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Programmed cell death in plants and caspase-like activities

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Citation
Gaussand, G. M. D. J. (2007, April 25). *Programmed cell death in plants and caspase-like activities*. Retrieved from https://hdl.handle.net/1887/11864

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/11864

Note: To cite this publication please use the final published version (if applicable).

Chapter 1

Involvement of caspases and caspase-like proteases in programmed cell death

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Abstract

Programmed cell death (PCD) plays an important role in development and maintenance of tissue homeostasis, and in defense against pathogens and environmental stresses. The molecular details of the signaling pathways underlying PCD, apoptosis in particular, have been well studied in animals. Cells undergo apoptosis through two major pathways controlled by complex regulatory networks: the extrinsic pathway (death receptor pathway) or the intrinsic pathway (mitochondrial pathway). Caspases are the key executioners of apoptosis in animals. In the past few years, the understanding of PCD mechanisms in plants has deepened. In plants, PCD plays an important role in development, response to pathogens and abiotic stress. The main question, still unanswered, is which proteases are the key executioners of PCD in plants.

Introduction

Cell death is divided in animal species into programmed cell death (PCD) and necrosis. PCD is an important process for multicellular organisms. As it removes superfluous, damaged or infected cells in an organized manner, PCD plays an important role in development, in tissue homeostasis, in defense against pathogens and to cope with adverse environmental stresses (Steller et al. 1995; Meier et al. 2000; Lawen 2003; Jin & El-Deiry 2005). In the past decades, PCD was held synonymous with apoptosis. The term apoptosis is derived from the Greek word for the process of leaves falling from trees or petals falling from flowers. It was introduced in the 1970s to differentiate a morphologically distinctive form of cell death associated with normal physiology (Kerr et al. 1972). Apoptosis is associated with activation of caspases, executioners of cell destruction. There are four mechanistic classes of proteases that have been recognized by the International Union of Biochemistry and Molecular Biology in 1984, including serine, cysteine, aspartic and metallo proteases (Table 1). Caspases belong to an evolutionary conserved family of cysteine proteases (Kroemer & Martin 2005). Necrosis is associated with acute injury to cells, leading to loss of membrane integrity, swelling and disruption of the cells. During necrosis, cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist & Jaatela 2001). Since, other types of PCD have been proposed for which cell death was found to occur in a programmed fashion but in complete absence and independent of caspase activation. PCD has been

classified into three main types according to Clarke's classification based on lysosomal involvement (Clarke 1990; Chipuk & Green 2004; Kim 2005):

- apoptosis (or type I cell death),
- autophagic cell death (also known as cytoplasmic, or type II cell death),
- necrosis-like cell death (also known as type III or non-lysosomal cell death).

Type I cell death, apoptosis, is a form of PCD morphologically defined by condensation of the nucleus and cytoplasm, association of chromatin with the nuclear periphery, DNA fragmentation, membrane blebbing, and engulfment and lysosomal degradation of the dying cell by a phagocyte (Kerr et al. 1972). Biochemical evidence has indicated the caspase family of cysteine protease as well as certain proteins of the mitochondria to be mediators of type I PCD. Type II, autophagic cell death, is characterized by sequestration of bulk cytoplasm and organelles in double or multi-membrane autophagic vesicles and their delivery to and subsequent degradation by the cell's own lysosomal system before the nucleus is destroyed (Bursch et al. 2000; Levine & Klionsky 2004). The sequestered cytoplasmic components may be degraded prior to heterophagocytosis of cellular remains. Type III, non-lysosomal or necrosis-like cell death, is characterized by breakdown of the plasma membrane, swelling of organelles, lysosome-independent formation of 'empty spaces' in the cytoplasm and disintegration of the cytoplasm (Gozuacik & Kimchi 2004).

Types I and II cell death have been observed in many animal species during development, whereas type III cell death is common in pathological conditions. Types II and III cell death are genetically regulated and often have morphological features resembling necrosis, yet their underlying molecular mechanisms are unclear. The various types of PCD have in common that they are executed by active cellular processes that can be intercepted by interfering with intracellular signaling. Those types can not be categorized because they might overlap since they share the same activation intermediaries or they can be activating each other (Lockshin & Zakeri 2004). The cellular components are safely isolated by membranes, and then consumed by adjacent cells and/or resident phagocytes without inflammation. The elimination of PCD debris may remain virtually unnoticed by the body (Fietta 2006). This distinguishes them from "accidental" necrosis. The caspase-independent cell death pathways - type II and type III cell death - are important safeguard mechanisms to protect the organism against unwanted and potential harmful cells when caspase-mediated routes fail but can also be triggered in response to cytotoxic agents or other death stimuli. In the case of accidental necrosis, cytosolic constituents chaotically spill into extracellular space through damaged plasma membrane and provoke an inflammatory response. The necrotic cell removal induces and amplifies pathological processes.

