A Biotechnological Approach to Apoptosis in Living Organisms: An Overview

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ABSTRACT

Background: Living organisms use apoptosis for resistance and progressive mechanisms. A noteworthy commonality has been advocated between the hallmarks of apoptosis in plants and animals, both at cellular and molecular level though very few governing proteins or protein domains have been identified as conserved across all eukaryotic apoptotic forms, biochemical and biotechnological topographies viz., chromatin condensation, nuclear DNA fragmentation and contribution of caspase like proteases in plant apoptosis look like across the board and in consonance with their process in metazoa as well. Transgenic expression of mammalian anti- and pro-apoptotic proteins in plants has been shown to influence the regulatory pathways of cell death processing, indicative of the existence of functional counterparts of such genes in plants, several of which have now been cloned and characterized extensively, suggesting that despite variances, there may be a rational level of functional resemblance between the mechanistic components of apoptosis of biosystems. Results: An overview of the existing data concerning with mechanism of apoptosis in plants is at best inclined to support an inherited association with animal apoptosis rather than any common governing approaches. Conclusion: This overview provides a brief insight into some of the relative features of Programmed Cell Death (PCD) in plants and animals.

Key words: Apoptosis, caspases, conserved domain, programmed cell death

INTRODUCTION

Programmed cell death (PCD) pronounces a biochemical and pathological process of cell removal that theatre's a significant role in upholding tissue homeostasis. It is an extremely controlled cellular suicidal process indispensible for growth and existence in all eukaryotes. The foundation of the phenomenon appears to be as old as the very first cell, as cellular homeostasis and averting self-destruction would have been incredible to accomplish without such machinery. Therefore, this apparatus appears to have existed in all cells from the very origin. It has, indeed, been recognized in several prokaryotes and unicellular eukaryotes and related to numerous phenomena. Only later, during evolution of multicellular organisms, PCD is believed to have ‘fine tuned’ for purposes such as the social control of cell members. PCD is an integral part of embryogenesis. In plant embryos, PCD functions during terminal differentiation and elimination of the temporary organ, suspensor, as well as during establishment of provascular system. Embryo abortion is another example of embryonic PCD activated at pathological situations and in poly-embryonic seeds. The studies identified the sequence of cytological events leading to cellular self-destruction in plant embryos. As in most if not all the developmental cell deaths in plants, embryonic PCD is hallmark by autophagic degradation of the cytoplasm and nuclear disassembly that includes breakdown of the nuclear envelope and DNA fragmentation. The optimized setup of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) allows the routine in situ analysis of nuclear DNA fragmentation in plant embryos. This chapter provides step-by-step procedure of how to process embryos for TUNEL, and how to combine TUNEL with immunolocalization of the protein of interest. Multicellular organisms use the physiological mechanisms of cell death to regulate developmental morphogenesis and remove infected, mutated or damaged cells from healthy tissues. This phenomenon is characterized in detail, especially in animal apoptosis systems, by a stereotypical set of morphological and biochemical changes such as condensation or shrinkage of the cell, reorganization of the nucleus, membrane blebbing, formation of apoptotic bodies and chromatin condensation. This process finally results in activation...
of certain endonucleases, leading to the fragmentation of chromatin in multiples of 180 bp nucleosomal units, a process known as DNA laddering. Most, but not all, of the above apoptotic features are commonly observed during PCD in a wide range of biological systems.

In plants, PCD occurs during development, such as during xylegenesis, embryogenesis, parenchyma formation, several plant reproductive processes, seed development and leaf senescence. In addition, PCD is well documented in relation to manifestation of Hypersensitive Response (HR) caused by the interaction between the host plant and an incompatible pathogen. This Hypersensitive Response (HR) is thought to directly kill invaders and/or to interfere with their acquisition of nutrients. In contrast to animal system, signaling pathways and molecular mechanism of PCD are largely unknown in plants. Although a number of morphological and biochemical changes such as cell shrinkage, blebbing of the plasma membrane, condensation and fragmentation of the nucleus and inter-nucleosomal cleavage of DNA which are commonly observed during animal apoptosis, appear to be conserved in plant cells undergoing PCD, very little is known about the execution process that leads to cell death in plants.

This overview provides a brief insight into some of the relative features of PCD in plants and animals. Furthermore, this article endeavors to critically review some of the atypical and specific topographies and consistencies of apoptosis in living organisms.

**Fundamental hallmarks of PCD:** The major features of PCD have been intensively studied in animals. PCD in animal systems is described to result in the disassembly of cells including the condensation, contraction and disintegration of cytoplasm and nuclei into several sealed packets (often known as apoptotic bodies) which are then phagocytosed, by the neighboring cells or the macrophages. Therefore, there are no scraps of the cell corpses left. Nuclear disintegration is headed by chromatin condensation and sidelined in the nucleus. Disintegration of DNA at the nucleosome linker sites then takes place and the fragmented oligonucleosomal bits are reported to be 180 bp. Fragmentation is effected by endonucleases such as NUC 1, DNase I and DNase II which are present in the nucleus and are activated by Ca²⁺ and Mg²⁺ but inhibited by Zn²⁺ and by several caspase activated nucleases such as CAD (Caspase activated DNase) or DFF-40 (DNA fragmentation factor 40 Kda). The DNA fragments can be cytotoxicly determined by Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) of DNA at 3-OH group. When all these events are pooled to consequent in a distinct morphological as well as biochemical/molecular expression then PCD is designated as apoptosis. In other words, apoptosis is a distinct form of PCD. However, there are others who consider apoptosis and PCD as one and the same.

