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Programmed cell death or apoptosis: Do animals and plants share anything in common

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Plants, animals and several unicellular eukaryotes use programmed cell death (PCD) for defense and developmental mechanisms. While cell death pathways in animals have been well characterized, relatively little is known about the molecular mechanism of such a strategy in plants. Although, very few regulatory proteins or protein domains have been identified as conserved across all eukaryotic PCD forms, still plants and animals share many hallmarks of PCD, both at cellular and molecular levels. Morphological and biochemical features like chromatin condensation, nuclear DNA fragmentation, and participation of caspase like proteases in plant PCD appear to be similar across the eukaryotic kingdom and in conformity with the process in metazoans as well. Transgenic expression of mammalian anti- and pro-apoptotic proteins in plants has been shown to influence the regulatory pathways of cell death activation and suppression, indicating the existence of functional counterparts of such genes in plants, several of which have now been cloned and characterized to various extents. This suggests that despite differences, there may be a fair level of functional similarity between the mechanistic components of plant and animal apoptosis. Although genome scan of Arabidopsis thaliana seems to rule out the existence of major mammalian apoptotic counterparts in plants, the identification of caspase like proteins and other structural homolgs (metacaspases) together with mildly conserved apoptotic players like Bax-1 inhibitor may seemed to suggest some degree of common grounds both in execution and in the regulation of the cell death phenomenon. The overall review of the available data pertaining to mechanism of PCD in plants primarily supports an ancestral relationship with animal apoptosis rather than any common executional or regulational strategies. The establishment of mechanistic details of the phenomenon in plants is certain to throw up many surprises to necessitate a fresh review of this intriguing phenomenon. Metacaspases and Paracaspases having been ruled out to possess caspase activity is the beginning for this surprise to unfold.

Key words: Programmed cell death, apoptosis, caspases.

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INTRODUCTION

Programmed cell death (PCD) describes a physiological and pathological process of cell deletion that plays an important role in maintaining tissue homeostasis (Wertz and Hanley 1996). It is a highly regulated cellular suicide process essential for growth and survival in all eukaryotes. The origin of the phenomenon seems to be as old

as the very first cell, because cellular homeostasis and preventing self-destruction would have been impossible to accomplish without such machinery (Ameisen, 2002). 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 (Gray, 2004). Multicellular organisms use the physiological mechanisms of cell death to regulate developmental morphogenesis and remove infected, mutated or damaged cells from healthy tissues (Vaux and Korsmeyer, 1999). 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 (Kerr et al., 1972), and chromatin condensation (Earnshaw, 1995; O'Brien et al., 1998). 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 (Earnshaw, 1995; Fath et al., 1999; McCabe et al., 1997; Wang et al., 1996b; Wyllie, 1980). Most, but not all, of the above apoptotic features are commonly observed during PCD in a wide range of eukaryotic organisms.

In plants, PCD occurs during development, such as during xylogenesis, embryogenesis, parenchyma formation, several plant reproductive processes, seed development and leaf senescence (Pennel et al., 1997; Gray, 2004). 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 (Hatsugai et al., 2004). This hypersensitive response (HR) is thought to directly kill invaders and / or to interfere with their acquisition of nutrients (Heath, 2000). 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.

In this review we provide a brief insight into some of the comparative features of PCD in plants and animals. Additionally, this article attempts to review some of the peculiar and specific features and regularities of apoptosis in plants.

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MORPHOLOGICAL HALLMARKS OF PCD

The morphological features of PCD have been intensively studied in animals. PCD in animal systems is reported to result in the disassembly of cells involving the condensation, shrinkage and fragmentation of cytoplasm and nuclei into several sealed packets (often called apoptotic bodies), which are then phagocytosed, by the neighboring cells or the macrophages. Thus, there are no remnants of the cell corpses left. Nuclear fragmentation is preceded by chromatin condensation and marginalization in the nucleus. Fragmentation of DNA at the nucleosome linker sites then takes place and the fragmented oligonucleosomal bits are reported to be 180 bp (Danon and Gallois, 1998). Fragmentation is effected by endonucleases such as NUC 1, DNaseI and DNaseII (Peitsch et al., 1993), which are present in the nucleus and are activated by Ca^{2+} and Mg^{2+} but inhibited by Zn^{2+} and by several caspase activated nucleases such as CAD (Caspase activated DNase) or DFF40 (DNA fragmentation factor 40KDa) (Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1998). The DNA fragments can be cytochemically determined by Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling (TUNEL) of DNA at 3'-OH group. When all these events are combined to result in a distinct morphological expression then PCD is termed as apoptosis (Cohen 1993). In other words, apoptosis is a distinct form of PCD (D'Silva et al., 1998; Danon and Gallois, 1998). However there are others who consider apoptosis and PCD as one and the same (White, 1996; Miller and Marx, 1998; Hengartner, 1998; Chinnaiyan and Dixit, 1996).

