REVIEW

Programmed cell death in plants resembles apoptosis of animals

Cyrelys Collazo[1], Osmani Chacón[2], ✉ Orlando Borrás[1]

[1]Plant Division, Center for Genetic Engineering and Biotechnology
P O Box 6162 Havana, 10600, Cuba
[2]Tobacco Research Institute, Havana, Cuba
E-mail: orlando.borras@cigb.edu.cu

ABSTRACT

The selective and regulated elimination of certain cells is an effective mechanism to ensure the maintenance of homeostasis in both plants and animals. This is possible through a genetically regulated process called programmed cell death (PCD). Morphological and biochemical hallmarks of PCD or apoptosis in animals can also be observed in PCD of plants. Furthermore, genetic similarities between plant genes and apoptosis-related animal genes have been observed. The data tend to consider both processes of cell death as two expressions of the same type of process. However, distinctive characteristics of plant cells including the existence of a cell wall, imply dissimilarities in the execution of PCD. The cell wall precludes phagocytosis, establishing a different mechanism for corpse management. Similarly, the vacuole can be transformed into a hydrolytic compartment, with hydrolases and toxin profiles that establish the processing of the apoptotic body. The apoptosis machinery can be successfully employed to control plant diseases through the transgenic expression of animal anti-apoptotic or pro-apoptotic genes. However, the apoptotic nature of PCD in plants as well as the perspectives of the therapeutic modulation of this process for plant disease management is still under discussion.

Key words: apoptosis, programmed cell death, caspases, mitochondria, reactive oxygen species, ion fluxes, mitogen-activated protein kinases

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RESUMEN

La muerte celular programada en plantas y su similitud con la apoptosis de animales. La eliminación regulada y selectiva de determinadas células es un mecanismo eficiente que garantiza el mantenimiento de la homeostasia en las plantas y los animales. Este proceso genéticamente regulado se denomina muerte celular programada (MCP). Algunos rasgos morfológicos y bioquímicos de la MCP o apoptosis de los animales también se observan durante la MCP en las plantas. Además, existen genes vegetales semejantes a genes relacionados con la apoptosis en animales. Estos datos sugieren que pudiera tratarse de dos manifestaciones de un mismo tipo de proceso. No obstante, características distintivas de las células vegetales como la pared celular, implican divergencias en la ejecución de la MCP. La pared celular impide la fagocitosis estableciéndose otro mecanismo de procesamiento del cuerpo apoptótico. Igualmente, la vacuola puede transformarse en un compartimiento hidrolítico, cuyo perfil de hidrolasas y toxinas define el modo de procesamiento del cuerpo apoptótico. La maquinaria apoptótica puede ser empleada para el control de fitopatógenos mediante la expresión de genes pro o antiapoptóticos de animales. No obstante, la naturaleza apoptótica de la MCP de las plantas, así como la utilidad de su modulación terapéutica para el tratamiento de fitopatologías son todavía temas discutidos.

Palabras claves: Apoptosis, muerte celular programada, caspasas, mitocondria, especies reactivas del oxígeno, flujos iónicos, proteínas kinasas activadas por mitogenos

*I*ntroduction

The suicide of individual cells is an efficient and conserved mechanism to achieve and maintain homeostasis in multicellular organisms as a response to pathogen attack and abiotic stress, as well as in normal development [1]. The selective elimination of certain cells is carried out by a gene-directed process called programmed cell death (PCD). This is an energy-dependent asynchronic process that comprises the loss of cell-to-cell contacts, cytoplasmic shrinkage, membrane blebbing, DNA fragmentation, disassembly of the nuclei and formation of apoptotic bodies. The execution of PCD requires the participation of a complex cell suicide machinery that involves several molecules regulated by the expression of a certain set of genes. The self-contained nature of PCD contrasts with necrosis, which is an unregulated process of traumatic destruction, followed by the release of intracellular components without the active participation of the cell [2].

In animals, the active study of PCD began in 1972 when Kerr *et al.* introduced the term apoptosis as "a basic biological phenomenon with a wide range of implications in tissue kinetics" [3]. However, it took more than a decade to realize the biological importance of PCD in plant pathogenesis and development [4]. As in animals, PCD plays a key role in numerous vegetative and reproductive phases of plant development, including the senescence of leaves, xylogenesis, death of petals after fertilization, post-embryonic decay of aleuronic layers, root cap development, somatic and zygotic embryogenesis and sex determination. Similarly, PCD in plants occurs in response to biotic and abiotic stimuli. In plant-pathogen interactions, PCD serves not only as a defense mechanism of the plant in incompatible interactions, but also to promote the dissemination of the pathogen in compatible interactions [5].

✉ Corresponding author

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Avirulent infections are usually characterized by a localized cell death known as hypersensitive response (HR) which results in the formation of necrotic lesions around the infection sites [6]. On the other hand, there is the abiotic stress response, and the best example is aerenchyma development under low oxygen conditions, in which root cortical cells are induced to die and form larger airspaces, enabling a greater diffusion of air from the upper parts of the plant [7]. PCD in plants has also been characterised in response to high temperature [8].

Finally, some of the morphological features of apoptosis as well as transduction pathways and signal molecules have been shown to be similar in both animals and plants. However, differences in the execution of PCD have also been observed. A comparison of common and different features of PCD between animal and plant systems is the aim of this review.

Morphology of apoptosis in animals and PCD in plants

Morphological features of apoptosis may be detected by various cytochemical and microscopic methods. The use of intercalatory agents such as propidium iodide (PI) and Hoechst 3325 enables the detection of the condensation and marginalization of chromatin in the nucleus by measuring the level of fluorescence, which is reduced during apoptosis [9]. Fluorescent microscopy using acridine orange produces a characteristic chromatin clumping, observed soon after staining. A further refinement is the comet assay, which shows DNA degradation. In addition, modern video microscopy allows the visualization of the temporal sequence of events that occur over 15 ± 60 min to 24 h.