In the last few years and due to new concern in a possible apoptosis like phenomenon existing in plants, morphological and biochemical fluctuations have been explored during plant PCD. Only certain number of the hallmarks is akin to those described in animal apoptosis. Condensation and contraction of the cytoplasm and nucleus have been pronounced in carrot cell culture, after cell death persuaded by heat shock. The DNA processing reported earlier for the animal PCD is believed to exist in the dying cells of plant as well. In plants, DNA ladders have been reported during development characterized by death of monocot aleurone layer and endosperm, senescence of petal, carpel tissue and leaves during anther development as well as during death induced by different stresses such as: Cold, nutrient deprivation, salt or D-mannose stresses, UV radiation, pathogens or a pathogen toxin. In aleuronic cells of grass species such as barley, in dying root cap cells and in tobacco cells subjected to HR, nuclear condensation and contraction as well as oligonucleosome sized DNA wrecks have been documented through the presence of 3-OH group detected by TUNEL experiments. The foremost problem concerning with nuclear alterations in plant PCD is that there is no uniformity vis-à-vis the size of DNA fragments during DNA fragmentation: Fragments of more or less 50 kb in some cases and as small as 0.14 kb in others. It is believed that the activation of some endonucleases leads to 50 kb DNA fragments followed by a different set of endonucleases causing the fabrication of oligonucleosomal length of DNA fragments. The first type of cleavage is thought to be the consequence of the discharge of chromatin loops and is perceived in almost all cases of apoptosis and the following nucleosomal laddering occurs less often and is considered to be not vital for apoptosis.

The overall mechanisms of enzymes convoluted in nuclear dismantling in plants are still obscure. Several DNase activities and nuclease genes have been documented to be upregulated in diverse models of plant PCD. However, confirmation on their involvement in the cell death has been reported only in some of them. Recent studies described the stimulation of the activity of a 28 kDa endonuclease (p28) activity in tobacco treated oat leaves and this preceded the DNA laddering and chromatin condensation. The p28 activity also significantly augmented in parallel with the rate of DNA fragmentation and cell death. Besides p28, an inducible nuclease, p24 (24 kDa) and four constitutive nucleases,
p22 (22 kDa), p31 (31 kDa), p33 (33 kDa) and p55 (35 kDa), have been detected in oat cell lysates using an in-gel assay for nuclease activity22,23. An Mg²⁺ dependent nuclease activity has been identified in the intermembrane space of mitochondria responsible for the generation of 30 kb DNA fragments in Arabidopsis24. ZEN1, a Zn²⁺ dependent endonuclease, has been identified in the degradation of the nuclear DNA in Zinnia tracheary elements25. ZEN1 is confined to vacuoles which collapse before DNA is degraded26. However, ZEN1 activity does not produce the characteristic DNA laddering shown by the nuclease executing DNA fragmentation in apoptotic animal cells22,23,26. Based on the biochemical differences of ZEN1 and the nuclease involved in apoptosis27, it has been proposed that plants and animals have evolved independent systems of nuclear DNA degradation during cell death. In contrast with tracheary elements, the tissues undergoing PCD in cereal grains show the characteristic DNA laddering indicative of internucleosomal fragmentation of DNA26,27 which is a hallmark of apoptosis in animal cells28. Recently, a Ca²⁺/Mg²⁺ endonuclease localized in the nucleus wheat aleurone cells undergoing PCD has been identified which is detected prior to DNA laddering27. A cell-free system used to analyse nucleus degeneration in nucellar cells in wheat grains29, shows that a different wheat tissue, the nucellus which undergoes PCD at early stage of grain development30, presents a nucleus localized nuclease with identical cation requirements, but with a different electrophoretic mobility than the aleurone nuclease. These results suggest that, as in animal apoptosis4, there is more than one nuclease involved in plant PCD. Nuclear extracts from such cells have been shown to be capable of triggering DNA fragmentation in both plant and human nuclei, demonstrating that similar features of nucleus degradation could be shared between plant and animal cells.

Apoptotic bodies have not been shown to form during plant cell death. These bodies may be absent in plant PCD because they are functionally irrelevant due to the absence of possible phagocytosis by adjacent cells in the presence of cell wall. Instead, the plant pathway might involve autolysis. Although, cells that die as part of the HR typically exhibit features of an oncotic cell death which is characterized by the retention of a dead protoplast containing swollen organelles31, many other plant cell suicide programs include cellular disassembly via autophagy and/or autolysis. The degree of processing of dead and dying cells ranges from that apparently limited to nucleus or nuclear DNA to complete autolysis that includes the extracellular matrix. Degradation of nucleus and nuclear DNA has been evaluated in several recent investigations of plant PCD. The results are consistent with earlier work in a variety of systems. They include reports nuclear blebbing and fragmentation32,33 and the detection of oligosomal DNA ladders34,35,36. and labelled fragmented DNA in nucleus34,35,36,37,38. Autophagy has been observed as engulfment and degradation of nucleus and other organelles by vacuoles, vacuoles and other autophagic organelles derived from leucoplast67. Autolysis does not require engulfment and contributes to the degradation of organelles and soluble cellular components. Unlike autophagy, autolysis can continue after cell death, as occurs during tracheary element differentiation. In most cases, autolysis and autophagic mechanisms cooperate to yield cellular disassembly, such as that occurring during embryo suspensor death39. So if using a strict morphological definition, the term apoptosis-like phenomenon in plants should be used instead of apoptosis since some of the terminal hallmarks of apoptosis are absent.

