochondria are thought to have originated from bacteria that took up residence in host eukaryotic cells, it is intriguing to speculate that an ancient bacterial system of pore-forming proteins was adapted for control of cell suicide in higher eukaryotes. The anti-apoptotic members inhibit oligomerization of pro-apoptotic *Bcl-2* proteins in the mitochondrial membrane to prevent pore formation (Antonsson *et al.*, 1997).

Mammalian caspase activation pathways

In mammals, two major signalling pathways leading to caspase activation have been defined and are commonly known as the 'extrinsic' and 'intrinsic' pathways in reference to the origin of the cascade signal (reviewed in Rupinder *et al.*, 2007). Even though each pathway is capable of functioning independently, cross-talk between pathways is common. Importantly, both pathways converge, leading to the activation of the executioner protease, Caspase-3.

Extrinsic activation pathway

The extrinsic pathway involves the activation of a family of cell surface death receptors typified by the tumour necrosis factor receptor family (TNFRs). Upon external recognition by TNFR ligands, receptor activation occurs in the cytoplasm where TNFRs aggregate and form a complex comprising pro-Caspase-8 through the action of adapter proteins which interact with the Caspase-8 N-terminus. The formation of TNFR, Caspase-8 and adapter protein complexes is mediated via homo-typic domains known as death domains (DDs) or death effector domains (DEDs) (Ashkenazi and Dixit, 1998). These domains also represent a source of function for regulation of the pathway with many mammalian proteins such as FLIP, BAR and Bap31 competing for access to the DED-containing caspases. Additionally, several animal viruses encode DED-containing suppressors of the Fas/ TNF-family signalling proteins and contain *Bcl-2* homologues to allow maximum replication levels (Roulston *et al.*, 1999).

Intrinsic activation pathway

The intrinsic or mitochondrion (mt) pathway involves the activation of apoptotic proteins by signals including oxidative stress [nitrogen oxide, NO or hydrogen peroxide (H₂O₂)], DNA damage, voltage changes (see above) or growth factor withdrawal (starvation), resulting in the dissipation of mitochondrial membrane potential and increased permeability; this has a range of effects (reviewed in Rupinder *et al.*, 2007).

Normally, the mitochondrial inner membrane is impermeable to most metabolites. While the exact mechanism by which the mitochondria releases apoptotic regulators is not entirely defined, current theories indicate that during times of stress a non-selective pore, the mitochondrial permeability transition

pore (MPTP), develops to allow the free translocation of molecules that normally reside in the mt inter-membrane space across the inner mt membrane (Vieira *et al.*, 2000). This passage of solutes but not proteins through the MPTP causes an increased osmotic pressure, an inflow of solutes and ultimately the swelling of the mt. The inner mt membrane remains intact by unfolding its cristae. In contrast, the outer membrane, which does not contain cristae, cannot tolerate such dramatic permeability changes and bursts to release an array of proteins including caspases, cytochrome C, apoptosis inducing factor (AIF), endonuclease G (a mitochondrion nuclease) and SMAC/Diablo into the cytoplasm, resulting in both caspase-dependent and caspase-independent PCD (reviewed in Hengartner, 2000).

The release of SMACs into the cytoplasm results in the inactivation of IAPs, leading to the activation of caspase-3 and cell death. Additionally, enhanced permeability of the outer mitochondrial membrane releases cytochrome C into the cytosol where it binds with Apaf-1 (human homologue of *C. elegans* CED-4) and dATP, to form an oligo-protein complex termed the 'apoptosome'. Once formed this complex recruits and cleaves pro-caspases, which activate caspase-3, leading to cell death (Adams and Cory, 2002). In effect, the apoptosome serves as an adapter for the recruitment and activation of the necessary machinery required for the cell death process (cytochrome C, pro-caspases).

Cytochrome c—a multi-faceted signalling molecule

Traditionally, cytochrome C has been associated with electron transport; however, cells are resourceful in developing multi-functional uses for the limited repertoire of available molecules at their disposal. In mammals, cytochrome C has the additional and important function of being involved with PCD induction and is a positive regulator of cell death via the intrinsic pathway. The change in mt membrane permeability during apoptosis results in the free passage of protons across the inner membrane; this uncouples oxidative phosphorylation and results in hydrolysis rather than synthesis of ATP and production of ROS. The presence of lowered ATP levels in the cell in turn activates degradative enzymes such as proteases, lipases and nucleases, leading to irreversible cell damage.