In plants, in addition to these techniques, the use of Green Fluorescent Protein-Nitrilase 1 (GFP-Nit1) fusion and other markers to image plant cell death in vivo has revealed subcellular responses such as nuclear envelope separation, the formation of nuclear lobes and the release of nuclear contents into the cytosol [10].

DNA cleavage

Table 1 provides a detailed comparison of PCD in animals and plants. The fragmentation of the DNA occurs at the nucleosomal linker sites and the fragments are reported to be of 140-180 base pairs in animals [11]. Electrophoretic separation exhibits DNA fragments as a ladder formation of which the rungs are multiples of 180 bp. The DNA fragments can be cytochemically determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) of the 3'OH groups [12]. The DNA processing reported for animal PCD is also believed to exist in the dying cells of plants. Nuclear condensation as well as oligonucleosome sized DNA fragments have been detected by TUNEL and electrophoresis analysis in aleurone cells of barley [4], in dying tobacco roots cap cells [13], and also in plants exhibiting HR resistance such as cowpea leaf cells infected with *Uromyces vignae* and Tobacco Mosaic Virus (TMV) infected tobacco [14]. DNA ladders were also observed during cell death in Alternaria Alternata (AAL) toxin treated tomato protoplasts and leaflets. The intensity of the DNA ladders was enhanced by Ca^{2+} and inhibited by Zn^{2+} [15]. The DNA fragments have been seen to range from sizes as high as 50000 bp in some cases and of 140 bp in others [16]. Moreover, DNA fragmentation may be a marker feature of certain cell deaths of plants such as root cap cells, aleurone cells, etc; but it is not likely to be involved either in tracheary elements and fibers [17] or in epidermal and mesophyl cells of lace plant leaves during development [18].

Cell membrane components

Another well characterized morphological feature of apoptosis is the lost of membrane phospholipid asymmetry that results in phosphotidylserine (PS) exposure on the outer and the inner surface of the plasma membrane. Externalized PS appears to serve as an important signal for targeting the recognition and elimination of apoptotic cells by macrophages. PS externalization has been suggested to originate from the balance between its inward and outward translocations driven by two enzymatic activities: aminophospholipid translocase (APT) and a non-specific phospholipid scramblase (PLSCR). Selective PS oxidation may affect one or both of these activities-APT or PLSCR-hence shifting the balance in favor of PS egression to the cell surface [19]. The potential interactions between the anionic phospholipid phosphatidylserine and the redox-active cationic protein cytochrome c is presented as a potential mechanism to account for the selective oxidation of PS during apoptosis [20]. It is generally assumed that the exposure of PS on the plasma membrane is a consequence of caspase activation. However, a caspase-independent mechanism has been observed which occurs in primary T cells during apoptosis and is induced by stimuli that do not trigger death receptors; it is probably mediated by the release of the apoptosis inducing factor (AIF) from the mitochondria [21].

Changes in PS asymmetry, analyzed by measuring Annexin V bound to the cell membrane, were detected in tobacco PCD induced by a number of chemical agents [16] and in apple suspension cells under a low oxygen culture [22]. The physiological role of PS exposure in plants is still unknown since phagocytosis does not occur.

Cytoplasmic events

Morphological changes in the cytoplasm of animal cells during apoptosis include condensation, shrinkage and fragmentation. However, cell membrane integrity is preserved allowing the packaging of nuclear and cytoplasm components in apoptotic bodies. Condensation and shrinkage of the cytoplasm were reported in dying aleurone cells [23], onion and tomato root cap cells [15], HR lesion cells in *Arabidopsis* [24] and wound and herbicide induced PCD [10].The cytoplasm of differentiating treachery elements (TE) is reported to become lobed, condensed, shrunken and finally broken into small packages [25]. The formation of membrane-bound structures or apoptotic- like bodies in tomato has been observed in response to AAL toxin from Alternaria alternata f. sp. lycopersiti, and also to arachidonic acid, an inducer of HR [15]. In contrast to animals, hallmarks

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Table1. Comparison of PCD in animals and plants