In the last few years and due to new interest in a possible apoptosis like phenomenon existing in plants, morphological changes have been investigated during plant PCD. Only some of the hallmarks are similar to those reported in animal apoptosis. Condensation & shrinkage of the cytoplasm and nucleus have been described in carrot cell culture, after cell death induced by heat shock (McCabe et al., 1997). The DNA processing reported earlier for the animal PCD is believed to exist in the dying cells of plant as well (Wang et al., 1996a; Wang et al., 1996b; Mc Cabe et al., 1998; Orzaez and Granell, 1997a). In plants, DNA ladders have been reported during development represented by death of monocot aleurone layer (Wang et al., 1996a) and endosperm (Young et al., 1997), senescence of petal, carpel tissue and leaves (Orzaez et al., 1997a; Orzaez et al.1997b; Yen et al., 1998) or during anther development (Wang et al., 1999) as well as during death induced by different stresses such as: cold (Koukalova et al., 1997), nutrient deprivation (Callard et al., 1996), salt or D-mannose stresses (Katsuhara et al., 1997; Stein et al., 1999), UV radiattion (Danon et al., 1998), pathogens or a pathogen toxin (Navarre et al., 1999; Ryerson et al., 1996; Wang et al., 1996b).In aleuronic cells of grass species such as barley, in dying root cap cells and in tobacco cells subjected to HR, nuclear condensation and shrinkage as well as oligo-

nucleosome sized DNA fragments have been recognized through the presence of 3'-OH group detected by TUNEL experiments (Pennel and Lamb, 1997; Mittler and Lam, 1997). Although in case of animals, one of the major hallmarks of PCD is the fragmentation of DNA at the nucleosome linker sites into oligonucleosomal bits of precisely 180 bp, the major problem relating to nuclear changes in plant PCD is that there is no consistency regarding the size of DNA fragments during DNA fragmentation: fragments of more or less 50Kb in some cases (Mittler and Lam, 1997) and as small as 0.14 Kb in others (Cohen, 1993; O'Brien et al., 1998). It is believed that the activation of some endonucleases leads to 50 Kb DNA fragments followed latter by a different set of endonucleases causing the production of oligonucleosomal length of DNA fragments (Pandey et al., 1994). The first type of cleavage is believed to be the result of the release of chromatin loops and is observed in almost all cases of apoptosis and the subsequent nucleosomal laddering occurs less often and is considered to be not essential for apoptosis (Oberhammer et al., 1993).

The enzymes involved in nuclear dismantling in plants are still poorly known. Several DNase activities and nuclease genes have been recognized to be up regulated in different models of plant PCD (Sugiyama et al., 2004). However, molecular and biochemical evidences on their involvement in the cell death has been reported only in some of them. Recent work reported the induction of the activity of a 28 KDa endonuclease (p28) activity in victorin (Tada et al., 2001) treated oat leaves and this preceded the DNA laddering and heterochomatin condensation. The p28 activity also markedly increased in parallel with the rate of DNA fragmentation and cell death (Tada et al. 2001). In addition to p28, an inducible nuclease, p24 (24 kDa) and four constitutive nucleases, p22 (22 kDa), p31 (31 kDa), p33 (33 kDa) and p35 (35 kDa), have been detected in oat cell lysates using an in-gel assay for nuclease activity (Tada et al., 2001; Kusaka et al., 2004). A Mg^{2+} dependent nucleolytic activity has been identified in the intermembrane space of mitochondria responsible for the generation of 30Kb DNA fragments in Arabidopsis (Ito and Fukuda, 2002). ZEN1, a Zn^{2+} dependent endonuclease, has been directly impli-cated in the degradation of the nuclear DNA in Zinnia tracheary elements (Ito and Fukuda 2002). ZEN1 is localized to vacuoles, which collapse before DNA is degraded (Obara et al., 2001). However, ZEN1 activity does not produce the characteristic DNA laddering shown by the nucleases executing DNA fragmentation in apoptotic animal cells (Enari et al., 1998; Halenbeck et al., 1998; Liu et al., 1998). Based on the biochemical differences of ZEN1 and the nucleases involved in animal apoptosis, 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 inter-nucleosomal fragmentation of DNA

(Dom´ınguez et al., 2001; Young and Gallie, 1999; Young and Gallie, 2000; Dom´ınguez et al., 2004; Wang et al., 1996a), which is a hallmark of apoptosis in animal cells (Earnshaw, 1995). Recently, a Ca^{2+}/Mg^{2+} endonuclease localized in the nucleus wheat aleuron cells undergoing PCD has been identified which is detected prior to DNA laddering (Dom´ınguez et al., 2004). A cell-free system used to analyze nucleus degeneration in nucellar cells in wheat grains (Domı´nguez and Cejudo, 2006), shows that a different wheat tissue, the nucellus, which undergoes PCD at early stage of grain development (Dom´ınguez et al., 2001), presents a nucleus localized nuclease with identical cation requirements, but with a different electrophoretic mobility than the aleuron nuclease. These results suggest that both animal apoptosis (Samejima et al., 2005) and plant PCD involves more than one nuclease. Nuclear extracts from such cells have been shown to be capable of triggering DNA fragmenation 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 organelles (Jones, 2000), 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 includes reports of nuclear blebbing and fragmentation (Schussler and Longstreth, 2000; Yamada et al., 2000; Filonova et al., 2000), and the detection of oligosomal DNA ladders (Xu and Hanson 2000; Yamada et al., 2000; Filonova et al., 2000; Delorme et al., 2000) and labelled fragmented DNA in nucleus (Jordan et al., 2000; De Jong et al., 2000; Yamada et al., 2000; Filonova et al., 2000; Simeonova et al., 2000).