Intriguingly, a number of APAF proteins harbour NBS-LRR regions and bear striking similarity to plant resistance ('R') genes (van der Biezen and Jones, 1998). For example, the human APAF, CARD-4, has more similarity to the N gene for resistance in tobacco and RPS2 in Arabidopsis than it does with its sibling family members (Bertin *et al.*, 1999). Recent findings suggest that plant R genes may function in a similar manner to APAF, recruiting the necessary players and inducing a programmed cell death [the hypersensitive response (HR)]. Programmed cell death may also occur independently of caspases; during caspase-independent PCD, AIF and endonuclease G, are released from the mt and translocate to the nucleus to trigger DNA fragmentation

and caspase-independent cell death (Wang *et al.*, 2002). The intrinsic pathway is not always focused on the mitochondria. Recent studies have identified the endoplasmic reticulum (ER) as a relevant player in mammalian PCD. In humans, Capase-12 is found in the ER (Nakagawa *et al.*, 2000); additionally, BI-1 (Bax inhibitor-1) homologues have been identified in plants, including Arabidopsis, tomato and rice. Preliminary studies suggest that plant BI-1 is involved in stress tolerance and structurally may localize in the ER (Chae *et al.*, 2003; Watanabe and Lam, 2006, *In press*).

Autophagy

Autophagy is a highly conserved intracellular degradation system that utilizes the lysosomal pathway of PCD. As suggested by its Greek derivatives, 'auto—self' and 'phagy—eating', autophagy is a catabolic process for the bulk degradation of cytoplasmic cell components for homeostasis and response to a wide variety of environmental and physiological conditions (Levine and Klionsky, 2004). The process is ubiquitous, present in all eukaryotic cell types, and typically involves specific autophagy gene (*atg*) products, the formation of a double-membrane vesicle called the autophagosome, and the subsequent fusion of an autophagosome with a vacuole or lysosome for the destruction of cytoplasmic material (Klionsky, 2005). Autophagosomes are formed by the engulfment of small portions of the cytoplasm by a double-membrane vacuole and mature upon receiving hydrolases from either lysosomes or elements of the Golgi complex. Therefore, once mature, the autophagosome represents a hydrolase-enriched cellular compartment for the cleavage and degradation of proteins, lipids, carbohydrates or even organelles (macroautophagy) (Shintani and Klionsky, 2004).

Numerous processes have been attributed to autophagy, including both pro-life measures (resistance to nutrient starvation) and pro-death (cellular differentiation, ageing); thus the precise role of autophagy (cyto-protective versus cyto-destructive) may vary. Autophagy can protect cells against death, but paradoxically can also mediate cellular demise. For example, autophagy is cytoprotective during nutrient starvation by promoting survival via the generation of nutritional building blocks and maintaining energy homeostasis. Under different physiological conditions, however, autophagy promotes cell death in a process distinct from apoptosis. Careful note should be taken as the distinction between apoptosis and autophagy can overlap (reviewed in Shintani and Klionsky, 2004). Autophagy and apoptosis may be triggered by the same upstream signals to result in combined autophagy and apoptosis responses. These observations show that apoptotic and autophagic responses can share common pathways that either link or coordinate distinct cellular responses. Furthermore, autophagy can indirectly trigger cell death by induction of apoptosis (Scott *et al.*, 2007).

Necrosis

In contrast to apoptosis and autophagy, necrotic or non-lysosomal cell death is 'accidental' and traumatic, typically occurring after exposure to excessive physiological conditions greater than the innate tolerance levels of the cell. Key features of necrosis include loss of membrane integrity and ionic homeostasis, random degradation of DNA, de-compartmentalization of subcellular organelles, swelling and eventual burst of the organelle/cell (reviewed in Proskuryakov *et al.*, 2002). A distinct difference between apoptosis and necrosis, at least in mammals, is the association of an inflammatory response and release of toxic cell remnants during necrosis.