Animals	Plants
DNA fragments of more or less 180 bp.	DNA fragments of 50 kb (50,000 bp) in some cells and 0.14 kb (140 bp) in other cells are reported. DNA fragmentation is not likely to occur in differentiating TEs, fibres and sclerids.
Ca^{2+}-dependent endonucleases are shown to be responsible for DNA processing and fragmentation; in almost all instances (except one case in *C. elegans*) the nuclease is the product of the dying cell itself.	Nucleases are reported in some dying plant cells; but there is yet no direct evidence of their involvement in PCD. Plant nucleases are either Ca^{2+} or Zn^{2+}-dependent. Some nucleases are activated by both Ca^{2+} and Zn^{2+}. Nucleases may be the product of the dying cell itself or may be transported from adjacent cells.
There is an increased exposure of the acidic phospholipids phosphotidyl serine (PS) on the outer surface of the plasma membrane, which can be demonstrated by Annexin V binding.	So far PS exposure has been shown through Annexin V binding, only in the protoplasts of tobacco subjected to abiotic stresses.
Cytoplasmic condensation, shrinkage and fragmentation are always noticed.	Condensation and shrinkage of cytoplasm noticed, but not fragmentation. Reports of fragmentation are either due to artefacts or uncritical observation.
Cells shrink.	Cells shrink in most plant cell categories but not in differentiating TEs, fibres and sclerids.
PCD is due to a syndrome of well organized and executed mechanisms involving effectors, adaptors, regulators and signals.	The mechanism underlying plant PCD is incompletely known; so far no adaptor or regulator molecule has been known; only effectors, signal and regulator molecules have been found.
Effector caspases (cysteine proteases) and Granzymes are activated and expressed; they specifically cleave after the aspartate residues in proteins.	Expression of cysteine proteinases is reported in some cases, but not exclusively during cell death; their role in scavenging the proteins of dead cytoplasm cannot be ruled out; substrates for some of the expressed cysteine proteinases are not yet definitely shown; their specificity is also unknown; their homology to animal caspases is also very low.
Cell corpses are engulfed and eliminated through phagocytosis by neighbouring cells or macrophages.	Cell corpses persist due to the persistence of the cell wall; in TEs, fibres and sclerids cell corpses not only persist in a distinctive manner, but start functioning only then. Dead cytoplasm is almost always eliminated by vacuolar auto-phagy; elimination by plastolysomes is also likely.
The antiapoptotic protein BCl-xL suppresses PCD at least in some cells.	Bd-xL does not suppress PCD associated with HR.
ROS like O_2 and H_2O_2 as signalling molecules are required to activate PCD.	O_2 and H_2O_2 are implicated in cell death, especially in HR responses due to biotic and abiotic stresses; evidence, however, is not conclusive. The reported involvement of H_2O_2 in TE death and HR should be viewed with caution.
Increase in cytosolic Ca^{2+} can activate PCD through the activation of endonucleases and caspases.	Increase in cytosolic Ca^{2+} can activate PCD, probably through the activation of endonucleases; there is no report as yet of Ca^{2+} activated proteinase in plant PCD.
The role of mitochondria in executing PCD is well known.	The role, if any, of mitochondria in PCD is to be substantiated, although there are one or two reports implicating it.
Plasma membrane blebbing is common	Plasma membrane blebbing has so far not been reported.
Protein phosphorylation/ dephosphorylation changes are common.	Protein phosphorylation/dephosphorylation changes reported only in cells subjected to hypoxia and HR, and in aleurone cells.
Growth factor deprivation (GFD) promotes cell death.	There are reports of GFD-promoting cell death in some categories of plant cells; contradictory results are obtained in certain cells (where the supply of growth regulators promotes cell death).
Chromatin condensation noticed.	Chromatin condensation noticed in some categories of dying cells, but not in all.
Systematic DNA cleavage and fragmentation demonstrated through electrophoretic ladder formation and through TUNEL staining; Fragmentation takes place at the nucleosome linker sites to result in oligonucleosomal fragments.	DNA cleavage and fragmentation demonstrated through electrophoretic ladder formation and through TUNEL staining only in some categories of cells but not in all.
Typical apoptotic bodies each consisting of some cytoplasm and an oligonucleosomal DNA bit are formed.	There is no instance where typical apoptoic bodies are yet reported.
Stress proteins are not synthesized during cell death.	Stress proteins such as hydroxyproline, glycine, arabinogalactan, and theonine-rich proteins are often synthesized and become integral components of cell walls of some categories of cells undergoing death.

of a typical HR-PCD involve membrane dysfunction, vacuolization of the cytoplasm, vacuolar disruption (oncosis) and changes in gross mitochondrial morphology characterized by swelling and cristae disorganization [26]. The final, preeminent step of TE PCD as well as in the formation of lace plant leaf perforations is a rapid collapse of the vacuole occurring after completing of secondary cell wall synthesis [27].

Corpse management in plant cells

Corpse management is a feature that is remarkably different between plants and animals since there is a cell wall in plant cells in contrast to animal cells and plants do not have an immune system. The cell wall precludes phagocytosis, the process of engulfing apoptotic bodies by neighboring cells or macrophages in animals, preventing their lysis and the release of toxic or immunogenic intracellular components to the nearby tissue with a consequent inflammation [28]. Instead of that, corpse processing in plants is autolytic, it is carried out by vacuoles.

Decisions on corpse management based on the integration of various signals such as auxins, cytokinins, ethylene and elicitors, are probably made

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by the living cell long before death takes place and probably even well before the point of no return in the plant cell. The ability to make these decisions is especially relevant in plant cells due to the absence of macrophages and neutrophils to decide for them [29]. The way the cell corpse is managed is a function of the profile of hydrolases and toxins that are loaded into the vacuole and these profiles are established by the original set of signals. The cell must be metabolically active to synthesize the destructive hydrolases it needs to process its corpse; it therefore sequesters these hydrolases and toxins into the vacuole and releases them when the vacuole collapses. This collapse is an irreversible step towards death which results in the immediate cessation of cytoplasmic streaming and requires a calcium flux [29]. Several genes encoding hydrolases that are up-regulated are common to all plant PCDs, whereas some genes are unique to each type of 'preparation' for death [30].

For example, auxins and cytokinins induce the *de novo* synthesis of vacuole-sequestered nucleases and proteases in treachery elements, leading to the complete degradation of the cellular content during the onset of autolysis, while leaving behind the extracellular matrix and the secondary cell wall built before death [18]. During the formation of lysigenous aerenchyma, induced by ethylene, the entire corpse is removed and the cell wall hydrolases such as cellulase are included to fulfill the need to remove not only the protoplasm but the extracellular matrix as well, thus resulting in gas spaces [31]. In the hypersensitive response, signals from pathogens, in most cases, induce the production of phytoalexins, polyphenols and chitinases and these are released when the vacuole collapses. The corpse is not significantly autolysed, but it is subsequently crushed by the expanding tissues [32].

In addition to autolysis, another way of processing dead and dying cells is called autophagy. This process has been observed as the engulfment and degradation of the nucleus and other organelles by provacuoles, vacuoles or other autophagic organelles derived from leucoplasts [33]. Autophagy has been described in animals as a form of non-apoptotic, non-necrotic cell death by which long lived proteins and organelle components are directed to and degraded within lysosomes [34]. Although the components of the autophagic machinery have been identified in *Drosophila*, *Xenopus*, mammals and yeasts, the physiological role varies [35]. In plants, in most cases, autolytic and autophagic mechanisms cooperate to yield cellular disassembly, such as that occurring during embryo suspensor death [33].

The role of the cell wall

The plant cell wall may or may not be degraded along with the protoplast, depending on the type of PCD [29]. During treachery element differentiation, the primary wall and a rigid secondary wall are required for cell function and are not hydrolyzed, except for the portion of the primary wall between the adjacent treachery elements that is degraded to form a perforation [36]. In most other forms of developmental PCD, collapsed primary cell walls are left behind, whereas nutrients from dismantled protoplast are recycled [37]. When the hypersensitive response is induced by pathogen invasion, the protoplast dies, leaving collapsed or crushed primary cell walls behind [13]. By contrast, cell walls must be degraded to form the extensive lysigenous air spaces in hypoxia induced aerenchyma tissues in maize and rice roots [38]. During leaf perforation in lace plants, cell walls are also degraded [18].