Autophagy has been observed as engulfment and degradation of nucleus and other organelles by provacuoles, vacuoles and other autophagic organelles derived from leucoplast (Filonova et al., 2000). 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 treachery element differentiation. In most cases, autolysis and autophagic mechanisms cooperate to yield cellular disassembly, such as that occurring during embryo suspensor death (Filonova et al., 2000). 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 gran-enzymes. 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 (C. 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 occur (Ellis et al., 1991). 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 fruit fly Drosophila melanogaster, for example, are lethal early in development (White et al., 1994) as are mutations in mice that inhibit apoptosis mainly in the developing brain (Kuida et al., 1996).The protein encoded by the *ced-3* gene was found to be very similar to a human protein called interleukin-1-converting enzyme (ICE) (Yuan et al., 1993). ICE is an intracellular protein cleaving enzyme (a protease) that cuts out interleukin-1, a signalling protein that induces inflammation, from a larger precursor protein (Nicholson, and Thornberry, 1997).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 to 15 different caspases that play a role in inflammation (group1 caspases) and apoptosis (group 2 caspases) have been identified in animals (Rudel, 1999). All these are believed to share a fair level of sequence homology and similarity in sequence specificity (Rudel, 1999; Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998). Up till 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 (Garcia-Calvo, 1998). 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 with same peptide substrate coupled with aldehyde (CHO: reversible inhibitor) or methyl ketone radical [Chlorometylketone (CMK), flouromethylketone (FMK): irreversible inhibitor).

Caspases are made as a large, inactive precursor (pro-caspase), which is itself activated by cleavage at aspartic acids, usually by another caspase (de Murcia et al., 1994; Figure1). 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 (Kumar, 2007; Timmer and Salvesen, 2007). 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 (Enari 1998). The other important substrates include PARP [poly(ADP-ribose)polymerase], DNA dependent protein kinase (DNA PK), Serum response element binding protein (SRE/BP), p21(CDKN1A)-activated kinase 2 (PAK2), 70KDa components of U1Sn-RNP, procaspases and so on (Rudel, 1999). 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 (Lazebnik et al., 1994). It is believed to be involved in the regulation of the repair of DNA strand breaks and in cell recovery from DNA damage (de Murcia et al., 1994).

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 show induction of YVADase activity whereas no DEVDase activity was detected (del Pozo and Lam, 1998). 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 an 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. Danon et al. (2004) and Lincoln et al. (2002) reported that the heterologous expression of Baculovirus p35 protein, a broad range caspase inhibitor that can effect-tively suppress PCD in animals, blocked AAL (Arternaria alternata) toxin induced cell death in transgenic tomato plants and provided protection against the pathogen Arternaria alter-
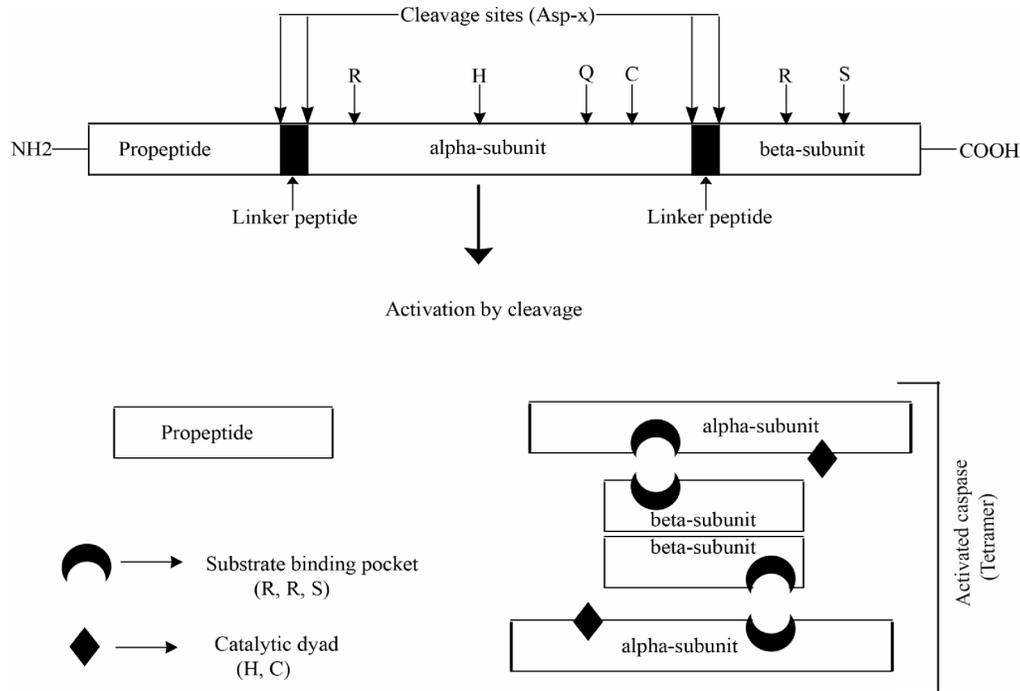
Figure1. Diagrammatic representation of the activation of human caspase 1: Caspases are activated by proteolytically removing the propeptide and the linker peptides from the inactive caspase and cleaving the rest of the caspase into larger α-subunit and smaller β-subunit. These subunits assemble into active tetramer containing two active sites (Nicholson, 1999; Grutter, 2000)