PCD IN PLANTS

PCD is known to play a critical role within many plant responses to a range of stimuli, including those of developmental, abiotic and biotic nature (reviewed in Lam *et al.*, 2001). A number of studies involving cell death in plants have observed striking similarities to the hallmark apoptotic features observed in animals, including DNA cleavage (ladders), DNA fragmentation (TUNEL positively reacting cells) (Ryerson and Heath, 1996), the activation of caspase-like proteases (Navarre and Wolpert, 1999) and formation of structures resembling apoptotic bodies (Li and Dickman, 2004; Wang *et al.*, 1996). By contrast, plant cells display several unique features as compared with their animal counterparts, including a lack of 'true' caspases, the presence of a rigid cell wall and more importantly the lack of an active phagocytosis system. Other unique feature of plant cells include totipotency, chloroplasts, non-motility and numerous and sometimes large vacuoles harbouring high levels of degradative enzymes. There is already ample evidence that several plant processes involve a form of PCD (e.g. senescence, xylem formation, HR), but whether these genetically programmed events are apoptotic-like is not entirely clear (Lim *et al.*, 2007).

Caspase-like proteases in plants

Despite the failure to identify bona fide caspases in plants (a fact all the more apparent with the completion of several plant genomes), caspase-like protease activities have been detected in plants upon activation of PCD (reviewed in Sanmartin *et al.*, 2005; Woltering *et al.*, 2002). Additionally, ectopic expression of mammalian pro-apoptotic proteins such as Bax in plants triggers cell death in a manner similar to the HR (Kawai-Yamada *et al.*, 2001). Several caspase-like proteases (metacaspases) have been identified in plants using bioinformatics and functional screen-based approaches, but searches for true caspase genes have proven elusive (Uren *et al.*, 2000).

Using informatic approaches, a family of genes exhibiting distant homology to mammalian-caspases have been identified

and designated 'metacaspases' in plants, fungi and protozoa (Uren *et al.*, 2000). Two classes of metacaspases have been categorized according to the presence (type I) or absence (type II) of an N-terminal pro-domain comprising a zinc-finger followed by either a proline-rich region or a glutamine and proline-rich region (Belenghi *et al.*, 2007; Vercammen *et al.*, 2004). Currently, nine (three type I and six type II) metacaspases have been predicted to reside in the Arabidopsis genome, all of which are reported to be up-regulated during bacterial infection. Consistent with their mammalian counterparts, ectopic expression of genes from both classes of the Arabidopsis metacaspases (AtMCP1b and AtMCP2b) successfully induced apoptosis-like PCD in yeast (Watanabe and Lam, 2005). Thus, metacaspases may function in the activation of other caspase-like proteases during PCD in plants.

The host selective toxin victorin, secreted from the pathogen *Cochliobolus victoriae*, induces PCD in oat plants containing the *Vb* allele which has recently been identified in Arabidopsis (Lorang *et al.*, 2007). Importantly, victorin-induced plant cell death exhibits several features similar to those observed during mammalian apoptosis, including DNA laddering, chromatin condensation and mitochondrial dysfunction via a mitochondrial permeability transition, thus implicating a role for PCD in plant susceptibility (Curtis and Wolpert, 2002; Navarre and Wolpert, 1999). Upon further investigation, two proteases displaying caspase-like activity were identified and characterized using synthetic peptides and peptide inhibitors (Coffeen and Wolpert, 2004). One of these activities was purified and characterized as a serine protease with caspase-like activity, and was therefore designated a 'saspase'. The saspase was involved in Rubisco cleavage (a marker for victorin-induced PCD). More importantly, the saspase was constitutively present in the cell, and was released in the extracellular fluid (ECF) during stress. Although no direct substrates have been identified, the saspases, like caspases, are proposed to activate other proteases in a signalling cascade leading to PCD.

Vacuolar processing enzyme and the plant vacuole—a self-cleansing, non-inflammatory plant-based PCD system?