Molecular bases of apoptosis in animals and plants PCD

Following the appropriate stimulus, the first stage or "decision phase" of apoptosis is the genetic control point of cell death. This is followed by the second stage or "execution phase" which is responsible for the morphological changes of apoptosis.

Much of the biochemistry and the genetics of the "effector mechanisms" of apoptosis have been well studied in the nematode *Caenorhabditis elegans* [39] and in mammals. However, relatively little is known on the details of many of the "signalling pathways" that can induce apoptosis in plants or animals.

Effectors of cell death

The key effectors of animal PCD are cysteine aspartate-specific proteases (caspases) that constitute a critical point in the execution phase of apoptosis. The activation of caspases follows two main pathways: extrinsic, which involves the interaction of a death receptor with its ligand, and intrinsic, with the participation of the mitochondrion and the release of cytochrome c (figure 1) [40]. Furthermore, other cysteine proteases, such as calpains and cathepsins, serine proteases, such as granzymes, and the proteasome/ ubiquitin pathway are all reported to be involved in apoptosis.

Caspases are a family of cysteine proteases that specifically cleave adjacent to an aspartate residue [41]. They are synthesized as inactive proenzymes and are activated by directed proteolysis that removes the N-terminal peptide (prodomain) and cleaves the proteolytic domain at specific recognition sites [42]. The t ertiary structure of human caspases, characterized by a unique topology designated as "caspase-hemoglobinase fold", is conserved from nematodes to humans [43]. In general, apoptotic cell death involves a sequence of caspase activation events in which initiator caspases such as Casp 8 and 9 activate downstream executioner caspases (Casp 3, 6, 7) that process a variety of target proteins eventually leading to the apoptotic phenotype [44]. In animals, at least 10 caspases have been identified that may activate or inactivate downstream various substrates during apoptosis execution [28]. One of the most widely studied inactivation events is the breakdown of poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair and genome integrity. Another report on cleavage is an inhibitor of caspase-activated DNAse (ICAD), leading to the activation of caspase-activated DNAse (CAD) and the fragmentation of DNA [45].

Although the existence of caspases in plants is controversial, cysteine protease activity has been reported to be induced in plant systems undergoing

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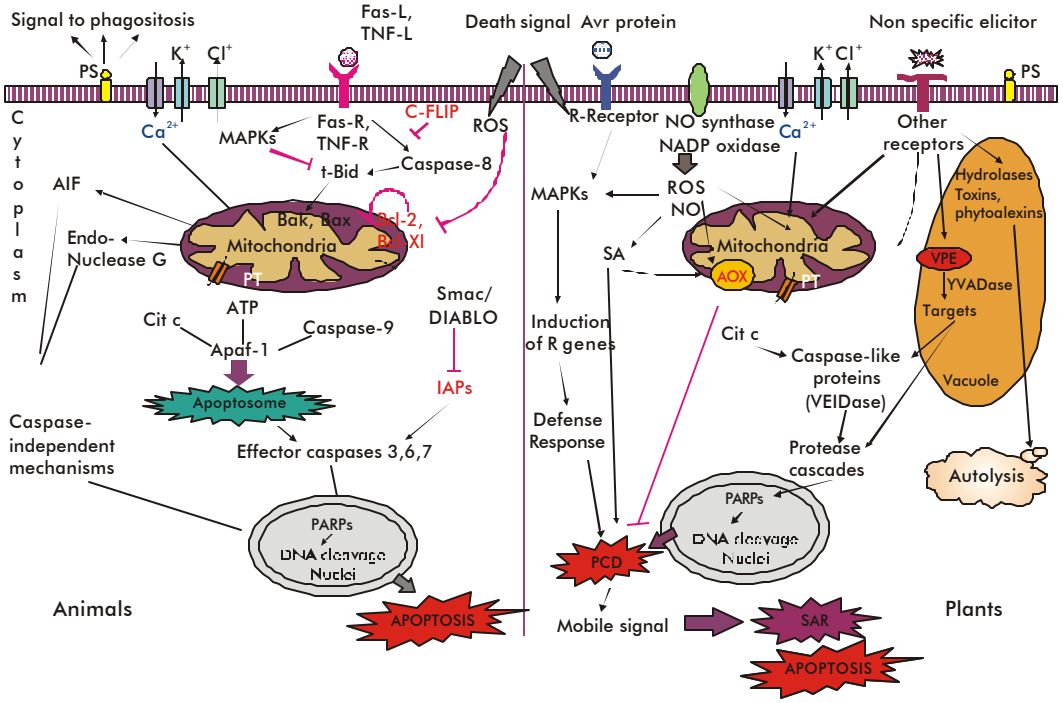

Figure 1. Main mechanisms for programmed cell death in plants and animals involving the participation of the mitochondrion or the activation of death receptors. Role of the vacuole in plants. The main proteins involved are shown, as well as the modulatory sites for selected modulatory proteins, (inhibitors in red). PS. Phosphatidylserine. MAPKs. Mitogen-activated protein kinases. NO. Nitric oxide. ROS. Reactive oxygen species. SA. Salicylic acid. IAPs. Inhibitors of apoptosis. Smac. Second-mitochondrial-derived activator of caspase. DIABLO. Direct IAP-binding protein with low pI. AIF. Apoptosis inducing factor. PT. Transient pore. APAF-1. Apoptosis protease activating factor-1. VPE. Vacuolar processing enzyme. AOX. Mitochondrial alternative oxidase. PARP. Poly (ADP ribose) polymerase. HR. Hypersensitive response. SAR. Systemic acquired resistance. R genes. Resistance genes. FLIP. FADD-like ICE (caspase 8) inhibitory protein.