Mitochondria play not only a key role in cellular metabolism and in signal transduction cascades but also an important role in the regulation of PCD (Ferri & Kroemer 2001). Mitochondrial alterations – following release of sequestered apoptogenic proteins, loss of transmembrane potential, production of reactive oxygen species (ROS), disruption of electron transport chain, and decreases in ATP synthesis - have been shown to be responsible for the different types of cell death (Bras et al. 2005). Thus, the mitochondria can be viewed as a central regulator of the decision between cellular survival and cell death.

During cell death, the ATP levels are determinant in directing toward PCD or necrosis (Leist al. 1997; Nicotera et al. 1998; Formigli et al. 2000). The disruption of the mitochondrial electron transport chain would result in diminished ATP production and consequently in a striking perturbation of the bioenergetic state of the cell. The inhibition of ATP production has been observed in both type I and type III cell death. However, this phenomenon occurs relatively late in type I cell death, as the complete apoptotic program involves the energy-dependent formation of the apoptosome (cytochrome c/ Apaf-1 / dATP complex) and hydrolysis of macromolecules. By contrast, type III cell death is characterized by an early loss of ATP synthesis and seems to proceed in conditions of low cytosolic ATP levels (Kim et al 2003). ATP dependency has been observed for the autophagic type II pathway, apparently at the lysosomal level (Plomp et al. 1989). Moreover, intracellular nucleotides can regulate apoptosis. They can directly block the cytochrome c initiated apoptosome formation and the caspase activation by interfering with Apaf-1 (Chandra et al. 2006).

Calcium is a key regulator of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis. Mitochondrial matrix Ca^{2+} overload can lead to enhanced generation of reactive oxygen species (ROS). ROS will trigger the opening of the mitochondrial permeability transition pore (MPTP). If the pore remains open, cells cannot maintain their ATP levels and this will lead to cell death by necrosis. When cells experience a less severe insult, the MPTP may open transiently. The resulting mitochondrial swelling may be sufficient to cause release of cytochrome c and activation of the apoptotic pathway rather than necrosis (Forte & Bernardi 2005; Green & Kroemer 2004).

The efflux of cytochrome c from mitochondria is also a pivotal event in apoptosis, as it drives the assembly of the apoptosome in the cytoplasm (Adrain & Martin 2001). Afterwards the activation of a proteolytic cascade involving caspase proteases is an irreversible step (Abraham et al. 2004). Caspases cleave a variety of proteins after specific aspartate residues, ultimately leading to cell death. The contents of dead cells are packaged into apoptotic bodies, which are recognized by neighboring cells or macrophages and cleared by phagocytes.

Table 1: Protease classification according to their catalytic mechanisms (IUBMB, 1984). The inhibitors described here are just some examples which are commercially available from several companies. Those inhibitors may be used separately or in inhibitory cocktails to identify the class of a protease.

Protease classes	Examples of proteases	Protease inhibitors
serine proteases	chymotrypsin	Trypsin inhibitor
	trypsin	TPCK (does not inhibit trypsin)
	elastase	TLCK (does not inhibit chymotrypsin)
	kallikrein	PMSF
	thrombin	Leupeptin-hemisulfate
	plasmin	Hirudin (inhibits thombin)
	urokinase	Elastatinal (inhibits elastase)
	subtilisin	DFP
		DCI
		Chymostatin
		Benzamidine-HCl (inhibits thrombin and trypsin)
		AEBSF-HCl
		Antipain-HCL
		Antithrombin III
		(alpha)1-Antitrypsin
		APMSF-HCl (does not inhibit chymotrypsin and acetylcholine esterase)
		Aprotinin (does not inhibit subtilisin and thrombin)
cysteine proteases	Papain	TPCK
	Actinidin	TLCK
	bromelain,	E-64
	cathepsins B, L, S,	PMSF (inhibits papain)
	calpains	Leupeptin-hemisulfate
		Chymostatin
		Cathepsin Inhibitor (inhibits papain and Cathepsins B, L and S)
		Calpain Inhibitor I (inhibits calpain I, cathepsins B and L)
		Calpain Inhibitor II (inhibits calpain II, cathepsins B and L)
		CA-074 (inhibits cathepsin B)
		Antipain-HCL

Table 1: continued

aspartic proteases	pepsin chymosin cathepsin D renin	Pepstatin
metallo proteases	Aminopeptidases Leucine aminopeptidase	1,10-Phenanthroline Phosphoramidon EGTA EDTA-Na_2 Bestatin-HCl Leuhistin (inhibits aminopeptidase M) Phebestin (inhibits aminopeptidase N) Amastatin-HCl (does not inhibit aminopeptidase B) Arphamenine A or B (inhibits aminopeptidase B)

De-regulation of apoptosis may lead to pathological disorders such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Thompson 1995).