Vacuoles represent important storage organelles in seeds as well as a haven for the containment of hydrolytic enzymes such as proteases, lipases and nucleases. Several recent reports, however, have postulated the added role of the vacuole in the turnover of unwanted organelles and cytoplasm during autophagy as part of a non-inflammatory, phagocytosis-independent, clean-up system for dead or dying cells (Hatsugai *et al.*, 2006). A prominent component of this system is the vacuolar processing enzyme (VPE) (Hatsugai *et al.*, 2004). Originally identified as a protease comprising processing activity required for maturation of seed storage proteins, VPE exhibits caspase-like (YVADase) activity in

tobacco and *Arabidopsis* (Hatsugai *et al.*, 2005). Since this initial discovery, VPE activity has been found to play a crucial role in plants for numerous PCD pathways, including senescence, lateral root formation and importantly in plant–pathogen interactions as part of the HR [e.g. *Tobacco mosaic virus* (TMV) (Hatsugai *et al.*, 2004)]. Upon recognition of an appropriate apoptotic signal, the processing functions of VPE may activate hydrolase zymogens which drive the degradation of the vacuolar membrane and the release of compartmental degradative enzymes into the cell cytoplasm, for the degradation of cell contents.

PCD in response to development and abiotic stimuli

Developmental PCD

Several model systems such as barley aleurone protoplasts, *Zinnia elegans* and *Arabidopsis* have been used to study PCD during plant development. Some of these processes (e.g. tracheal element formation) demonstrate traits characteristic of autophagy, such as vacuolar swelling, cell-wall modifications, gradual disappearance of organelles and cytoplasm followed by collapse of the vacuole and autolysis (reviewed in Turner *et al.*, 2007). In contrast, processes such as root cap formation and cell sloughing feature apoptotic-like 'hallmarks', including chromatin condensation and shrinkage, nuclear fragmentation, and the formation of apoptotic bodies (Giuliani *et al.*, 2002).

Tracheal element formation

At functional maturation, xylem vessels consist of a series of interconnected dead cells termed tracheal elements (TEs), which transport water and minerals from the roots to the shoots via ongoing evaporative loss from leaf pores. Utilizing an *in vitro* *Zinnia* mesophyll cell model system, (Fukuda *et al.*, 1980) manipulated plant hormone levels to differentiate mesophyll cells into TEs and define the major developmental steps of the TE cell death pathway (reviewed in Turner *et al.*, 2007). Initially, lytic enzymes including nucleases and proteases are synthesized followed by a thickening of the cell wall. During secondary wall thickening, autophagic vacuoles degrade the bulk of the cytoplasm. Following autophagic digestion of the cytosol, changes in cytosolic Ca^{2+} and extracellular serine protease activity trigger the collapse of the central vacuole, resulting in the degradation of nuclear DNA and remaining cellular material leaving only the rigid cell wall. Importantly, inhibitor studies using ICE inhibitors (caspase-1 inhibitors) failed to produce an effect upon TE differentiation, suggesting that TE-PCD differs from apoptotic-like PCD systems, which are sensitive to such caspase inhibitors (Fukuda, 1996; McCann *et al.*, 2000).

PCD in the root cap

Roots provide the plant anchorage in the soil as well as a means for the transportation of water and minerals into the shoots

where photosynthesis takes place. As the soil can be an extremely harsh environment, plant roots have developed a layer of protective cells termed the root cap (Laux and Jurgens, 1997). The root cap represents a specialized organ which provides at least two major functions. First, cells in the root cap provide mechanical protection to the highly important root meristem, the source of root tissue. Secondly, root cap cells accumulate a high concentration of polysaccharides, which upon bursting of the cell provide much needed lubrication to the root tip. Evidence suggests that bursting of root cap cells is programmed and is associated with characteristic apoptotic traits such as TUNEL-positive nuclei, and the formation of vesicles resembling apoptotic bodies which contain degraded DNA in root cap cells (Huh *et al.*, 2002; Wang *et al.*, 1996).

Abiotic induced PCD

PCD responses to abiotic stresses such as cold, drought, salt and excessive UV irradiation have long been recognized as important factors for limiting crop yield worldwide (Qiao *et al.*, 2002). Many of these responses have been well studied and extensively reviewed in the literature; here we discuss salt stress as an illustration.

Damage to plants from excess salt is thought to result from osmotic pressure (water loss) and interference with ion channels within the cell. In many crops, salt tolerance is maintained by sequestration of sodium ions in the vacuole via an active Na^+/H^+ vacuolar antiport system (Katsuhara and Shibasaka, 2000; Huh *et al.*, 2002). This has a two-fold effect; first, toxic Na^+ ions are removed from the cytoplasm where they can interfere with critical biochemical processes to cause membrane disorganization, an increase in ROS and metabolic toxicity. Secondly, the store of Na^+ can be used to create an osmotic pressure to drive H_2O into the cell to retain turgidity.