**Basic executors of PCD:** The real effector molecules of animal PCD are the cysteine aspartate specific proteases (caspases) and granzenzymes. The former are the conserved cysteine proteases, while the latter are serine proteases, both specifically cleave after the aspartate residues of many proteins. The studies in Caenorhabditis elegans identified two genes ced-3 and ced-4 required for apoptosis in the worm, if either gene is inactivated by mutation, the 131 cell deaths that normally happen during the development of the worm (which has only about 1000 cells when mature) fail to occur22. Remarkably, the mutant worms with 131 extra cells have a normal life span, showing that in this organism apoptosis is not essential for either life or normal ageing. By contrast, more complex animals cannot survive without apoptosis: Mutations that inhibit apoptosis in the fruitfly Drosophila melanogaster, for example, are lethal early in development as are mutations in mice that inhibit apoptosis mainly in the developing brain41. The protein encoded by the ced-3 gene was found to be very similar to a human protein called interleukin-1-converting enzyme (ICE)42. ICE is an intracellular protein cleaving enzyme (a protease) that cuts out interleukin-1, a signalling protein that induces inflammation, from a larger precursor protein42. The similarity between the CED-3 and ICE proteins was the first indication that the death programme depends on protein cleavage (proteolysis). Till date 14-15 different caspases that play a role in inflammation (group I caspases) and apoptosis (group 2 caspases) have been identified in animals33. All these are believed to share a fair level of sequence homology and similarity in sequence specificity33,43-45. Until now, the caspase family in animals is composed of 12 different proteases.
classified in 3 phylogenetic groups [Interleukin 1β converting Enzyme (ICE), ICH1 and cysteine protease 32 (CPP32)]. All these caspases have in common a highly conserved catalytic site, a stringent substrate specificity to cleave after an aspartic acid residue and requirement for at least 4 amino acids N terminal to cleavage site\(^\text{[8]}\). It is possible to classify these caspases on the basis of their affinity for different substrates including two tetrapeptides in particular: DEVD (ICH1 and CPP32) and YVAD (ICE caspases). Corresponding caspase activity can be blocked by specific substrate coupled with aldehyde (CHO) Reversible inhibitor or methyl ketone radical (Chloromethylketone (CMK), Fluoromethylketone (FMK): Irreversible inhibitor).

Caspases are made as large, inactive precursor (procaspase) which is itself activated by cleavage at aspartic acids, usually by another caspase\(^\text{[8]}\). In apoptosis, caspases are thought to be activated in an amplifying proteolytic cascade, cleaving one another in sequence. Once activated, the effector caspases ultimately cleave numerous substrates, thereby causing the typical morphological features of apoptosis\(^\text{[8,9]}\). They cleave proteins supporting the nuclear membrane (lamins) for example, thereby helping to dismantle the nucleus; they cleave protein constituents of the cell skeleton and other proteins involved in the attachment of the cell to their neighbors, thereby helping the dying cell to detach and round up making it easy to ingest; they cleave a protein inhibitor of caspase activated DNase (ICAD of CAD-ICAD complex) that normally holds the CAD- a DNA degrading enzyme in an inactive form, freeing the DNase to cut up the DNA in the cell nucleus\(^\text{[10,11]}\). The other important substrates include PARP [poly (ADP-ribose) polymerase], DNA dependent protein kinase (DNA PK), Serum response element binding protein (SRE/BP), ps1(CDK91A)-activated kinase 2 (PAK2), 70 kDa components of U1Sn-RNP, procaspases and so on. PARP is among the first target proteins shown to be specifically cleaved by caspases to a signature of 89 KDa apoptotic fragments during cell death\(^\text{[12]}\). It is believed to be involved in the repair of the DNA strand breaks and in cell recovery from DNA damage\(^\text{[13]}\).

Cell death in plants exhibits morphological features comparable to caspase mediated apoptosis in animals, suggesting that plant cell death is executed by (caspase like) proteases. The recent characterization of cell death associated plant proteases with aspartate specific cleavage activity demonstrates the involvement in plant PCD of proteolytic activities functionally resembling animal caspases. The result of a study carried out showed induction of YVADase activity whereas no DEVDase activity was detected\(^\text{[14,15]}\). Surprisingly, both inhibitor peptides (DEVD and YVAD) were efficient in blocking the HR and YVADase activity. Encouragingly, none of the classical protease activity could suppress the hypersensitive response or YVADase activity. This is cited as evidence for the presence of caspase like plant proteases that participate in hypersensitive response cell death. Different results were found during the plant response to UV-C radiation where both caspase inhibitors could prevent DNA digestion detected by TUNEL reaction and where UV-C induced DEVDase activity but no YVADase activity was found. The heterologous expression of Baculovirus p35 protein, a broad range caspase inhibitor that can effectively suppress PCD in animals\(^\text{[16,17]}\), blocked AAL (Artemisia annua) toxin induced cell death in transgenic tomato plants and provided protection against the pathogen Artemisia annua. Additionally, p35-expressing transgenic tomato plants displayed partial inhibition of cell death associated with non-host hypersensitive response cell death upon bacteria and virus challenge\(^\text{[18]}\). Because p35 shows a high degree of specificity towards caspases and it shows a little or no inhibitory activity towards other proteases, these physiological inhibitor studies support an important role for caspase like proteases during cell death in plants\(^\text{[19]}\). In addition, natural caspase substrates such as bovine and plant PARP are cleaved by plant proteases at caspase cleavage sites. Exogenous (bovine) PARP is endoproteolytically cleaved by extracts from fungus infected cowpea (Vigna unguiculata) plants that were developing a HR but not by extracts from noninfected leaves. This cleavage activity inhibited by caspase-3 inhibitor (Acetyl-DEVD-CHO) but not by caspase-1 inhibitor (Acetyl-YVAD-CHO)\(^\text{[20]}\). Interestingly, a polypeptide (GDEVGIDIVE) mimicking the PARP caspase-3 cleavage site (DEVD-G) partially inhibited PARP cleavage, whereas a modified peptide in which the essential Asp was replaced by Ala (GDEVAGIDVE) did not affect PARP cleavage. This cleavage activity was also inhibited by other Cys protease inhibitors (E-64, IA and N-ethylmaleimide). Inhibitors to other types of proteases (Ser-, metallo-, Asp proteases and calpain) were without effect in this system. In these experiments, PARP cleavage eventually yielded four different fragments of \(\text{g}^{21,22}\) and 45 KDa \(\text{g}^{23}\). Cleavage of endogenous (plant) PARP (116 KDa) reacting with a PARP antibody occur during endogenous-induced PCD in tobacco protoplasts and this cleavage of PARP and induction of DNA fragmentation has been shown to be inhibited by caspase-1 (Acetyl-YVAD-CHO) and caspase-3 (Acetyl-DEVD-CHO) inhibitors\(^\text{[24]}\). Also in heat shock induced PCD in tobacco suspension cells, endogenous PARP was cleaved, yielding a 89 KDa fragment\(^\text{[25]}\). This is similar to the cleavage of PARP described in animal apoptotic cells. In both mammals and plants, two different types of PARP exist and both types are presumably involved in DNA repair. The
Arabidopsis PARP-1 shows high homology to human PARP-1 including a conserved caspase-3 recognition site (DSVD-N). In plants, PARP genes have been cloned from *Zea mays* and *A. thaliana* and PARP activity has been identified in few species.