In plants, PCD plays normal physiological roles in a variety of processes in plants, including deletion of cells with temporary functions such as the aleurone cells in seeds and the suspensor cells in embryos; removal of unwanted cells, such as the root cap cells found in the tips of elongating plant roots and the stamen primordia cells in unisexual flowers; deletion of cells during sculpting of the plant body and formation of leaf lobes and perforations; death of cells during plant specialization, such as the death of TE cells which creates channels for water transport in vascular plants; leaf senescence; and responses to plant pathogens and abiotic stresses (Pennell & Lamb 1997; Danon et al. 2004; Kuriyama & Fukuda 2002). PCD in plants is an active suicidal process that removes unwanted or severely damaged cells (Dangl & Jones 2001; Kuriyama & Fukuda 2002). PCD in plant cells mostly resembles that of type II or autophagic cell death in animals (van Doorn & Woltering 2005; Liu et al. 2005). Some common features of type I cell death or apoptosis are conserved in both plants and metazoa (Danon & Gallois 1998; Yao et al. 2004). These include cytoplasm shrinkage, cytochrome c leakage out of mitochondria, chromatin condensation, altered nuclear morphology, DNA fragmentation in large fragments and DNA laddering. While the signalling events and cell death cascades have been well studied in animals, little is known about the regulation and execution of PCD in plants (Hoeberichts & Woltering 2003a). After

the elucidation of the complete Arabidopsis genome, it has become clear that no genes for the caspases and for the apoptotic regulators of the IAP and Bcl-2 families are present in plants. However, animal Bcl-2 members have been found to modify cell death processes in plants (Lam et al. 1999; Baek et al. 2004), indicating a possible identical apoptotic machinery in plants. Caspase-like activities have been measured in plant extracts and they seem to be involved in PCD processes, even though no sequence homologues have been found at the molecular level. Caspase-like activities cannot be inhibited by protease inhibitors other than animal caspase-specific ones (Korthout et al 2000; Lockshin & Zakeri 2002; Bozhkov et al 2004). Identification of such proteases is essential to reveal the molecular mechanism that operates in plant PCD and to provide some insights into differences between plant and animal PCD. The identification in plants of a class of putative proteases related to animal caspases and termed metacaspases (Uren et al. 2000) has stimulated research in this protein family. Two recent studies identified new types of substilisin-like proteases (named saspases-A and -B) from oats and a vacuolar processing enzyme (VPE) from tobacco that may play important roles as caspase-like proteases in the execution of PCD in plants (Coffeen & Wolpert 2004; Hatsugai et al. 2004; Hatsugai et al. 2006).

The aim of this review is to provide an overview of the molecular and cellular events that characterize PCD in animals and in plants. In addition, the recent developments concerning the possible involvement of proteases with caspase-like specificity in plant PCD will be described.

Caspases and programmed cell death in animals

The apoptotic cascade: discovery

The development of multicellular organisms involves an important balance between cell growth, cell division and cell death. The coordination of these cellular programs ensures appropriate organ and body size. Apoptosis has an important role in all animals, by forming and deleting structures, controlling cell numbers and eliminating abnormal damaged cells (Baehrecke 2002). Programmed cell death is a genetically encoded form of cell suicide that is central to the development and homeostasis of multicellular organisms.

The molecular mechanisms of apoptosis were discovered after intensive efforts (Jin & El-Deiry 2005). An early discovery was made in genetic studies of the nematode *Caenorhabditis elegans* which contains the basic components of the cell death machinery (as shown in figure 1). In *C. elegans*, cell death relies on the presence of CED-3 (ced, cell death

abnormal), CED-4, CED-9 and EGL-1 (egg-laying abnormal 1) (Liu & Hengartner 1999). The regulator of cell death in *C. elegans*, CED-3, was found to be related to the mammalian cysteine protease, interleukin-lβ converting enzyme, also named ICE or caspase-1 (Yuan et al. 1993). This finding suggested that the mechanism of cell death is conserved across species and that proteases are integral to the death program.