Application of high concentrations of salts such as sodium chloride has been linked to an increased number of cells containing TUNEL-positive nuclei and DNA laddering in the roots of plants, including barley and *Arabidopsis* (Huh *et al.*, 2002; Katsuhara and Shibasaka, 2000; Li and Dickman, 2004). In these plants, adaptation to high salt conditions is thought to occur via selective PCD, i.e. the plant sacrifices a few cells for the greater good (Greenberg, 1996; Havel and Durzan, 1996). The basis for this theory is that in contrast to necrosis, which releases toxins to neighbouring cells and occurs under high salt concentrations, PCD is self-contained (i.e. toxic compounds are not released), and thus protects surrounding cells from the toxic by-products of necrosis. Salt tolerance/adaptation is essentially a compromise between damage and recovery to a given stress. PCD in roots allows recycling of nucleotides and amino acids into the shoots, aiding in the recovery of the plant by providing a level of tolerance (Katsuhara and Shibasaka, 2000). Further evidence supporting a role for PCD during periods of excess salt was provided by studies

in our laboratory involving the transgenic over-expression of animal anti-apoptotic genes (e.g. *CED-9* and *Bcl-2*) in tobacco, which also provided salt tolerance. The production of ROS has been linked with virtually all plant stress responses and may serve as a signal to initiate PCD pathways in plants.

Autophagy, an emerging trend for cell death regulation

Plants lack a functional phagocytosis system for the removal of toxic by-products released from unwanted, pathogen-infected, dead or dying cells, many of which could trigger PCD in neighbouring, healthy cells. Therefore, an alternative strategy for the removal of toxic cell remnants and protection of neighbouring healthy cells must be present in plants.

In animals, autophagy is responsible for adaptation to nutrient stresses, including nitrogen and carbon starvation, as well as the normal turnover of cytoplasmic components required for cellular homeostasis. Similarly, *Arabidopsis* knockout mutants containing defective autophagy pathways demonstrate hypersensitivity to nitrogen and carbon starvation (Doelling *et al.*, 2002). These findings further validate the presence of a potential pro-survival role of some *atgs*; thus, *atgs* appear to be involved in plant cell survival. In tobacco, autophagy also plays a critical role in controlling/limiting the HR during tobacco N protein/TMV interactions (Marathe *et al.*, 2002). The TMV–tobacco interaction is a classic plant–pathogen system; the tobacco N resistance protein recognizes the 50-kDa helicase domain of the TMV replicase protein to trigger HR-PCD (local lesion) and limit TMV to the point of infection (Liu *et al.*, 2005). Studies have shown that *Beclin-1* (the orthologue of the human *beclin 1* gene), and other autophagy genes (*atg3* and *atg7*) are involved in controlling cell death for the limitation of the specific R-gene and elicitor-mediated HRs. Thus, surrounding cells are protected from runaway cell death (Torres *et al.*, 2005). During normal HR, PCD is induced in both pathogen-infected cells and distal ‘healthy’ cells. In contrast, silencing studies involving plants deficient in *beclin 1* demonstrated an uncontrolled and spreading HR response to TMV infection. Upon further investigation, these *beclin 1*-deficient cells were found to initiate PCD in the pathogen-infected cells, but not in distal uninfected neighbouring cells, thus resulting in uncontrolled HR. Current data suggest that autophagy-based protection of ‘innocent bystander cells’ during pathogen/HR interactions is based on negative regulation of signals or toxic remnants released from dead/dying cells or pathogen-infected cells (Liu *et al.*, 2005; Patel *et al.*, 2006). During stress, autophagy may protect the cell from damage by regulating ROS production, possibly through a combination of plant NADPH oxidase genes [also known as respiratory burst oxidase (RBO)] and mediation of cellular Ca^{2+} levels (Torres *et al.*, 2005). However, the exact details as to how autophagy accomplishes this feat await discovery.