nate. 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 (Del Pozo and Lam, 2003). 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 (Del Pozo and Lam, 2003). 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) (D'Silva et al., 1998). Interestingly, a polypeptide (GDEVDGIDEV) 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 (GDEVAGIDEV) 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

77, 52, 47, and 45 kD (D'Silva et al., 1998). Cleavage of endogenous (plant) PARP (116 KDa) reacting with a PARP antibody occur during menadione-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 (Sun et al., 1999). Also in heat shock induced PCD in tobacco suspension cells, endogenous PARP was cleaved, yielding a 89 kDa fragment (Tian et al., 2000). 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 *Arabodopsis thaliana* and PARP activity has been identified in few species (O'Farrell, 1995).

Although there have been numerous efforts to identify proteinases that exhibit caspase activities, plant caspases have remained unidentified (Woltering et al., 2002). Recent work, however, has unraveled this mystery. Vacuolar processing enzyme (VPE) has been shown to be a protease that exhibits caspase-1 activity and is essential for virus-induced hypersensitive cell death (Hatsugai et al., 2004). Direct evidence has been reported for the involvement of VPEs in plant cell death (Sanmartin et al., 2005; Hatsugai et al., 2004; Hatsugai et al., 2006; Kuro-
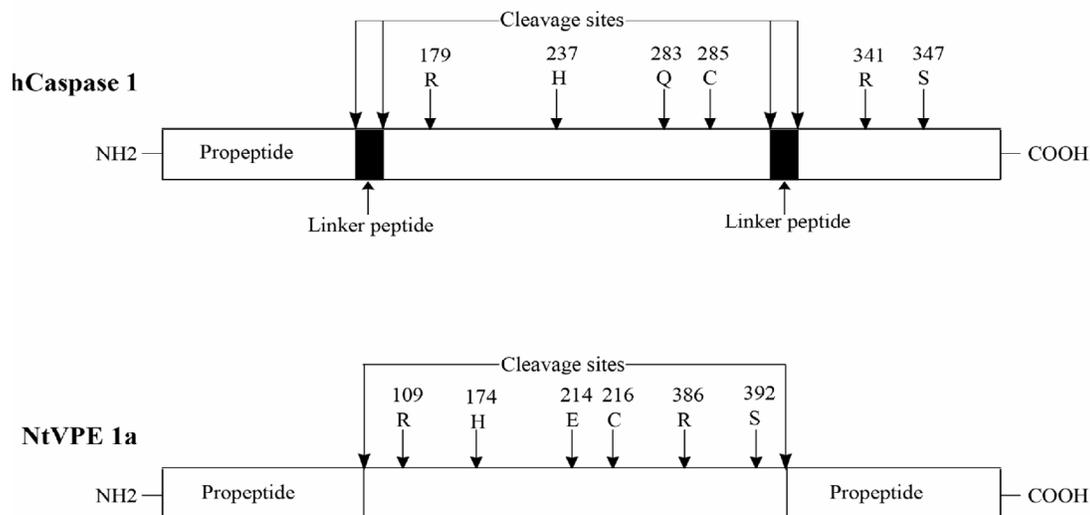
Figure 2. Schematic representation of active site residues among the human caspase1 (hCaspase1) and Nicotiana tabacum VPE (NtVPE1a). Histidine 237 and Cystein 285 that are involved in catalysis in hcaspase1 correspond to H174 and C216 of NtVPE1a and Argenine 179, Argenine 341, Serine 347 involved in substrate binding in hcaspase1 correspond to R109, R386 and S392 in case of NTVPE1a. Caspase1 and plant VPEs are activated by proteolytically removing the propeptides and the linker peptides from the inactive caspases which is then converted into active caspase.