Gene interaction studies have defined a genetic pathway in *C. elegans* and have ordered the functions of *egl-1*, *ced-9*, *ced-3* and *ced-4* (Liu & Hengartner 1999). Homologues of the nematode core apoptotic pathway genes have been identified and have been shown to be conserved throughout evolution, along with additional activators, effectors and inhibitors of cell death (Putcha & Johnson 2004; Chinnaiyan 1999; Riedl & Shi 2004). In *C. elegans*, CED-4 (Apaf-1 homologue) binds to and activates CED-3 (caspase homologue). In healthy cells, CED-4 remains inactive by its association with CED-9 (antiapoptotic Bcl-2 homologue). The protein EGL-1 is a trigger of cell death and is expressed in response to certain developmental cues. EGL-1 binds to CED-9, displacing CED-4, which in turn activates CED-3 to induce apoptosis (Liu & Hengartner 1999). Activation and regulation of apoptosis in higher organisms depends on components such as those found in *C.elegans*, but more complex. The first caspase, caspase-1, was identified due to its ability to convert the precursor of interleukin-1β (IL-1β) to its mature form, a potent mediator of inflammation (Cerretti et al. 1992; Thornberry et al. 1992). Subsequent cloning of *ced-3*, a pro-apoptotic gene in *C. elegans*, revealed that it encodes a protein highly homologous to caspase-1 (Yuan et al. 1993).

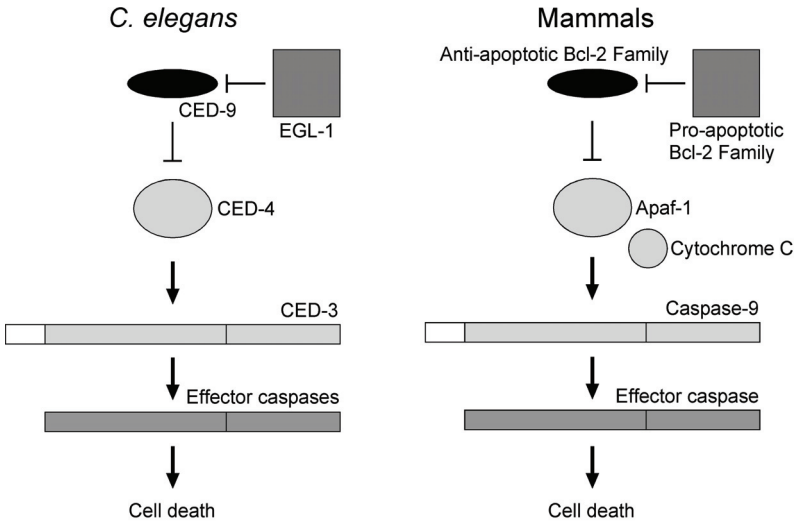

Figure 1: Evolutionary conserved cell death pathways in *C. elegans* and mammals. Functional homologues of caspases and their regulators across species are indicated by the same shape and shading.

A human sequence was identified, cloned and was shown to encode a 32 KDa cysteine protease, called CPP32 (Fernandes Alnemri et al. 1994). This identification was done with the DNA sequence encoding the active site of caspase-1 and CED-3 which was used to search an expressed sequence tag (est) database. Independently, two other groups identified a related caspase that was called caspase-3, one group naming it Yama (the Hindu god of death) and the other group Apopain (Tewari et al. 1995; Nicholson et al. 1995). Caspases (cysteine-dependent aspartate-specific proteases) are the key executioners of apoptosis and they belong to an evolutionary conserved family of cysteine proteases (Kroemer & Martin 2005). After discovery of caspase-1 and caspase-3 other caspases were found.

The apoptotic cascade: description

The apoptotic cascade can be initiated via two major pathways (figure 2). These pathways involve either the activation of death receptors in response to ligand binding (death receptor pathway), or the release of cytochrome c from the mitochondria (mitochondria pathway) (Ashkenazi & Dixit 1998; Hengartner 2000; Broker et al. 2005).

PCD can be triggered by the two pathways mentioned before. Both pathways involve a specific family of cysteine proteases, the caspases, that are activated to execute PCD. The execution of PCD results in the typical morphologic changes (Degterev et al. 2003).