PCD in plant/pathogen interactions: it’s all about context

Depending upon the pathogen, cell death may be either beneficial or detrimental to the plant. During biotrophic pathogen–plant interactions, PCD, in particular the HR, prevents infection as biotrophs by definition require living cells for growth and colonisation. In contrast, PCD in response to necrotrophic pathogens which feed upon dead or dying tissue is advantageous, in this case to the pathogen and not the plant. Recent studies suggest that the death associated with some necrotrophic pathogens is apoptotic-like, demonstrating features such as DNA fragmentation and the formation of apoptotic-like bodies (Dickman *et al.*, 2001; Tada *et al.*, 2004; Yao *et al.*, 2001). Why would the plant trigger PCD during necrotrophic infection when it is clearly detrimental to its own health? Recent research suggests the plant may not be a willing participant in the process.

As mentioned previously, *Cochliobolus victoriae*, the causal agent of victoria blight in oats, induces a PCD in plants containing *Vb*, a dominant susceptibility gene, via secretion of its host selective toxin, victorin (Navarre and Wolpert, 1999). Interestingly, the same *Vb* allele is genetically indistinguishable (tightly linked) to the resistance gene locus against the biotroph *Puccinia coronata*, the causal agent of crown rust. The failure to separate the two genes genetically suggests that the same gene may be responsible for conditioning both susceptibility and resistance depending on the pathogenic lifestyle. Recent data from the Wolpert laboratory are consistent with this idea (Lorang *et al.*, 2007). Importantly, victorin-induced cell death shares many similarities with apoptosis, including DNA ladders, the requirement of Ca^{2+} importation, caspase-like proteases and an enhanced mitochondrial permeability transition (MPT) (Navarre and Wolpert, 1999).

The oxidative burst is one of the earliest and most common plant responses to pathogen challenge and has been correlated with defence responses and host resistance (Mehdy, 1994). Regulation of ROS levels and redox homeostasis serve a broad and crucial function in the ability of plant cells to detect and respond to the environment (e.g. abiotic and biotic stimuli). Being directly toxic, ROS are commonly linked with forms of cell death. Recent research from a number of systems, however, suggests that like PCD, the effects of ROS may direct both beneficial and detrimental influences upon the cell (Gechev *et al.*, 2006). In low concentrations, ROS are not directly toxic and can act as second messengers in signalling pathways, including those within host–pathogen interactions (Apel and Hirt, 2004). In mammals, evidence for this idea includes the observation that ROS recapitulates gene expression identical to the stimulus (e.g. pathogen ligand). In contrast, high concentrations of ROS are toxic, leading to cell membrane damage and DNA degradation.

Sclerotinia sclerotiorum, a necrotrophic fungal phytopathogen of extremely broad host range (virtually all dicots), serves as an

additional example where the pathogen strategy is to subvert and induce plant PCD pathway(s) to cause inappropriate cell death in the host. Previous work in our laboratory established the importance of *Sclerotinia*-secreted oxalic acid (OA), a non-specific phytotoxin, as an important determinant of *Sclerotinia* pathogenicity (Cessna *et al.*, 2000). Mutants defective in OA synthesis are non-pathogenic and do not induce tissue cell death, a characteristic of the diseased state (Godoy *et al.*, 1990). OA is multifunctional, contributing to several processes (reduction in pH, elevation of Ca^{2+} , etc.) that augment fungal colonization of host plants. Exogenous application of physiological concentrations of OA recapitulated *Sclerotinia* disease symptoms (cell death). Furthermore, recent data suggest that OA is a fungal elicitor that induces apoptotic-like cell death features including DNA laddering and TUNEL-reactive cells in a time- and dose-dependent manner (Kim *et al.*, accepted). In contrast, OA-defective *Sclerotinia* mutant infections do not exhibit these characteristics. Importantly, oxalate also induces increases in reactive oxygen levels (H_2O_2), which correlate with PCD. When OA-induced ROS is inhibited, apoptotic-like cell death and disease does not occur. Therefore, fungal induced plant PCD is essential for *Sclerotinia* pathogenicity and appears to be mediated by ROS to trigger plant pathway(s) responsible for PCD (Kim *et al.*, accepted). In effect, OA is not directly toxic, but more subtly functions as a signalling molecule.

Identification of plant PCD regulators, the BAG proteins and more—Bio-informatic approaches

Although plant cells can undergo apoptotic-like cell death, plant homologues of mammalian core regulators of apoptosis have, in general, been scarce. In fact, examination of the completed *Arabidopsis thaliana* genome as well as other partial or near complete plant genomes by tools such as BLAST or FASTA have failed to reveal any apparent homologues to the core apoptotic regulators at the primary sequence level. This suggests a high level of sequence divergence for plant apoptosis proteins in relation to their animal counterparts. Therefore, if such plant genes exist then alternative strategies are necessary to identify candidate genes. Functional genomic screens and/or advanced bioinformatic approaches are used in our laboratory for this purpose.