PCD [46]. Recent reports indicate that at least two types of cysteine proteases with structural homology to caspases (metacaspases and legumains) as well as a class of caspase-unrelated serine proteases (subtilisin-like serine proteases), exhibit aspartate- specific cleavage activity in plants [47]. These proteases might function in a plant proteolytic network functionally equivalent to the animal caspase- mediated events involved in apoptotic cell death.

Metacaspases have been shown to have, in their tertiary structure, the so-called caspase-hemoglobinase fold present in animal caspases and a similar processing in a large and small subunit was observed in yeast [48]. Nine metacaspases have been reported in *Arabidopsis thaliana* genome and a type II metacaspase gene (*LeMCA1*) of *Lycopersicum esculentum* was found to be involved in PCD during the infection of *Botritis cinerea* [49]. VEIDase activity (equivalent to the activity of human caspase 6) have been reported to be the main caspase-like activity in embryo-genesis in *Picea abies*. It was located in the embryonic tubes and cells during the PCD stages. VEIDase activity was sensitive to Zn^{2+} and ionic strength and pH were comparable to caspase 6 [47].

Recently findings related to human caspase substrates and inhibitors contribute to the clarification of the specific role of metacaspases in PCD. Specific animal caspase inhibitors for caspase 1 (Ac-YVAD-cmk) and caspase 3 (Ac-DEVD-CHO) have been shown to attenuate HR in tobacco leaves induced by bacteria and TMV, as well as isopentenyladenine and menadione [50]. The baculovirus antiapoptotic protein p35, an inhibitor of apoptosis (IAP), which was shown to inhibit animal caspases, was also effective in preventing PCD induced by bacterial, fungal and viral infections [51]. Specific proteolytic processing of caspase substrates such as VirD2 protein from *A. tumefaciens* (substrate from human caspase 3), occurs early in the course of HR induced by TMV in tobacco leaves [52].

Experiments with PARP have also been carried out in plants. In both animals and plants there are two different types of PARP. The Arabidopsis PARP-1 shows high homology to human PARP -1 (usually cleaved by caspase- 3) and is endonucleolytically cleaved by extracts from fungus-infected cowpea plants developing HR. This activity is inhibited by the caspase- 3 inhibitor (Ac-DEVD-CHO) and not by the caspase 1 inhibitor (Ac-YVAD-cmk) [53].

Legumains, such as the vacuolar processing enzyme (VPE), also have the caspase-hemoglobinase fold (CHF) structure, which cleaves after an aspartic residue and is inhibited by the human inhibitor of caspase 1, showing a functional resemblance to animal caspases [54]. VPE have been shown to have caspase 1-like activity, which is essential to virus-induced hypersensitive response in tobacco plants [55].

Adaptors and regulators of cell death

The activation of caspase precursors or zymogens in animals is achieved by adaptor proteins that bind to

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them via shared motifs. Caspase 8 is activated when death effector domains (DED), in their prodomain, bind to the C-terminal DED in the adaptor molecule Fas-associated Death Domain (FADD). The C-terminus of FADD also contains a death domain (DD) allowing it to bind to an equivalent region within the cytoplasmic domain of the Fas receptor (CD95/APO-1) [56]. These adaptors that activate caspases in animals may be regulated by themselves. A protein called FLIP may bind to FADD preventing the activation of caspase 8 [57].

Caspase 9 activation follows the binding of its caspase recruitment domain (CARD) localized in its prodomain to the CARD of another adaptor protein: apoptosis protease activating factor-1 (Apaf-1). Apaf-1 combines with cytochrome c released from the mitochondrion causing conformational changes that allow it to process caspase 9. The combination of cytochrome c, Apaf-1 and caspase 9 in a multimeric active complex is termed apoptosome which directly activates effector caspases [58]. The domains DD, CARD and DED are structurally similar, suggesting a common evolutionary origin, and are associated to proteins with a role in cell death. Although such domains have not yet been identified in plants, structural similarities have been observed between the adaptor Apaf-1 and a number of Resistance (R) genes. The cytochrome c release may also be important in plant cell death. It has been shown that maize cells are induced to die by the addition of D-mannose, through the release of cytochrome c to the cytoplasm, possibly by the opening of a transient pore [59].

The mitochondrion is a key compartment of the cell where the adaptors and regulators of PCD are centered to integrate signals and trigger execution pathways. Pro-apoptotic (Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xl) members of the Bcl-2 family, important regulators of apoptosis, are present in the mitochondrial membrane. Bax can create a channel in the outer mitochondrial membrane, thus releasing cytochrome c and other caspase-activating molecules into the cytosol. Bcl-2 and Bcl-Xl inhibit this process through dimerization with Bak or Bax [60]. This process imbricates with the activation of death receptors by the cleavage of another protein of the BH3-domain- only subset of the Bcl-2 family, Bid to t-Bid, that induces the oligomerization of Bak and Bax [61]. Evidence for a similar complex regulatory control of cell death in plants has yet to appear. Nevertheless, a homologue of Bcl-2, located at the mitochondria, chloroplasts and nuclei, has been detected in tobacco leaf cells using antibodies [62]. The stable transformation of plants for the expression of the human and nematode anti-apoptotic proteins Bcl-2, Bcl-Xl and CED-9, suppressed cell death triggered by several fungal phytopathogens [63]. The expression of Bax in tobacco induced HR-like cell death [64]. Homologs of human Bax inhibitor -1of *Arabidopsis* (AtBI-1) and rice OsBH-1, can suppress Bax-induced cell death in yeast [65].