Although, there have been numerous efforts to identify proteinases that exhibit caspase activities, plant caspases have remained unidentified. Recent work, however, has unraveled this mystery. Vacuolar Processing Enzyme (VPE) has been shown to be a protase that exhibits caspase-1 activity and is essential for virus-induced hypersensitive cell death. Direct evidence has been reported for the involvement of VPEs in plant cell death. Hara-Nishimura and coworkers have used a temperature sensitive N-TMV tobacco plant-atrophy system to identify the protases responsible for caspase like activity. The temperature-sensitive N-TMV (tobacco mosaic virus) tobacco plant-pathogen system allows massive cell death to be synchronized. At 30°C, TMV can systemically infect tobacco plants because induction of cell death and defense gene expression is completely suppressed. Upon shifting the temperature to 25°C, cell death appears throughout the infected plant. A biotinylated caspase inhibitor (biotin-XVAD-FMK) was used to identify the proteins completing with this inhibitor. The inhibitor when infiltrated into tobacco leaves before temperature shift, effectively blocked cell death and specifically bound to protein fractions of 40 and 38 kDa. Using antibodies against the intermediate and mature forms of VPE these fractions were recognized as two forms of VPE, indicating that the caspase like activity was performed by VPE. Infiltration of leaves with XVAD-CHO as well as specific VPE inhibitor (ESEN-CHO) abolished lesion formation. In addition, in VPE silenced plants hypersensitive cell death response to TMV was greatly suppressed. These results clearly demonstrate the caspase like activity of VPE and its involvement in TMV induced cell death. Cell death is accompanied by an increase in XVADase activity but not by DEVase activity. This suggests the involvement of VPEs in cell death and shows that VPEs are among the targets of caspase inhibitors in plants. *Arabidopsis* has four VPE genes (αVPE, βVPE, γVPE, δVPE) which are separated into seed type and vegetative type. VPE is an asparaginyl endopeptidase. It cleaves peptide bonds on the C-terminal side of asparagine residues exposed on molecular surface of preprotein to generate the respective mature proteins. However, it also has been shown that VPE cleaves peptide bonds on the C-terminal side of aspartic acid residues. VPE recognizes aspartic acid when it is part of the XVAD sequence of a caspase-1 substrate, but does not necessarily recognize other aspartic acid residues, similar to caspase-1. VPE, like caspases, is a cysteine protease. Although, VPE is not related to the caspase family or the metacaspase family, VPE and caspase-1 share several enzymatic properties. Two residues of the catalytic dyad in VPE (histidine and cysteine) are comparable to His 237 and Cys 285 of the catalytic dyad in human caspase-1. The QACRG pentapeptide of the active site of caspase-1 is similar to the E(A/G)CES pentapeptide of the active site of VPEs. A similar comparison was also done for human caspase-8 and Arabidopsis γVPE. In addition, each of three essential amino acids, Arg179, Arg341 and Ser347 which form the substrate-binding pocket of caspase-1 are conserved in VPEs. This is the case for all of the more than 20 VPEs that are currently in databases. The substrate binding pocket of VPE might be similar to the substrate-binding pocket of caspase-1. Both VPE and caspase-1 are subjected to self-catalytic conversion/activation from their inactive precursors.

With the sequencing of the complete genome of the model plant *Arabidopsis thaliana*, these caspase like activities have ceased an intensive but frustrating search for caspase genes within plants. At the end of 2000, distant caspase relatives were discovered in silico in plants, the metacaspases that contain some of the structural features that are characteristic of the animal caspases. The *Arabidopsis thaliana* genome contains nine metacaspases. The function(s) and substrate specificity of the metacaspases from plants have not yet been investigated. The increased expression of one of the tomato metacaspases during infection with the necrotrophic pathogen *Botrytis cinerea* suggests a possible role for plant metacaspases in cell death. The recent findings by Peter Bozhkov and colleagues also indicate that a plant metacaspase might be involved in cell death. These authors studied proteolytic activity during embryogenesis in Norway spruce (*Picea abies*). Concomitant with massive cell death during shape remodelling, an increase in VEIDase activity (equivalent to activity of human caspase-6) was observed. Treatment with VEIDase inhibitor VEID-fluoromethylketone (VEID-FMK) inhibited cell death and prevented normal embryo development. The authors used a range of other caspase substrates but, apart from IETD-7-amino-4-methylcoumarin, these were cleaved poorly. The VEIDase activity was sensitive to pH, ionic strength and Zn²⁺ comparable to human caspase-6. The substrate specificity of the Norway spruce VEIDase appears similar to that of the yeast metacaspase YCA1, suggesting that the plant VEIDase involved in cell death is a metacaspase. This group also showed that silencing of a metacaspase gene (EMBL database Accession no. AJ534970) reduced VEIDase activity and cell death and inhibited embryonic pattern formation. These findings suggested that plant metacaspases were
among the targets of the human caspase inhibitors and perhaps metacaspases functionally resemble animal caspases. But later in vitro experiments have shown that mcl-2-Pa (type II metacaspase gene) had Arg but not Asp specificity. Because knocking down mcl-2-Pa not only disrupted cell death but also blocked embryonic differentiation, it was speculated that mcl-2-Pa might be primarily involved in cellular differentiation rather than in cell death. Possibly, mcl-2-Pa regulates the actin reorganization observed during cellular differentiation, like mammalian caspases do in the cytoskeletal rearrangements during apoptosis. In Arabidopsis, mere constitutive overexpression or disruption of metacaspase genes does not lead to an obvious phenotype and thus, a role for metacaspases in cell death or other processes has not been identified yet. Redundancy may exist between the various members of this family, or additional factors may be necessary to activate ectopically expressed metacaspases.