yanagi et al., 2005; Hara-Nishimura et al., 2005; Lam, 2005). Hara-Nishimura and coworkers (Hatsugai et al., 2004) have used a temperature sensitive N-TMV tobacco plant pathogen system to identify the proteases 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 30ºC, TMV can systemically infect tobacco plants because induction of cell death and defence gene expression is completely suppressed. Upon shifting the temperature to 23ºC, cell death appears throughout the infected plant. A biotinylated caspase inhibitor (biotin-XVAD-FMK) was used to identify the proteins complexing 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 YVAD-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 YVADase activity but not by DEVDase activity (Lam and Del Pozo, 2000). 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 (Yamada et al. 2005; Nakaune et al., 2005; Kinoshita et al., 1995). VPE is an asparaginyl endopeptidase (Hara-Nishimura et al., 1998; Hara-Nishimura et al., 2003). It cleaves peptide bonds on the C-terminal side of Asparagine residues exposed on molecular surface of proprotein precursors to generate the respective mature proteins (Hara-Nishimura et al., 1991; Hara-Nishimura et al., 1987; Hara-Nishimura et al., 1993). However, it also has been shown that VPE cleaves peptide bonds on the C-terminal side of aspartic acid residues (Becker et al., 1995; Hiraiwa et al., 1999). VPE recognizes aspartic acid when it is part of the YVAD sequence of a caspase-1 substrate, but does not necessarily recognize other aspartic acid residues (Hatsugai et al., 2004), similar to caspase-1 (Stennicke et al., 1998; Earnshaw et al., 1999). 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 (Figure 2). Two residues of the catalytic dyad in VPE (histidine and cysteine; Figure 2) (Hiraiwa et al., 1999) are comparable to His237 and Cys285 of the catalytic dyad in human caspase-1 (Nicholson, 1999; Cohen, 1997). 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 (Sanmatin et al., 2005). In addition, each of three essential amino acids, Arg179, Arg341 and Ser347, which form the substrate-binding pocket of caspase-1 (Wilson et al., 1994; Nicholson 1999) are conserved in VPEs. This is the case for all of the more than 20 VPEs that are currently in databases. The sub-

strate binding pocket of VPE might be similar to the substrate-binding pocket of caspase-1. Both VPE (Hiraiwa et al., 1997; Hiraiwa et al., 1999; Kuroyanagi et al., 2002) and caspase-1 (Cohen, 1997) are subjected to self-catalytic conversion / activation from their inactive precursors.

With the sequencing of the complete genome of the model plant *A. thaliana* (Arabidopsis Genome Initiative, 2000), these caspase like activities have steered 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 (Uren et al., 2000; Vercammen et al., 2004). 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 expre-ssion of one of the tomato metacaspases during infection with the necro-trophic pathogen *Botrytis cinerea* suggests a possible role for plant metacaspases in cell death (Hoeberichts et al., 2003). The recent findings by Peter Bozhkov and colleagues (Bozhkov et al., 2004) 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-methyl-coumarin, these were cleaved poorly. The VEIDase activity was sensitive to pH, ionic strength and Zn^{2+} comparable to human caspase-6. The substrate specificity of the Norway spruce VEIDase appears similar to that of the yeast metacaspase YCA1 (Madeo et al., 2002), 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 (Suarez et al., 2004). 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 *mcII-Pa* (type II metacas-pase gene*)* had Arg but not Asp specificity (Bozhkov et al. 2005). Because knocking down *mcII-Pa* not only disrupted cell death but also blocked embryonic differ-rentiation, it was speculated that mcII-Pa might be prima-rily involved in cellular differentiation rather than in cell death. Possibly, *mcII-Pa* regulates the actin reorga-nization observed during cellular differentiation (Smertenko et al., 2003), like mammalian caspases do in the cytoskeletal rearrangements during apoptosis (Mashima et al., 1999). In *Arabidopsis*, mere constitutive

over expression or disruption of meta-caspase genes does not lead to an obvious phenotype (Vercammen et al., 2006; Belenghi et al., 2007) 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 metacas-pases.

The proteins responsible for the activation of the executors of PCD: The adaptors

The controlled activation of caspase precursors (Zymo-gens) is achieved by adaptor proteins that bind to them through shared motifs. Tumour Necrosis Factor (TNF) receptors superfamily or apoptogenic cofactors released by the mitochondria can be mentioned 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 asso-ciation of Caspase Recruitment Domain (CARD) in its prodomain with the CARD in another cofactor protein, Apoptosis Protease Activating Factor-1 (APAF-1) (Vaux et al. 1999; Thornberry et al. 1998). In the worm *Caenor-habditis elegans,* Ced-4 acts as the adaptor molecule.

Database searches have identified several motifs of similarities between Ced-4, APAF-1 and proteins enco-ded by resistance genes regulating HR in plants. The conserved domain has been coined as NB-ARC (Van der Biezen and Jones, 1998; Rojo et al., 2004).

Intracellular controls: The regulators

A decision to die should not be taken lightly and so it is not surprising that the death programme is regulated in complex ways. 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 (Adams and Cory 1998). *ced-9* (*ced* for cell death abnormal) gene 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 (Kerr et al., 1972). Ced-9 prevents caspase activation by binding to adaptor Ced-4 (Vaux and Korsmeyer, 1999) 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 humans *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 x protein (Bax), BCL2 antagonist/ killer (Bak), BCL2-related ovarian killer (Bok), BCLxL/BCL2 associated death promoter (Bad) and BH3

interacting domain death agonist (Bid) promote it (proapoptotic) (Adams and Cory, 1998). 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 help determine a cell's susceptibility to apoptosis (Merry and Korsmeyer, 1997).