The plant mitochondrion also has unique components that alter mitochondrial function in comparison with animal cells and could hence, alter the specific mechanisms by which it could take part in PCD. One of these component is mitochondria alternative oxidase (AOX) that functions as a part of an alternative electron pathway. This enzyme has been identified in *Arabidopsis* as an early induced gene in HR [64]. An overexpression of AOX in transgenic tobacco plants carrying the R gene results in reduced HR lesions upon virus infection [66]. Furthermore, the treatment of tobacco cells with inhibitors of the cytochrome c pathway (Cys and antimycin A) is accompanied by a strong induction of the AOX capacity and the prevention of cell death [67]. The antisense suppression of AOX resulted in a hypersensitivity to antimycin A and a rapid cell death under this treatment, concomitant with the marked production of Reactive Oxygen Species (ROS) in the mitochondria [68]. These data indicate the role of AOX as a safety valve for the control of the ROS generated from the mitochondria, and are consistent with its activation during HR. Among the growing number of cellular proteins that have been shown to regulate caspase activation and activity are the IAPs (Inhibitors of apoptosis proteins). These proteins have been reported to block both death receptors and mitochondrially mediated apoptotic pathways by directly inhibiting the initiator and effector caspases [69, 70]. Smac/DIABLO, a mitochondrial protein released into the cytosol in response to apoptotic stimuli, was recently found to promote caspase activation by eliminating IAP function [71, 72].

Receptors of death signals

The initial perception of stimuli leading to apoptosis in animals is through a number of death receptors belonging to the Tumour Necrosis Factor (TNF) receptor superfamily or to the Fas receptors initially named APO-1 or CD95. They can activate caspases within seconds of ligand binding followed by a rapid activation of a cascade of events involving a large number of regulator and adaptor molecules. TNF death receptor superfamily mediates apoptosis in tumor cells and is activated by binding to the TRAIL (TNF-related-apoptosis-inducing ligand) ligand. The TNF receptor contains a DD that binds to the TNF-associated death domain (TRADD) activating a transduction cascade [28]. Fas receptors are localized in the cell membrane and are activated by binding to a Fas ligand (Fas-L) from outside the cell, conforming a death-signal reception and transduction system [73]. Fas receptors are found in epithelial tissues, tumors and haemopoietic tissues. This mechanism is important in controlling the immune response in animals. Signalling from these receptors may also lead to cell survival as opposed to cell death.

Plants also have receptors (encoded by R genes) that are specifically involved in response to pathogens. They are activated through specific interactions with avirulence proteins generated only by certain types of pathogens. The R genes isolated encode proteins that fall into several classes. R genes often encode domains including nucleotide binding sites (NBS), leucine-rich repeats (LRR), transmembrane domains (TM), and serine threonine protein kinases (PK) to produce proteins with different combinations that can be classified into various groups. The majority of these proteins have the NBS-LRR structure and are believed to be functionally confined to disease resistance. This

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class of R proteins may be further subdivided to the presence or absence of an N-terminal Toll/Il-1 Receptor (TIR) domain. The NBS domain shows homology to regions found in the pro-apoptotic regulator Apaf-1 [74]. Apaf-1 and these proteins also share a similar structural organization. Thus, the common nucleotide binding (NBS) domain shared by these proteins links an effector domain (CARD in Apaf-1 and TIR in these proteins) to a C-terminal domain likely to be involved in protein-protein interactions (WD domains in Apaf-1 and Leucine-rich repeats (LRR) domain in R proteins), and both are involved in cell death. Nod-1 is another protein reported in humans that like Apaf-1 activates caspase 9, and has a terminal CARD domain followed by an NBS domain and a c-terminal LRR domain, further increasing the similarity between R proteins and apoptosis adaptor proteins [75].

Signalling and signal transduction

In plants as in animals, signalling and signal transduction following the activation of a receptor involve a number of parallel and interacting pathways. Complex signalling networks are activated, involving changes in protein phosphorylation, the generation of ROS, lipid-mediated signalling and a modification in ion fluxes including an influx of Ca^{2+}. Over the past few years, different signalling requirements that are operated under equally diverse transduction pathways have been shown to be involved in HR-linked cell death control in plants initially activated by the avirulence (*avr*) - *R* gene product interaction [76].

Mitogen-activated protein kinases

A change in protein phosphorylation is one of the early stages in PCD. Similarly, it plays important roles in signalling the execution, regulation and spread of cell death. Mitogen-activated protein kinase (MAPKs) cascades have become one of the most widely studied pathways of phosphorylation signalling related to PCD. A direct evidence for the role of protein kinases in the HR-linked death in plants arise from a conditional gain-of-function study of NtMEK2, the upstream Mitogen-activated protein kinases kinase (MAPKK) of salicylic acid (SA)-induced protein kinase (SIPK) and wounding-induced protein kinase (WIPK). Expression of NtMEK2DD, a constitutively active mutant of NtMEK2, in a transient transformation analysis induces HR-like cell death in tobacco, which is preceded by the activation of endogenous SIPK and WIPK [77]. It has also been demonstrated that two Arabidopsis MAPKKs, AtMEK4 and AtMEK5, are functionally interchangeable with tobacco NtMEK2 in activating the downstream MAPKs. In transient transformation experiments performed in tobacco, the active forms of AtMEK4 and AtMEK5 activate endogenous tobacco SIPK and WIPK. These two MAPKKs, as well as tobacco NtMEK2 also activate two endogenous MAPKs in permanently transformed Arabidopsis, which is followed by the HR-like cell death of the *Arabidopsis* plants with the additional generation of H_2O_2 [78].

The activity of protein kinases is simultaneously regulated by cofactors and second messengers such as calcium. A MAPK phosphatase gene (NtMKP1), ortholog of Arabidopsis MKP1, was isolated as a candidate gene for a calmodulin (CaM)-binding protein from tobacco. The bacterially expressed NtMKP1 protein physically interacted with three plant-specific types of CaM in an overlay assay with labeled CaMs. In transgenic tobacco plants over-expressing NtMKP1, the wound-induced activation of SIPK, salicylic acid-induced MAPK and WIPK was inhibited. These results suggest that plant CaMs are involved in these stress-activated MAPK cascades via NtMKP1 [79].