In mammals, the cell surface death receptor-mediated pathway (figure 2, left) involves cell surface death receptors such as Fas, Tumor Necrosis Factor (TNF), or TRAIL receptors (Ashkenazi & Dixit 1998). Death ligand stimulation, via a series of protein-protein interactions, results in oligomerization of the receptors and recruitment of an adaptor protein and caspase-8 or -10, forming a death-inducing signalling complex (DISC). Autoactivation of caspase-8 at the DISC is followed by activation of other caspases, including caspase-3, -6 and -7. These activated caspases function as downstream effectors of the cell death program.

The other caspase activation pathway in mammals is the mitochondria pathway (figure 2, right). This pathway is characterized by a depolarization of the mitochondrial membrane and a release of mitochondrial proteins (Danial & Korsmeyer 2004) including pro-apoptotic proteins, such as cytochrome c, into the cytosol. A cytosolic complex, the apoptosome, is then formed. This complex consists of oligomerised Apaf-1 (apoptotic protease-activating factor 1), ATP/dATP, cytochrome c and the initiator caspase, pro-caspase-9 (Chinnaiyan 1999; Riedl & Shi 2004). Oligomerisation of Apaf-1 allows the

recruitment and autocatalytic activation of caspase-9, and consequently the propagation of a death signal by proteolytic processing and activation of effector caspases (Li et al. 1997).

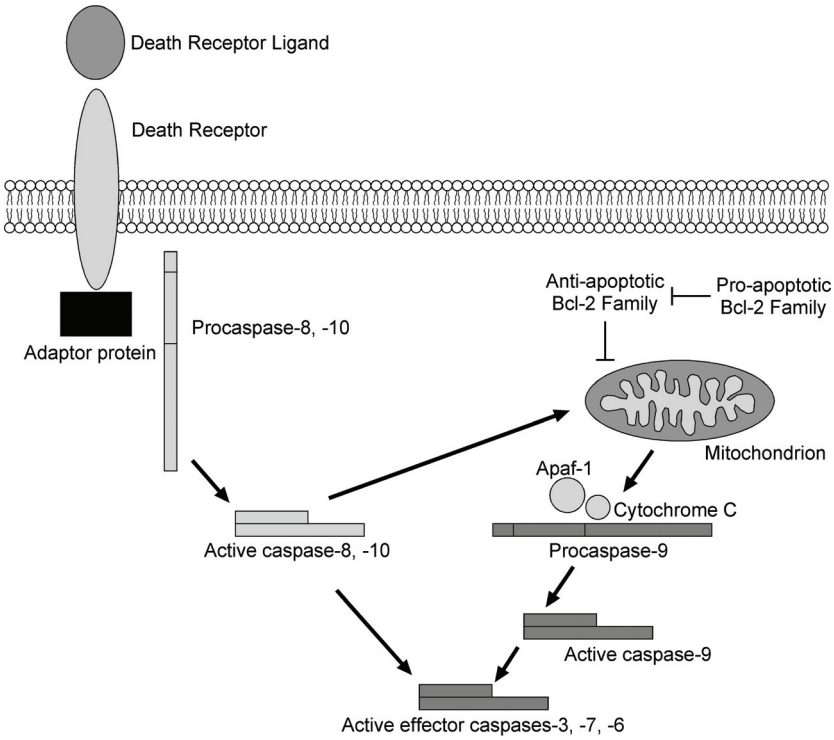

Figure 2: Apoptotic pathways leading to mammalian cell death. The extrinsic apoptotic pathway (left) is induced by a death receptor ligand (TNF, TRAIL, FasL etc) which results in the recruitment and formation of a multiprotein complex DISC that includes the death receptor, intracellular adaptor proteins (TRADD, FADD, RAIDD) and initiator caspases (procaspase-8 or -10). The complex leads to autocatalytic processing and activation of the initiator caspase. The intrinsic pathway (right) is initiated by the majority of apoptotic stimuli, including irradiation, cytotoxic drugs, DNA damage etc. Loss of mitochondrial membrane potential and release of pro-apoptotic cell death proteins results in the formation of another multiprotein complex, the apoptosome, that includes Apaf-1, cytochrome c, ATP/dATP and the initiator caspase, procaspase 9. That complex leads to autocatalytic activation of caspase-9 and subsequent effector caspases. Pro- and anti-apoptotic bcl-2 homologues regulate the release of pro-cell death mitochondrial proteins.