Specific proteins accountable for the triggering of the executors of PCD: The measured triggering of caspase precursors (Zymogens) is accomplished by specific adaptor proteins which bind to them through shared motifs. Tumour Necrosis Factor (TNF) receptors superfamily or apoptotic cofactors released by the mitochondria can be stated as examples of adaptors. Caspases-8 is activated when death effector domains (DEDs) in its pro domain bind to the C-terminal DED in adaptor molecule Fas-associated Death Domain (FADD); similarly Caspase-9 is activated after the connotation of Caspase Recruitment Domain (CARD) in its prodomain with the CARD in another cofactor protein, Apoptosis Protease Activating Factor-1 (APAF-1). In the worm C. elegans, Ced-4 acts as the adaptor molecule. Database searches have identified several motifs of similarities between Ced-4, APAF-1 and proteins encoded by resistance genes regulating HR in plants. The conserved domain has been coined as NB-ARC.

Intracellular switches: A pronouncement to die should not be taken casually and so it is not amazing that the death programme is regulated in complex ways, both from inside and outside the cell. A major class of intracellular regulators is the B-cell leukemia/lymphoma 2 (Bcl-2) family of proteins which like caspase family, has been conserved in evolution from Worms to humans. C-terminal (ced) for cell death abnormal protein in C. elegans encodes such a protein: If it is inactivated, most of the cells in the developing worm die and worm, therefore, dies early in development, but if ced-4 is also inactivated so that apoptosis cannot occur, the worm and all of the cells live. Ced-9 prevents caspase activation by binding to adaptor Ced-4 in the worms. So, it seems that the only reason any cells in the developing worm live is that ced-9 normally keeps the death programme suppressed in these cells. Ced-9 gene is similar to the human's bcl-2 gene. Fifteen bcl-2 family members have been identified so far in the mammals. Some such as Bcl-2, Bcl-XL, Bcl-W etc. suppress apoptosis (anti-apoptotic); Others such as BCL2 associated protein (Bax), BCL2 antagonist/killer (Bak), BCL2-related ovarian killer (Bok), BCLXL/BCL2 associated death promoter (Bad) and B3H interacting domain death agonist (Bid) promote it (pro-apoptotic). Some of these proteins can bind to each other: When an apoptosis suppressor forms a complex with an apoptosis promoters, each protein inhibits the others function. The ratio of suppressor to promoters helps determine a cell's susceptibility to apoptosis.