It is now an established fact that mitochondria which are called the powerhouses of the cell, not only generate energy for cellular activities but also play an important role in cell death (Green et al., 1998; Shah et al., 2000) 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, Ca^{2+} & 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 pro-apoptotic proteins (Bernardi et al., 2001; Kroemer and Reed, 2000). The first involves Bax that could simply oligo-merise in outer mitochondrial membrane (OMM) to form a channel. Alternatively, Bax, in association with either the voltage-dependent anion channel (VDAC) or truncated Bid (tBid), 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 (Bernardi et al., 2001; Kroemer and Reed, 2000). This process is mediated by either VDAC or the opening of a permeability transition pore (PTP) (Desagher and Martinou, 2000). 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 benzodiazepin receptor and Bcl-2, hexokinase bound to VDAC, and intermembrane creatine kinase (Bernardi, 1999; Zoratti and Sza`bo, 1995). Recent evidence indicates that the mitochondria-associated hexokinase plays an important role in the control of apoptosis in mammals (Downward, 2003; Birnbaum, 2004; Majewski et al., 2004). Hexo-kinase is an integral component of the PTP through its interaction with porin or the voltage-dependent anion channel (VDAC) (Wilson, 2003), and hexokinase binding to the VDAC interferes with the opening of the PTP, thereby inhibiting cytochrome c release and apoptosis (Pastorino et al., 2002; Azoulay-Zohar et al., 2004).Thus, detachment of hexokinase from the mitochondria potentiates, and its over expression inhibits mitochondrial dys-function and cell death induced by various stimuli (Gottlob et al., 2001; Bryson et al., 2002; Majewski et al., 2004). Recent studies have shown that cyclophilin D, another compo-

nent 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 (Baines et al. 2005; Nakagawa et al., 2005). However, these observations do not exclude the possibility that certain forms of apoptosis are mediated by the mitochondrial permeability transitions, because some forms of apoptosis are significantly inhi-bited by cyclosporin A, a specific inhibitor of cyclophilin activity (Green and Kroemer, 2004). Additionally, cyclophilin D–overexpressing mice exhibited an increase in apoptotic heart muscle cells (Baines et al. 2005). Furthermore, in cancer cells, cyclophilin D over expression suppresses apoptosis via the stabilization of hexokinase II binding to the mitochondria (Machida et al., 2006). 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 atractylate) and inhibitors [CyclosporinA (CsA)], Adenosine diphospate H^+, 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 & intracellular ATP levels (Green and Reed, 1998; Adams and Cory, 1998). 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 & ANT & brings about a conformational alteration to form a megachannel leading to the release of Cyt-C (Martinou, 1999; Bernadi et al., 2001). 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 (Shimizu et al., 2000). Bax and Bim interact with VDAC and lead to the release of Cyt-C, whereas Bcl-XL blocks this release (Shimizu et al., 1999; Sugiyama et al., 2002). The permeabilization of the IMM to solutes with molecular mass up to 1.5 kDa, caused by the aperture of the PTP results in the complete dissipation of mitochondrial electrical potential. Consequently, the high concentration of solutes present in the matrix induces an osmotic swelling that could ultimately lead to OMM rupture and the consequent release of proteins from the intermembrane mitochondrial space (IMS) (Kroemer and Reed, 2000).

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 (van Gurp et al., 2003). 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 cas-

pase 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 (Kaufmann and Hengartner 2001). The most common hallmark used to identify the involve-ment 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 (Stein and Hansen, 1999), and in maize cells infected by Agrobacterium sp. (Hansen, 2000). In addition, harpin (a bacterial proteinaceous elicitor)–induced HR in tobacco cells is associated with an alteration of mitochondrial functions (Xie and Chen, 2000). 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 (Krause and Durner, 2004). Consistent with this, induction of PCD in *A. 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 (Yao et al., 2004). 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 (Balk and Leaver, 2001; Lin et al., 2005; Tiwari et al., 2002). This contention seems to be confirmed by the observation that Nitric Oxide-induced programmed death in Citrus sinensis cell cultures is also prevented by CsA (Saviani et al., 2002). Plant mitochondria have the main components probably involved at the contact sites of OMM and IMM, e.g. ANT, VDAC (Godbole et al., 2003) and cyclophilin (Yokota et al., 2004), Hexokinase (Kim et al., 2006). 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, Hxk1, induced the spontaneous formation of necrotic lesions in leaves (Kim et al., 2006). Hxk1 was associated with the mitochondria, and its expression was stimulated by various cell death–inducing stresses. VIGS of Hxk1 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 (Lam et al. 2001). 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 (Lacomme and Santa Cruz, 1999). 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 (Godbole et al., 2003; Swidzinski et al., 2004). In agreement, VDAC expression increases during HR, senescence and heat-induced PCD in A. thaliana cells (Lacomme and Roby, 1999; Swidzinski et al., 2004). This evidence indicates a putative dual role for VDAC, as a component of PTP 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 Apaf-1 and several plant resistance genes. Although these resistance genes do not contain CARD but may be assumed to function as controlling adaptors in plant protein complexes which are activated during HR (Vander Biezen and Jones 1998). 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, a further evidence supporting the mitochondrial involvement in plant PCD is provided by the reports of strong increase in HSP during harpin HR in *A. thaliana* cells (Krause and Durner, 2004). 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 (Hoeberichts and Woltering, 2002).