Apart from the pathways now described, there is another cell death pathway. That pathway involves the endoplasmic reticulum (ER), and it is known as the apoptotic pathway of ER stress-mediated cell death (Momoi 2004). The endoplasmic reticulum (ER) is the site of assembly of polypeptide chains that are destined for secretion or routing into various subcellular compartments. The ER-initiated PCD pathway comprises the activation of caspase-12 and/or the cytochrome c-dependent apoptotic pathway.

The caspases

Caspases are present in the cytosol of most cells. Caspases are expressed as inactive single-chain zymogens of 30-50 kDa (figure 3) with three domains: a pro-domain, a large subunit domain (*ca.* 20 kDa), and small subunit domain (*ca.* 10 kDa). The domains are separated by aspartate cleavage sites in interdomain linkers (Chang & Yang 2000). The pro-domain is important for the regulation of activation, which results in proteolytic cleavage of the linkers, usually accompanied by loss of the pro-domain. Activation can be by autoactivation, transactivation, or proteolysis by other peptidases (Wolf & Green 1999).

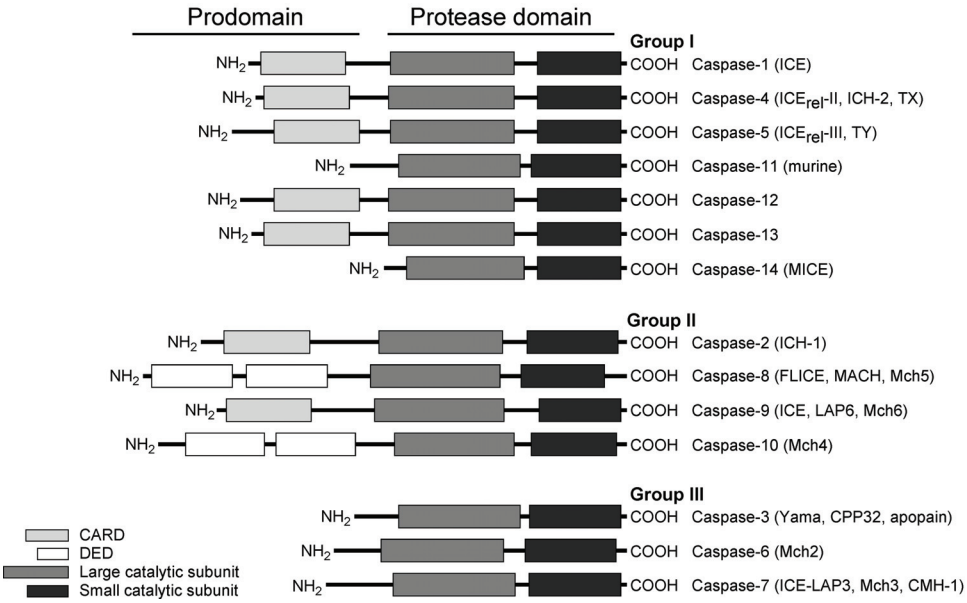

Figure 3: Mammalian caspase family. All mammalian caspases shown are of human origin except for murine caspase-11 and -12. Three major groups of caspases are presented. Group I: inflammatory caspases; group II: apoptosis initiator caspases; group III: apoptosis effector caspases. The CARD, the DED, the large (p20) and small (p10) catalytic subunits are indicated.

Based on their function, caspases can be classified into three groups (figure 3, Thornberry & Lazebnik 1998). The first group consists of inflammatory caspases. This group includes caspase-1, -4, -5, -11, -12, -13 and -14, which are involved in inflammation. The second group consists of apoptotic initiator caspases. Initiator caspases possess long pro-domains that contain either a death effector domain (DED) (caspase-8 and -10) or a caspase activation and recruitment domain (CARD) (caspase-2 and -9). Both domains interact with upstream adaptor molecules. The third group consists of effector caspases. This executioner

class (caspase-3, -6 and -7) is characterized by the presence of a short pro-domain. These caspases are processed and activated by upstream caspases. The caspases of the executioner class perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates such as regulators of apoptosis, housekeeping and structural proteins (reviewed in Cohen 1997; Nunez et al. 1998; Thornberry & Lazebnik 1998; Wolf & Green 1999).

Initiator but not effector caspases have long pro-domains that contain modules which mediate protein-protein interactions between the caspases themselves and with regulatory adaptor molecules. Two types of modules have been defined: death effector domains (DEDs) and caspase recruitment domains (CARDs). These modules allow for the assembly of proteins into oligomeric structures, and this assembly induces autoprocessing and activation of the initiator caspases (reviewed in Nunez et al. 1998; Wolf & Green 1999; Stennicke & Salvesen 2000).