It is now an established fact that mitochondria which are called the powerhouse of the cell not only generate energy for cellular activities but also play an important role in cell death in animals. They release several death promoting factors such as cytochrome C (Cyt-C) (which contribute to caspase activation), Apaf-1, Apoptosis Inducing Factor (AIF), procaspase-3, Ca²⁺ and reactive oxygen species (ROS) in response to various stimuli. Different mechanisms have been suggested to explain the release of apoptogenic factors from mitochondria, induced by proapoptotic proteins. The first involves Bax that could simply oligomerise in Outer Mitochondrial Membrane (OMM) to form a channel. Alternatively, Bax, in association with either the Voltage-dependent Anion Channel (VDAC) or truncated Bid, could promote the formation of pores allowing the passage of soluble proteins. Alternative models have been suggested in which, during early stages of apoptosis, the Inner Mitochondrial Membrane (IMM) plays a key role. The first one implies that water and solutes enter the mitochondrial matrix, inducing swelling of mitochondria. This process is mediated by either VDAC or the opening of a permeability transition pore (PTP). The PTP may be defined as a voltage-dependent, cyclosporin A (CsA)-sensitive, high conductance inner membrane channel. The molecular structure of PTP is still unknown, although evidence suggests that it may be formed of several components, including matrix cyclophilin D, the outer membrane VDAC, the inner membrane Adenine Nucleotide Translocase (ANT), peripheral benzodiazepine receptor and Bcl-2, hexokinase bound to VDAC and innermembrane creatine kinase evidence indicates that the mitochondria-associated hexokinase plays an important role in the control of apoptosis in mammals. Hexokinase is an integral component of the PTP through its interaction with porin or the
voltage-dependent anion channel (VDAC)\(^{145}\) and hexokinase binding to the VDAC interacts with the opening of the PTP, thereby inhibiting cytochrome c release and apoptosis\(^{144,145}\). Thus, detachment of hexokinase from the mitochondria potentiates and its overexpression inhibits mitochondrial dysfunction and cell death induced by various stimuli\(^{145,147,148}\). Recent studies have shown that cyclophilin D, another component of the PTP, is a key factor in the regulation of PTP function and that cyclophilin D-dependent mitochondrial permeability transitions are required to mediate some forms of necrotic cell death but not apoptotic cell death\(^{149,150}\). However, these observations do not exclude the possibility that certain forms of apoptosis are mediated by the mitochondrial permeability transitions as some forms of apoptosis are meaningfully inhibited by cyclosporin A, a particular inhibitor of cyclophilin activity\(^{151}\). Furthermore, cyclophilin D-overexpressing mice revealed an elevation in apoptotic heart muscle cells\(^{149,150}\). Furthermore, in cancer cells, cyclophilin D overexpression suppresses apoptosis via the stabilization of hexokinase II binding to the mitochondria\(^{152}\). The pore open-closed transitions are highly regulated by multiple effectors at discrete sites. Factors affecting PTP can be subdivided into matrix and membrane effectors. The former include both openers (Ca\(^{2+}\), phosphate, oxidizing agents, −OH and arachidonic acid) and inhibitors (Cyclosporin A(CsA)), Adenosine diphosphate (ADP), bongkrekate and reducing agents). Among the latter, a high (inside-negative) membrane potential tends to stabilize the PTP in a closed conformation, whereas depolarisation by different uncouplers determines its aperture. PTP is also regulated by quinones which prevent Ca\(^{2+}\)-dependent pore opening. In addition, the Pore is regulated by Bcl-2 proteins and intracellular ATP levels\(^{151,153,154}\). The Bcl-2 proteins are membrane spanning and have at least one of the four Bcl-2 Homology (BH) domains. It is shown that the proapoptotic Bax interacts with VDAC and ANT and brings about a conformational alteration to form a megachannel leading to the release of Cyt-C\(^{155,156}\). The pore conductance of VDAC has been shown to increase in the presence of Bax in artificial membranes and this increase is blocked by Bcl-XL\(^{157}\). Bax and Bim interact with VDAC and lead to the release of Cyt-C, whereas, Bcl-XL blocks this release\(^{158,159}\). The permeabilization of the IMM to solutes with molecular mass up to 1.5 kDa, initiated by the aperture of the PTP outcomes towards the complete interdependence of mitochondrial electrical potential. As a result, the high concentration of solutes present in the matrix induces an osmotic swelling which could eventually lead to OMM rupture and the resultant release of proteins from the intermembrane mitochondrial space (IMS)\(^{150,152,156}\).

Cyt-C, the most investigated protein involved in caspase activation, binds the scaffolding protein, named apoptotic protease activating factor-1 (Apaf-1), leading to an ATP- or dATP-dependent conformational change that induces Apaf-1 oligomerisation\(^{157}\). This high molecular mass complex, called the apoptosome, is assembled by binding Cyt-C and Apaf-1 with procaspase-9 through the interaction between their caspase recruitment domains (CARDs). Procaspase-9 activity is greatly enhanced in the apoptosome that, in turn, proteolytically activates caspase-3, finally resulting in the morphological and biochemical changes associated with apoptosis\(^{158}\). The most common hallmark used to identify the involvement of plant mitochondria in PCD is the release of Cyt-C. The release of Cyt-C from mitochondria has been detected in different plant systems, in which PCD was induced. In particular, the release of Cyt-C precedes the appearance of PCD symptoms and has been recognized in A. thaliana cells treated with mannose, where the effect is also associated to endonuclease activation\(^{159,160}\) and in maize cells infected by Agrobacterium sp.\(^{161}\). In addition, harpin (a bacterial protease in elicitor)-induced HR in tobacco cells is associated with an alteration of mitochondrial functions\(^{162}\). The initial steps of cell death are accompanied by an oxidative burst, depletion of ATP, collapse of the mitochondrial electrical potential and release of Cyt-C. A strong stimulation of the expression of the Alternative Oxidase (AOX) and small Heat-shock Proteins (HSPs) has also been described\(^{163}\). Consistent with this, induction of PCD in Arabidopsis thaliana cell cultures by ceramide, protoporphyrin IX and an elicitor of HR (AvrRpt2) leads to the dissipation of mitochondrial electrical potential followed by morphological changes and Cyt-C release\(^{164}\). By analogy with animal mitochondria, several authors have correlated the detected release of Cyt-C to the activity of PTP, on the basis of the inhibitory effect exhibited by CsA\(^{165,166,167}\). This contention seems to be confirmed by the observation that Nitric Oxide-induced programmed death in Câ©rus sinesis cell cultures is also prevented by CsA\(^{168}\). Plant mitochondria have the foremost components probably convoluted at the contact sites of OMM and IMM, e.g., ANT, VDAC\(^{169}\) and cyclophilin\(^{166}\). Hexokinase\(^{165}\). A functional genomic screen to assess the functions of various signaling genes in Nicotiana benthamiana revealed that a Tobacco Rattle Virus (TRV)-based Virus Induced Gene Silencing (VIGS) of a hexokinase gene, Hsk1, induced the spontaneous formation of necrotic lesions in leaves\(^{170}\). Hsk1 was associated with the mitochondria and its expression was stimulated by various cell death-inducing stresses. VIGS of Hsk1 resulted in apoptotic cell death in leaves, indicating that depletion of mitochondrial hexokinases activated Programmed Cell Death (PCD). Conversely,
overexpression of the mitochondria-associated Arabidopsis hexokinases, HXk1 and HXk2, conferred enhanced resistance to oxidative stress-induced cell death. Finally, the exogenous addition of recombinant Hxk1, but not Hxk1DN which lacks the membrane anchor, inhibited clotrimazole (CTZ)/H2O2-induced Cyt-C release from mitochondria. These results suggest a direct link between plant hexokinases and the PCD process. In any case, the opening of this channel would determine the entry into mitochondria of osmotically active solutes and water. This would cause a mitochondrial swelling with the consequent rupture of the OMM and release of Cyt-C.