The underlying premise of the informatic approach is that functional similarity can be predicted from structural similarity. This becomes important as distantly related proteins may have limited (undetectable) overall sequence homology, but key features (e.g. catalytic sites, folds, helical structure, hydrophobicity, water accessible surfaces, electrostatic potential) may be conserved to the extent that functional predictions can be made independently of the primary sequence. For example, we previously discussed that human Bcl-2 family proteins share three-dimensional (3D) structural similarity to the pore-forming bacterial colicins; however, the primary amino-acid sequences of these

proteins lack significant homology. Indeed, human pro-apoptotic proteins such as BID were found to have 3D structures extremely similar to other human Bcl-2 family proteins, but lacked significant primary amino-acid sequence homology (Janjusevic *et al.*, 2006). As such, diverse primary amino-acid sequences can result in a common protein-fold of relevance for cell death regulation, perhaps explaining the inability to detect Bcl-2 homologues in plant genomes based on sequence comparisons. A similar case may occur in plants such that the genomes may possess functional equivalents of cytoprotective Bcl-2 family proteins that share little or no sequence homology with their counterparts in animal cells. An example more in the context of this review is the recent demonstration that *AvrPtoB* (a bacterial avirulence gene) exhibits E3 ubiquitin ligase activity with functional consequences for disease (Abramovitch *et al.*, 2006). The primary sequence of this gene did not suggest that such activity was present, but X-ray crystallography of the protein unexpectedly yielded this important component of the protein.

In agreement with this approach, we uncovered and characterized the BAG protein family of Arabidopsis by profile-sequence (PFAM) and profile-profile (FFAS) algorithms (Doukhanina *et al.*, 2006). The mammalian BAG proteins are a family of chaperone regulators that modulate a number of diverse processes ranging from proliferation to growth arrest and cell death, and were originally discovered in a screen for Bcl-2 interactors (Takayama *et al.*, 1995). BAG proteins are distinguished by a conserved 'BAG' domain, which directly interacts with Hsp70/Hsc70 proteins to regulate activity (Doukhanina *et al.*, 2006). Our searches of the *A. thaliana* genome sequence revealed seven homologues of the BAG protein family with limited sequence similarity to their human counterparts. Structurally, however, these proteins were highly similar and contained putative Hsp 70 binding sites (Doukhanina *et al.*, 2006).

The functional versatility of this family is maintained in Arabidopsis and quite possibly other plants. Akin to their mammalian counterparts, the Arabidopsis BAGs function in cell protection during both abiotic (e.g. cold, UV light, drought), biotic (pathogen challenge) and developmental (plant hormone) stimuli (Doukhanina *et al.*, 2006). Not surprisingly, subsequent bioinformatic analysis of plant BAG promoters in our laboratory has revealed the presence of numerous *cis*-acting elements typically associated with stress regulation (our unpublished data). Thus, plant BAG members are also multi-functional and remarkably similar to their animal counterparts, as they regulate apoptotic-like processes ranging from pathogen attack, to abiotic stress, to development.

The second approach we have taken uses unbiased screens for cDNAs and gene fragments that function within cell suicide pathways. Novel genes that suppress cell death pathways have been discovered by using these methods in animal cells, and attempts to extend this approach to the genomes of plants have begun. The major function-based screen we have used is predicated on

the ability of ectopically expressed mammalian Bax to kill yeast conditionally, and on the ability of cytoprotective proteins to rescue yeast from the lethal phenotype conferred by Bax. A yeast strain was constructed expressing Bax under direction of the *Gal10* promoter, which drives expression according to the carbon source (Chen *et al.*, 2004). On glucose, yeast grows normally, although, when placed on galactose, Bax is expressed and the yeast dies. Using such a system researchers in our laboratory successfully identified a tomato (*Lycopersicon esculentum*) gene encoding a phospholipid hydroperoxide glutathione peroxidase (LePHGPx) which functioned as a cytoprotector preventing Bax, H₂O₂ and heat-induced apoptosis in yeast and tobacco mesophyll cells (Chen and Dickman, 2004). Thus, using both bioinformatic-based approaches coupled with heterologous functional screens, candidates have been identified that may participate in cell death regulation in plants.