In animals, the participation of MAPKs in apoptosis is controversial. Stimulation of the p38-MAPK cascade by oxidative stress leads to the consequent phosphorylation of heat shock protein (HSP27) and prevents cell death in perfused amphibian heart [80] while TNF-induced p38 MAPK-mediated phosphorylation of Bcl-x(L) in endothelial cells leads to degradation of the Bcl-x(L) in proteasomes and the subsequent induction of apoptosis [81]. The activation of other MAPK (p42/ p44 and pI3K) cascades are implicated in the Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) induced rescue from apoptosis in neutrophils [82]. p42/ p44 MAPK is linked to the inhibition of caspase 3 and to the phosphorylation of Bad, freeing in this way the Bcl-xL, leading to an anti-apoptotic milieu [83]. It has also been observed that MAPK or Extracellular Signal-Regulated Kinase (ERK) signalling suppresses CD95-mediated apoptosis in T-Jurkat and T-cells by preventing the activation of Bid and caspase-8 [84].

Lipid-based signalling

Sphingolipids are essential components of eukaryotic membranes that not only serve as modulators of extracellular interactions, as cell surface receptors, but also have critical functions as intracellular signalling messengers. The sphingolipid signalling pathway generates three metabolites known to function in intracellular signalling; ceramide, sphingosine, and sphingosine-1-phosphate. These metabolites play important roles in cell growth and differentiation not only in animals but also in plants [85, 86].

A ceramide kinase (CERK) from humans was identified and shown to act on a number of ceramide substrates with a much greater activity than the related sphingosine substrate [87]. The Arabidopsis CERK mutant, called *acd*5, accumulates CERK substrates and shows apoptosis-like PCD and enhanced disease symptoms upon pathogen attack [86]. Besides, the phosphorylation of ceramides by the *acd*5 CERK directly dampens the pro-apoptotic effects of unphosphorylated ceramides. A similar scenario likely exists in animals as well [87].

Experiments involving the influence of sphinganine analogue mycotoxins (SAM) derived from plant pathogenic fungi such as AAL from *Alternaria alternata* fsp and fumonisins from *Fusarium moniliforme*, have contributed to elucidate sphingolipid signalling in animals and plants. The most abundant and best studied SAM: Tannic acid (TA) and FB1m are functionally potent inhibitors of ceramide synthase in plants resulting in the activation of PCD [88]. Characteristics observed in SAM-induced PCD in plants include TUNEL positive

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cells, DNA ladders, Ca^{2+}-activated DNA cleavage, the deposition of phenolic compounds and callose, the production of Reactive Oxygen Intermediates (ROIs), the expression of Protein Resistance (*PR*) genes, the accumulation of free long-chain bases (LCBs) and the formation of apoptotic- like bodies [89]. Disease response to SAMs in tomato plants is mediated by a codominant locus Asc, an apoptosis-associated speck-like protein containing a CARD (Caspase Recruitment Domain) [74]. Recently, it was discovered that the single-copy gene *Asc-1* present at the Asc locus mediates SAM insensitivity in tomato. AAL toxin blocked sphingolipid biosynthesis in *asc/asc* leaf discs leading to PCD, and the presence of Asc-1 is able to release the AAL toxin avoiding death [90]. The *Asc-1* gene is homologous to the yeast longevity assurance gene LAG1 and has no homology to known plant resistance genes. Sensitivity to SAMs is associated with a dysfunctional *Asc-1* gene, and insensitivity presumably functions as a salvage pathway for ceramide-depleted plant cells [91]. The genus *Nicotiana* phenotypically and genotypically behaves like the genus *Lycopersicon* with respect to SAM insensitivity [92].

Reactive oxygen species

The involvement of ROS and nitric oxide (NO) in HR-triggered PCD is well characterized in plants [93, 94]. Within and between-cellular generation of ROS (O_2^- and H_2O_2) results in oxidative burst trigger HR reactions [76]. Signalling responses of ROS include the activation of mitogen MAPK and the up- and down gene regulation related to HR [95]. Oxidative stress-activated MAP triple-kinase 1 (OMTK1) is a more specific MAPK kinase kinase that can be activated only by H_2O_2 and not by abiotic stresses or hormones in alfalfa [96]. OMTK1 can specifically activate the downstream MAP kinase MMK3, which results in cell death. MMK3 can also be activated by ethylene and elicitors, thus serving as a convergence point of the cell death network [96]. Recent experiments show that Acid Tolerance Response 1 (Atr1) mutants tolerant to AAL toxin are also resistant to H_2O_2-induced death, suggesting the involvement of ROS with sphingolipid metabolism in the regulation of cell death [97].

In animals, NO cooperates with ROS to kill tumor cells possibly through unregulated NO levels, driving a diffusion/limited reaction with O_2^- to generate peroxinitrite ($ONOO^-$), a mediator of cell injuries in many biological systems [5]. Delledonne *et al* clearly demonstrated that NO and ROS produced during a pathogen-induced HR cooperate to activate cell death in plants. They proposed that the reaction of NO with H_2O_2 is required to elicit cell death, whereas peroxynitrite is not an essential signal for cell death in soybean cells [93]. In contrast, urate, a natural scavenger of $ONOO^-$ has been shown to compromise cell death induced by the avirulent *Pseudomonas syringae* pv. *phaseolicola* in *Arabidopsis* leaves [98], suggesting the involvement of $ONOO^-$ in PCD. Moreover, in oat plants infected with crown rust fungus, the data indicate that NO and O_2^- may not trigger hypersensitive cell death but, rather, play an important role in the destruction of the infected cells [99]. Taken together these data suggest that the role of ROS and NO in inducing hypersensitive response may differ in different plant-pathogen interactions.

NO has been suggested to have a role in cell-cell signalling and the spreading of cell death during the course of infection [100, 101]. NO can exert pro-apoptotic and anti-apoptotic functions depending on its concentration, for example, low equimolar concentrations of NO and O_2^-, or a $ONOO^-$ generating agent SIN-1, inactivate alcohol dehydrogenase (ADH) which is a target for cellular oxidants. Interestingly, an excess level of NO abolishes the significant oxidations induced by SIN-1 or the co-treatment with NO and O_2^-, suggesting that NO acts as an antioxidant interacting with $ONOO^-$ to form nitrogen dioxide [102]. In plant-pathogen interactions, the rate of NO or ROS generation may be different even among adjacent cells, and the difference leads to the establishment of distinct signal networks in individual cells. Alteration of the NO/ROS ratios may induce a variant set of defense responses [100].