A further model refers to the non-swelling mechanism involving the OMM. In this mechanism, a crucial role is performed by VDAC which interacts with Bax, forming a pore through which Cyt-C is released. The first evidence derives from a study in which the overexpression of mammalian Bax gene in tobacco plants causes hypersensitive-like lesions and induces defence genes. Recent experimental findings seem to corroborate this mechanism, suggesting that VDAC can play a crucial role in PCD pathway being a conserved element in both plants and animals. In agreement, VDAC expression increases during HR, senescence and heat-induced PCD in A. thaliana cells. This evidence indicates a putative dual role for VDAC, as a component of PIP or as a channel that interacts with Bax.

In animal cells, the significance of the release of Cyt-C is the subsequent assembly of the apoptosome complex that is followed by the activation of the executioner caspases. Although, evidences are lacking for the formation of apoptosome in plant cells, sequence alignments have revealed significant similarities among regions of C. elegans cell death gene that encodes a protease activating factor (Ced-4), human Apat-1 and several plant resistance genes. Although, these resistance genes do not contain CARD but may be assured to function as controlling adaptors in plant protein complexes which are activated during HR. Thus, the subsequent fate of Cyt-C is still problematic, because the formation of the complex like the apoptosome, is largely speculative. If an apoptosome-like complex exists in plants, it may interact with caspase-like proteases (Metacaspases, VPE) by analogy with the system in animal cells. In addition, further evidence supporting the mitochondrial involvement in plant PCD is provided by the information of strong upsurge in HSP during harpin HR in A. thaliana cells. HSPs are considered to partially suppress apoptosis in animal cells, by preventing Cyt-C release and disrupting the apoptosome. Plant HSPs are considered to accomplish comparable effects. It has been demonstrated that Bax inhibitor (BI-1) protein inhibits Bax induced apoptosis in mammalian cells and when ectopically expressed in yeast, BI-1 contains six or seven predicted transmembrane domains. As an integrate membrane protein, the localization of BI-1 is found to be similar to Bcl-2 exhibiting a nuclear envelope and endoplasmic reticulum association pattern. Moreover BI-1 has been isolated as one of the candidate suppressors of TNF-related apoptosis inducing ligand (TRAIL), an apoptosis-inducing member of the Tumour Necrosis Factor (TNF). The fundamental features of PCD are believed to be conserved throughout metazoans and plants. In support of this, a study carried out on tobacco shows that the expression of Bax which is a mammalian pro-apoptotic protein, triggered cell death in tobacco leaf. Moreover, overproduction of animal cell death suppressors Bcl-XL and Ced-9 conferred enhanced resistance to UV-b and pararquat treatment and salt, cold and wound stresses tobacco plants. However, recent research demonstrated that homologous plant genes for cell death suppressors such as bcl-2, Bcl-XL from humans and ced-9 from C. elegans are not found in Arabidopsis genome whose sequence has been presented as the first complete genomic sequence of higher plants. Recently, it has been reported that a gene encoding a homolog of Bax inhibitor (BI-1) from Arabidopsis inhibits mammalian Bax action in plants. This is the first report on the direct contribution to plant cell death of a plant originating gene that is a homolog of animal cell death related gene. An Arabidopsis homolog AtBI-1 (Arabidopsis Bax inhibitor 1) was identified from the genome sequencing project. The identity level is 57.5% AtBI-1 shares 41% amino acid identity with mammalian BI-1 (mBI-1). Plant homologs of BI-1 gene for several plant species including Oryza sativa (OsBI-1), Arabidopsis thaliana (AtBI-1) have been cloned and characterized to various extents. Ectopic overexpression of OsBI-1 leads to the elimination of cell death caused by Bax protein in Budding yeast, S. cerevisiae. Intriguingly enough, however, when AtBI-1 was transfected into the mammalian cell systems, it did not suppress Bax induced cell death in the human cells. Infact, AtBI-1 induced cell death comparable to Bax. The possibility exists that AtBI-1 might directly damage the mitochondrial structure causing Cyt-C release. However, co-transfection of the cells with both human BI-1 and AtBI-1 crippled cell death, suggesting preferably a dominant-negative mechanism in which AtBI-1 induced apoptosis is minimized by overexpressed mBI-1. It may, thus, be speculated that AtBI-1 competitively interacts with endogenous mBI-1 or BI-1 target protein, interfering with its function and thereby triggering cell death. In this regard, in vitro binding of BI-1 with Bcl-2 but not with Bax has been
demonstrated\textsuperscript{72,78}. Thus, it remains unclear how BI-1 suppresses Bax's function given that yeast and plants contain no obvious Bcl-2 homologs\textsuperscript{167,64,65,170}. Interestingly, downregulation of a tobacco BI-1 homolog using an antisense RNA approach resulted in accelerated cell death of tobacco BY-cells upon carbon starvation\textsuperscript{171,78,79,170,181,182,183,184,185,186}. Downregulation of rice BI-1 in cultured rice cells upon challenge with a fungal elicitor from 	extit{Mucor polymorpha} was concomitant with the progression of cell death and conversely, overexpressed rice BI-1 can improve cell survival against the elicitor\textsuperscript{186}. Another study found that decreased BI-1 expression correlated with chemical-induced resistance of barley to the infection of a biotrophic fungal pathogen powdery mildew (Blumeria graminis) and overexpression of barley BI-1 at a single-cell level induces hyper susceptibility and could reverse the fungal resistance that is conferred by the loss of MLO, a negative regulator of cell death and defense response in barley\textsuperscript{187}. Although, these observations support the idea that BI-1 homologs of yeast and plants have an anti-PCD function, the physiological importance of BI-1 and the impact of its loss of function in plants are still unclear at the whole plant level as clear genetic evidence is absent. However, a study carried out more recently\textsuperscript{188} identified and characterized two independent Arabidopsis homologues with T-DNA insertion in the 	extit{AtBI-1} gene. The phenotype of atb1-1 and atb1-2, with a C-terminal missense mutation and a gene knockout, respectively, is indistinguishable from wild-type plants under normal growth conditions. However, these two mutants exhibit accelerated progression of cell death upon infiltration of leaf tissues with a PCD-inducing fungal toxin fumonisin B1 (FB1) and increased sensitivity to heat shock-induced cell death. Under these conditions, expression of AtBI1 mRNA has been shown to be upregulated in wild-type leaves prior to the activation of cell death, suggesting that increase of AtBI1 expression is important for basal suppression of cell death progression. Over-expression of AtBI-1 transgene in the two homozygous mutant backgrounds rescued the accelerated cell death phenotypes. Together, these results provide direct genetic evidence for a role of BI-1 as an attenuator for cell death progression triggered by both biotic and abiotic types of cell death signals in 	extit{Arabidopsis}.