It has been demonstrated that Bax inhibitor1 (BI-1) protein inhibits Bax induced apoptosis in mammalian cells and when ectopically expressed in yeast (Xu and Reed 1998). BI-1 contains six or seven predicted trans-membrane 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 asso-

ciation pattern (Xu and Reed, 1998). 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) (Burns and El-Deiry, 2001). The fundamental features of PCD are believed to be conserved throughout metazoans and plants (Pennel and Lamb, 1997). 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 (Lacomme and Santa Cruz, 1999). Moreover, over-production of animal cell death suppressors Bcl-XL and Ced-9 conferred enhanced resistance to UV-b and paraquat treatment and salt, cold and wound stresses in tobacco plants (Mitsuhara et al., 1999; Qiao et al., 2002). 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 (Lam et al., 2001). Recently, it has been reported that a gene encoding a homolog of Bax inhibitor (*BI-1*) from *Arabidopsis* inhibits mammalian Bax action *in planta* (Kawai-Yamada et al., 2001). 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 37.5% 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*), *A. thaliana* (*AtBI-1*) have been cloned and characterized to various extents (Bolbuc and Brisson, 2002; Bolduc et al., 2003; Eichmann et al., 2004; Huckelhoven et al., 2003; Kawai et al., 1999; Kawai-Yamada et al., 2004; Matsumara et al., 2003; Sachez et al., 2000). Ectopic overexpression of OsBI-1 leads to the elimination of cell death caused by Bax protein in Budding yeast *S. cerevisiae* (Kawai et al., 1999). 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 (Yu et al., 2002). 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 (Yu et al., 2002). It may, thus, be speculated that AtBI-1 competitively interacts with endogenous mBI-1 or BI-1 target protein, interfering with its function & thereby triggering cell death. In this regard, *in vitro* binding of BI-1 with Bcl-2 but not with Bax has been demonstrated (Xu and Reed, 1998). Thus, it remains unclear how BI-1 suppresses Bax's function given that yeast and plants contain no obvious Bcl-2 homologs (Lam et al., 2001).

Interestingly, down regulation of a tobacco BI-1 homolog using an antisense RNA approach resulted in accelerated cell death of tobacco BY-cells upon carbon starvation (Bolduc and Brisson 2002). Down regulation of rice BI-1 in cultured rice cells upon challenge with a fungal elicitor from *Megnaporthe grisea* was concomitant with the progression of cell death and, conversely, overexpressed rice BI-1 can improve cell survival against the elicitor (Matsumara et al., 2003). 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 (Huckelhoven et al., 2003). 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 (Watanabe and Lam, 2006) identified and characterized two independent Arabidopsis mutants with T-DNA insertion in the AtBI-1 gene. The phenotype of atbi1-1 and atbi1-2, 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 Arabidopsis. Plant homologs of the animal anti-apoptotic defender against Apoptotic Death 1 (DAD1) gene have been reported in the cells of *A. thaliana* (Gallois et al., 1997), pea (Orzaez and Grannel, 1997b) and rice (Tanaka et al., 1997). DAD 1 has been discovered in hamster cells where the corresponding mutant cell line dies via apoptosis (Kusaka et al. 2004). The suppressor function of this protein was further suggested in *C. elegans* via it's over expression protects some of the cells destined to die by apoptosis during development (Sugimoto et al., 1995). At least two 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
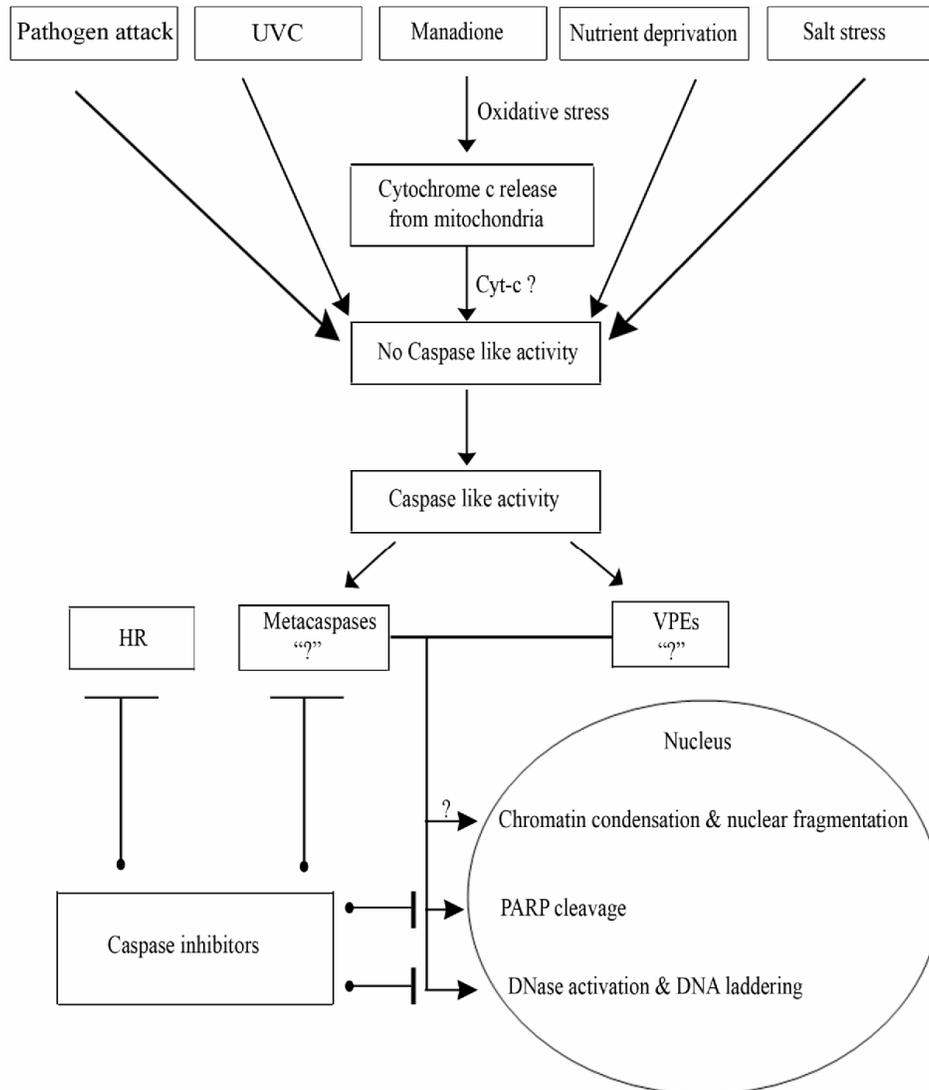
Figure 3. Chosen examples of treatments triggering different animal or plant PCD hallmarks in plant cell**?** Indicates events that have been shown in animals and have not been detected in plants and **"?"** Indicates arbitrary functional assignment in plants.