Ion fluxes

Ion channels play an important role in regulating HR-induced PCD [76]. Ca^{2+} influx is one of the hallmarks of apoptosis in animals and plants. It is crucial in the regulation of the release of cytochrome c from the mitochondria and DNA degradation in animals. The over-expression of the two- pore channel 1 (OsTPC1), a putative voltage-gated Ca^{2+}-permeable channel in rice cells, provoked enhanced sensitivity to a proteinaceous fungal elicitor to induce an oxidative burst, the activation of MAPK, OsMPK2, as well as a hypersensitive response [103]. Apoptotic cell shrinkage has been demonstrated to correlate also with an increased efflux of K^+ and Cl^- and the activation of K^+ channels [104]. K^+ channels play a key role in regulating cell death, being involved with cytochrome c released from the mitochondria, the activation of caspases and DNA fragmentation in animals [105].

Anion channels also participate in PCD signalling by initiating or amplifying plasma membrane depolarization, which in turn may activate Ca^{2+} voltage-dependent channels or K^+ channels. Mitochondria voltage-dependent-gated anion channels, is involved in the release of cytochrome c during apoptosis in mammals by the formation of a transient pore (TP) associated with the adenine nucleotide transporter (ANT). The opening of TP may be induced directly by high levels of calcium inside the mitochondria or indirectly by ROS at reduced ATP levels [30]. This mechanism is also presumed to operate in the early stages of HR in plants [64]. Cryptogein, an elicitin from *Phytophtora* sp, induces a fast efflux of NO_3^- sensitive to anion channel blockers and regulated by phosphorylation and Ca^{2+} influx, during the signalling pathway leading to cell death [94].

Applications of PCD in disease resistance

With an increase in the understanding of PCD mechanisms, genetic based and signal molecule gene-specific therapies have become a strong alternative for combating diseases in animals and plants. Degenerative diseases such as Alzheimer's, Parkinson's and AIDS as well as proliferative diseases such as cancer that involve apoptosis in one way or another, are recent targets for the selective manipulation of PCD [106]. Plants may

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also be modified for resistance to a wide range of pathogens, considering the fact that there are no specialized cells as in mammals, dedicated exclusively to defense regulation. Therefore, the modulation of PCD to alter the development and progression of diseases in plants must be site-specific at the location of the infection [5].

The modulation of apoptosis for disease control in plants can be achieved by either the inhibition or induction of PCD. For compatible obligate pathogens (necrotrophic) the inhibition of apoptosis may result in a broad-spectrum disease resistance. For example, the expression of the anti-apoptotic gene p35 from baculovirus in transgenic tomato plants provided resistance to *Alternaria alternata*, *Colletotrichum coccode* and *Phytophtora syringae* pv. tomato [51]. Transgenic tobacco plants expressing animal genes: human Bcl-2 and Bcl-xl, nematode CED-9, or baculovirus Op-IAP, negative regulators of apoptosis, exhibited heritable resistance to several necrotrophic fungal pathogens [63].

For incompatible-obligate pathogens (biotrophic), disease resistance can be achieved through the induction of HR-linked cell death. Genes with distinct roles in the induction of apoptosis during HR have been identified and characterized. For example, an *Arabidopsis thaliana* gene, AtMYB30, has been identified as a positive regulator of HR-linked cell death following incompatible interactions in response to bacterial pathogens [107]. Another strategy to control biotrophic pathogens is to introduce avr/R gene pairs as two-component sensor systems which could be introduced into crop plants with the avr gene under the control of a pathogen-inducible promoter so that infection by any pathogen will trigger an HR, or to fuse such a promoter to gene coding for a non-specific death elicitor [108]. Another possibility is to use such gene pairs to induce low levels of cell death that trigger Systemic Acquired Resistance (SAR) [109]. Furthermore, the manipulation of signal transduction pathways that lead to the HR such as those related to second messengers, such as calcium, is an attractive strategy that has been used. The expression of three types of tobacco calmodulins lead to the activation of HR in response to wound and infection with TMV [110].

Concluding remarks

Many features of plant PCD resemble those seen in animals, and similar mechanisms have been observed during the recognition of the pathogen and up to the killing process of the cell. Resistance genes encoding specific receptors of pathogens have similar domains that are conserved in animals, signalling/signal transduction in each involve changes in protein phosphorylation, lipid metabolism, ion fluxes, the participation of similar second messengers and regulators and the production of ROS. Similar processing of the DNA and the rupture of the nuclei are shared by both types of cell death processes. Similarities have also been observed between plant DNA sequences and apoptosis-related genes. Moreover, cysteine proteases with a certain degree of conservation in their tertiary structure as well as in substrates and inhibitors from animals to plants appear to be involved.

Although an apoptotic morphotype has been described for plant cell death in association with the hypersensitive response, there is a general view that typical HR morphology is oncotic-like. These opinions are based on the observations of striking dissimilarities in the execution of processes of HR-linked cell death. Dying cells have been seen to leak, the organelles swell and the corpse is unprocessed and left to be invaded by the surrounding cells. However, there is clear evidence that the death pathway is programmed; thereby other denominations such as "programmed oncosis" have been proposed for this process [29].

In conclusion, despite significant recent development discoveries in this field, there is still a lack of information on several genes such as caspases and Bcl family-like genes that have been found in plants. The many intriguing similarities with PCD in animals will need to be rigorously tested to demonstrate that they are conserved, and are derived from a common ancestral origin. Whether PCD in plants, such as HR- related death, should be called apoptosis depends on whether the participation of caspases and the execution of phagocytosis are important to the definition of this form of cell death. However, besides the comparisons, the applications of PCD to achieve resistance to pathogens in plants should be taken into account more seriously in the future as an effective strategy within the biotechnological improvement programs.

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