Plant homologs of the animal anti-apoptotic Defender against Apoptotic Death 1 (DAD1) gene have been reported in the cells of 	extit{Arabidopsis thaliana}\textsuperscript{189}, pea\textsuperscript{79} and rice\textsuperscript{172}. DAD 1 has been discovered in hamster cells where the corresponding mutant cell line dies via apoptosis\textsuperscript{89}. The suppressor function of this protein was further suggested in C. elegans via its over expression protects some of the cells destined to die by apoptosis during development\textsuperscript{189}. At least two 	extit{A. thaliana} homologues have been found and transformation of the mutant hamster cell line with one of them, demonstrates that the function of the protein is conserved between plants and animals\textsuperscript{189}. Other experiments have shown that DAD 1 is a subunit of mammalian Oligosaccharyl transferase complex\textsuperscript{183,184}. Furthermore, cell competition is a homeostatic mechanism that regulates the size attained by growing tissues\textsuperscript{188}. Performance of an unbiased genetic screen for mutations that permit the survival of cells appeared to be competed due to haplo-insufficiency for Rpl36. Mutations which protect Rpl36 heterozygous clones comprise the tumor suppressors expanded, hippo, saldanta, mats and warts which are members of the Warts pathway, the tumor suppressor fat and a novel tumor-suppressor mutation. Other hyperplastic or neoplastic mutations did not rescue Rpl36 heterozygous clones. Most mutations that rescue cell competition elevated Dpp-signaling activity and the Dsmurf mutation that uplifts Dpp signaling was also hyperplastic and rescued. Two nonlethal, nonhyperplastic mutations prevent the apoptosis of Minute heterozygous cells and suggest an apoptosis pathway for cell competition. Besides rescuing Rpl36 heterozygous cells, mutations in Warts pathway genes were supercompetitors that could eliminate wild-type cells nearby\textsuperscript{189}. The outcomes show that variances in Warts pathway activity can lead to competition and incriminate the Warts pathway, certain other tumor suppressors and novel cell death modules in cell competition, in addition to the Dpp pathway connected by previous studies. It is therefore suggested that cell competition might occur during tumor development in mammals\textsuperscript{189,191,192}. All these outcomes together make the role of this gene in animal apoptosis unclear, whereas its putative suppressor role in plants and microorganisms is still obscure.

CONCLUSION

Conclusively, there are probable inherent alterations in the functioning mechanism of PCD between plants and animals; there is also the probability for the participation of different functioning mechanisms of PCD in diverse plant cell types, i.e., more than one pathway of PCD is expected to be operational in plants, whereas, in animals there appears to be only one programme. No plant system is yet pronounced showing all features common to animal PCD. The comparison of plant PCD and apoptosis referring to animal system spectate key differences regarding morphological fluctuations happening in the dying cells and enzymes involved in the process. However, the ending execution of the process, DNA fragmentation and nuclear disorganization, has resemblances in animal and plant cells, proposing that it might have developed from a common ancestor. Extensive studies have delivered
evidence that PCD in plants and animals share components which include caspase-like activity and these caspase-like activities, could be inhibited with caspase inhibitors but not caspase-unrelated protease inhibitors. Additionally, the caspase inhibitors have been revealed to eliminate these PCDs. The existence of plant proteases which recognize and process the natural caspase substrate PARP seemingly at caspase recognition site and the functionality of natural caspase substrates (p35) in plants corroborates the involvement of caspase like activity in plant programmed cell death. VPEs and metacaspases seem to be the prime candidates shown to be responsible for the caspase like activities witnessed. Nevertheless, the role of metacaspases in cell death still remained enigmatic and both up- and down-regulation of metacaspases have yielded contradictory data. Conversely, such approaches bear the risk that a constitutive perturbation of genes which are crucial for normal cellular homeostasis. Overproduction of the cleavage fragments and/or of uncleavable mutant proteins would help in an elucidation of the practical mechanisms of substrate cleavage by metacaspases. The many intriguing similarities with PCD in animals will require to be scrupulously tested to exhibit that they are conserved and are derived from a common ancestral origin. Despite the significant evolution in our understanding of plant PCD in recent years, its regulatory mechanism is still obscure. Deployment of reverse genetic tactics such as PTGS/RNAi strategies and knockout screens by means of T-DNA or transposon insertion collections, coupled with global expression tactics like microarray analysis should help speed up the first essential step of identifying the important players involved in plant cell death activation. This approach would be complementary to forward genetic approaches that are revealing new regulator(s) that may not have counterparts in other organisms.

REFERENCES


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