animals (Gallois et al., 1997). Other experiments have shown that DAD 1 is a subunit of mammalian Oligosaccaryl transferase complex (Kelleher et al., 1997). In mice, overexpression of DAD 1 does not have any protecting function against apoptosis but affects cell cycle (Hong et al., 1999). All these results together make the role of this gene in animal apoptosis unclear, whereas its putative suppressor role in plants is still not established.

CONCLUSIONS AND FUTURE PERSPECTIVES

There are likely to be inherent differences in the operational mechanism of PCD between plants and animals; there is also the possibility for the involvement of different operational mechanisms of PCD in different plant cell types, that is, more than one pathway of PCD is likely to be operative in plants, while in animals there seems to be only one programme. No plant system is yet described which shows all features common to animal PCD. The comparison of plant PCD and apoptosis in animals show important differences regarding morphological changes occurring in the dying cells and enzymes involved in the process. However, the final execution of the process, DNA fragmentation and nuclear disorganization, has similarities in animal and plant cells, suggesting that it might have evolved from a common ancestor. Extensive studies have provided evidence that PCD in plants and animals share components that include caspase-like activity (Lam et al., 2001; Hatsugai et al., 2004; Suarez et al., 2004; Sanmartin et al., 2005; Kuroyanagi et al., 2005; Hara-Nishimura et al., 2005; Lam, 2005; Hatsugai et al., 2006) and these caspase-like activities could be inhibited

with caspase inhibitors but not caspase-unrelated protease inhibitors (del Pozo and Lam, 1998). Furthermore, the caspase inhibitors have been shown to abolish these PCDs (del Pozo and Lam, 1998; De jong et al., 2000). The existence of plant proteases that recognize and process the natural caspase substrate PARP apparently at caspase recognition site (Sun et al., 1999; Tian et al., 2000; O'Farrell, 1995) and the functionality of natural caspase substrates in plants (Danon et al., 2004 and Lincoln et al., 2002) substantiates the involvement of caspase like activity in plant programmed cell death (Figure 3)

VPEs and metacaspases appear to be the prime candidates that seem to be responsible for the caspase like activities observed. However, until now, the role of metacaspases in cell death still remained enigmatic, and both up- and down-regulation of metacaspases have yielded conflicting data. However, such approaches bear the risk that a constitutive perturbation of genes that are essential for normal cellular homeostasis leads to over-interpretation. Alternative routes toward unraveling the function of metacaspases could involve the identification of their substrates by using technologies that allow direct characterization of *in vivo* protein processing on a proteome-wide scale (Gevaert et al., 2006). Knowing the degradome specificity of metacaspases could reveal their role in cellular and developmental processes, including cell death. Overproduction of the cleavage fragments and/or of uncleavable mutant proteins would help elucidate the functional consequences of substrate cleavage by metacaspases. 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. In spite of significant progress in our understanding of plant PCD in recent years, its control mechanism remains unclear. Deployment of reverse genetic approaches such as PTGS/RNAi strategies (Wang and Waterhouse, 2002) and knockout screens using T-DNA or transposon insertion collections (Bouche and Bouchez, 2001), coupled with global expression approaches such as micro-array analysis should help speed up the first essential step of identifying the important players involved in plant cell death activetion. This approach would be complementary to forward genetic approaches that are revealing new regulator(s) that may not have counterparts in other organisms.

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