The other members of the apoptotic cascade

The caspase-cascade signalling pathway is regulated by various molecules, receptors, enzymes and gene regulating proteins such as the Bcl-2 family proteins and the inhibitor of apoptosis protein (IAP) (Launay et al. 2005; Fan et al. 2005). A few of them are here described.

Bcl-2 derives its name from B-cell lymphoma 2, a tumor which was the consequence of a reciprocal gene translocation in chromosomes 14 and 18 in follicular lymphomas including the Bcl-2 gene (Vaux et al. 1988). The members of the Bcl-2 family are a group of regulatory factors crucial in apoptosis. According to functional and structural criteria, the members can be divided into two groups:

- Group I proteins are all anti-apoptotic proteins, including A1/Bfl1, Bcl-2, Bcl-w, Bcl-xL, Boo/Diva, Mcl-1, NR-13 and Nrf3 in mammals, BHRF-1, E1B19K, Ks-Bcl-2, LMW5-HL, and Ced-9 in *C. elegans* (Fu & Fan 2002; Milosevic et al. 2003; Gross et al. 1999). They all have four short Bcl-2 homology (BH) domains: BH1, BH2, BH3 and BH4. Their function is to inhibit pro-apoptotic proteins of the Bcl-2 family by binding to them.
- Group II proteins are all pro-apoptotic proteins, including Bad, Bak, Bax, Bcl-rambo, Bcl-xS, Bid, Bik, Bim, Blk, BNIP3, Bok/Mtd, Hrk and Nip3 in mammals, and Egl-1 in *C. elegans* (Milosevic et al. 2003). Bax and Bak, originally localized in the cytoplasm, can translocate to the mitochondrial outer membrane after an apoptotic program starts. Following the translocation, they will undergo conformation changes, oligomerization and insertion into

the mitochondrial outer membrane to elevate the permeability of mitochondrial permeability transition pores (MPTPs).

Group I proteins bind to the active conformation of Bax to prevent it from inserting into the mitochondrial outer membrane. The binding maintains the normal permeability of MPTPs, and it prevents the release of mitochondrial pro-apoptotic factors such as cytochrome c, AIF and Smac/DIABLO (Lü et al. 2003; Fan et al. 2001). Through cytochrome c, AIF, and others, the Bcl-2 family proteins indirectly regulate the activity of caspases in related apoptotic pathways (Fan et al. 2001). There are a number of theories concerning how the Bcl-2 family proteins exert their pro- or anti-apoptopic effect. According to one such theory, the effect depends on activation or inactivation of an inner MPTP, which is involved in the regulation of matrix Ca^{2+}, pH, and voltage. It is also thought that some Bcl-2 family proteins induce (pro-apoptopic members) or inhibit (anti-apoptopic members) the release of cytochrome c in to the cytosol which, once there, activates caspase-9 and caspase-3, leading to apoptosis. Zamzami et al. (1998) suggest that the release of cytochrome c is in fact mediated by effects of the permeability transition pore on the inner mitochondrial membrane, linking the theories. The site of action for the Bcl-2 family is mostly on the outer mitochondrial membrane (OMM). There are apoptogenic factors (cytochrome c, Smac/DIABLO, Omi) within the mitochondria. If these factors are released, they activate the executioners of apoptosis: the caspases. Depending on their function, once activated, Bcl-2 proteins either promote the release of these factors, or keep them sequestered in the mitochondria.

The Inhibitor of Apoptosis Protein (IAP) was first identified in insect cells infected by *baculovirus*. Encoded by a viral gene, IAP can inhibit infected host cells from executing the apoptotic program. So far, in humans, the identified members of the IAP family include cIAP1, cIAP2, XIAP (X-linked mammalian inhibitor of apoptosis protein), NAIP (neuronal apoptosis inhibitory protein), survivin and livin (Fan et al. 2005). The activity of mammalian IAP can be inhibited by Smac/DIABLO released from mitochondria (Costantini et al. 2002). IAP family proteins may also have other functions besides caspase inhibition. As reported by Uren et al. (1998), IAP family members in yeast could neither unite caspases nor induce apoptosis. Caspases are suppressed by IAP (Fesik & Shi 2001). When a cell receives an apoptotic stimulus, IAP activity is relieved after SMAC (Second Mitochondria-derived Activator of Caspases), a mitochondrial protein, is released into the cytosol. SMAC binds to IAPs, effectively preventing them from arresting the apoptotic process.