Chloroplasts—a rich source of ROS

A distinct difference between plant and animal cells, the chloroplast is a major source of ROS, and evidence suggests it is a likely player of cell death responses (Zapata *et al.*, 2005). Light is required for many plant PCD responses, including those induced by developmental, abiotic and biotic stimuli. In accordance, chloroplast degradation is one of the earliest and most dramatic changes in the cell prior to leaf senescence. The actions of chloroplast-specific herbicides have provided clues supporting the involvement of the chloroplast in PCD, in particular the electron transport chain and ROS production that occur in at least some PCD responses (Chen and Dickman, 2004).

Cell death induced by the exogenous application of chloroplast-targeted, ROS-generating herbicides such as methyl viologen (paraquat) and acifluorfen appears to be programmed with affected cells displaying typical apoptotic traits. In contrast, the treatment of plants with glyphosate (Roundup), a chloroplast-specific, non-ROS-generating herbicide did not trigger apoptotic-like cell death. Furthermore, the transgenic over-expression of animal anti-apoptotic genes *Bcl-2*, *Bcl-xl* and *CED-9* in tobacco provided protection against ROS-eliciting herbicides (Chen and Dickman, 2004). Of particular interest for this review is the observation that chloroplasts may play a key role in many plant–pathogen interactions (Genoud *et al.*, 2002; Mittler *et al.*, 1997).

Chloroplasts have also been linked to pathogen-induced PCD responses via association with salicylic acid production, an established hormone and signalling molecule in plant defence responses, and a mediator of PCD. Arabidopsis plants grown in low light conditions displayed compromised local and systemic defence responses when challenged with *Pseudomonas syringae* (Genoud *et al.*, 2002). Additionally, light is a requirement for the N-gene-mediated HR and resistance to TMV and *Turnip crinkle virus* (Chandra-Shekara *et al.*, 2006; Gray *et al.*, 2002). Finally,

the identification of two chloroplast transit peptide cleavage sites (11s1 and acd1) in lesion mimic mutants of maize and Arabidopsis further supports the role of chloroplasts during plant–pathogen interactions (Gray *et al.*, 1997). Therefore, chloroplasts may play a crucial role in PCD by acting as a link between two signalling molecules involved in PCD, salicylic acid and ROS (Genoud *et al.*, 2002).

CONCLUSIONS

There is compelling evidence that plants contain a functional PCD mechanism for the regulation of biological processes ranging from development to abiotic and biotic stress responses. Additionally, recent research has suggested that the impact of many PCD responses may be either beneficial or detrimental depending on context, and more importantly the source of the signal. Historically, necrotrophic pathogens were thought to kill the cell via the secretion of potent toxins; however, studies have shown, at least in several cases, characteristic 'apoptotic' traits during the infection process. *Cochliobolus victoriae* and, more recently, *Sclerotinia sclerotiorum* interactions serve as examples of such a phenomenon. These fungi do not kill the cell directly but instead hijack plant PCD pathways. Evidence from *Sclerotinia* also suggests that ROS are important components of this pathway. As a mediator of PCD responses, ROS possibly function as signalling molecules. A major difference between animal and plant cells, the chloroplast is a key source of ROS levels and is thought to play a role in PCD. We envisage that ROS regulation and modulation of the redox environment will prove to be a key factor in plant PCD.

Other unique features of plant cells include a prominent vacuole, the cell wall and absence of phagocytosis. The vacuole is known to be a key player in non-apoptotic cell death and along with autophagy may represent the 'plant alternative' to non-inflammatory phagocytotic clean-up by eliminating pro-death signals, including ROS.

In summary, PCD is a complex, highly regulated process required for the normal physiology of all multicellular organisms and, importantly, if inappropriately regulated (too much/too little) can be detrimental. Further insight into PCD modulation and the overall cell/death process in plants is important not only for our fundamental understanding of plant stress and developmental responses but also for an applied perspective. Exploitation of the cell/death pathways in plants may lead to the generation of pathogen-resistant or stress-tolerant crops, the production of fruit with an extended shelf life, or aid the transformation of recalcitrant plants for use within bio-production/bio-farming applications.

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