The death substrates

The list of putative proteins that are cleaved by caspases either *in vivo* or *in vitro* is growing (Earnshaw et al. 1999; Nicholson 1999; Fischer et al. 2003). However, only a few of these proteins have been established as biologically relevant death substrates, and many others may just represent 'innocent bystanders' (Hengartner 2000).

Caspases are synthesized as proenzymes that are activated through cleavage at internal aspartate residues by other caspases or by autoactivation. The proteolytic cleavage of a caspase can induce a dramatic conformational change that exposes the catalytic pocket of the enzyme, and therefore results in the enzyme's activation. The proteolytic activation of caspases can be achieved either by autocatalysis or by an upstream protease. A caspase that cleaves and activates itself is called an initiator caspase. Once an initiator caspase is activated, it triggers a cascade to activate downstream executioner caspases. Subsequently, the activated executioner caspases cleave numerous cellular targets to destroy normal cellular functions, activate other apoptotic factors, inactivate anti-apoptotic proteins, and eventually lead to apoptotic cell death. The executioner caspases recognize specific tertiary motifs in their substrates and cleave their substrates after specific aspartate residues, ultimately leading to cell death. The cleaved substrates are cytoskeletal proteins including lamins, α-fodrin and actin, proteins involved in DNA repair and cell-cycle regulation such as poly(ADP-ribose) polymerase (PARP) and retinoblastoma protein (Launay et al. 2005; Ruchaud et al. 2002; Zhivotovsky 2003).

Table 2: the substrate specificity of caspases

	Caspase	Substrate specificity
Group I	Caspase-1	WEHD YVAD
	Caspase-4	(W/L)EHD
	Caspase-5	(W/L)EHD
	Caspase-13	WEHD
	Caspase-14	WEHD
Group II	Caspase-2	DEHD
	Caspase-8	I/LETD
	Caspase-9	LEHD
	Caspase-10	LEXD
Group III	Caspase-3	DEVD
	Caspase-6	VE(H/I)D
	Caspase-7	DEVD

The specificity requirements of the caspases were derived from studies with short synthetic peptides. The abundant nuclear enzyme PARP catalyses the attachment of poly (ADP-ribose) to several acceptor proteins, including itself, in response to DNA strand breaks. PARP cleavage by caspases-3 and -7 bisects a bipartite nuclear localization signal (Schreiber et al. 1992), and results in a form that cannot attach ADP-ribose polymers in response to damaged DNA (Lazebnik et al. 1994). PARP was one of the first identified examples of a substrate processed by an effector caspase much more efficiently than by either the inflammatory caspase-1 (Gu et al. 1995) or the initiator caspase-8 (Nicholson et al. 1995). Based on the cleavage site of PARP (DEVD↓G), a synthetic model substrate was developed: Ac-DEVD-AMC. Ac-DEVD-CHO and its biotinylated derivative (biotin-DEVD-CHO) were synthesized as specific inhibitors of PARP cleavage and as affinity ligands for purification of the protease.

Caspase-3 turned out to be one of the key executioners of apoptosis; this caspase bears either full of partial responsibility for the proteolytic cleavage of many key proteins, each of which contains an Asp-Xaa-Xaa-Asp (DXXD) motif that is similar to the one in PARP. Because all substrates of caspase-3 contain DEVD sequences, artificially synthesized tetra peptides Ac-DEVD-AMC and Ac-DEVD-CHO are used as the specific substrate and inhibitor of caspase-3, respectively. The inhibitors described in table 1 are used individually or in inhibitory cocktails to inhibit specific protease or protease classes. Caspases can only be inhibited with specific caspase inhibitors. Most of the synthetic peptide caspase inhibitors were developed based on the tetrapeptide caspase recognition motif. Therefore, the selectivity of inhibitors matches the caspase substrate specificities described above (Table 2). The introduction of an aldehyde group at the C terminus of the tetrapeptide results in the generation of reversible inhibitors (Graybill et al. 1994), whereas a fluoromethyl ketone (fmk), a chloromethyl ketone (cmk) (Estrov et al. 1995), or a diazomethyl ketone (dmk) (Thornberry et al. 1992) at this position irreversibly inactivates the enzyme.

Developmental defects, autoimmune diseases, neurodegeneration or cancer

Balance between cell division and cell death is of utmost importance for the development and maintenance of multicellular organisms. Deregulation of apoptosis may lead to pathological disorders such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Broker et al. 2005). Understanding of cell death signaling pathways is relevant to understanding cancer and to developing more effective therapies.

Numerous diseases have been attributed to apoptotic machinery malfunction. Altered expression and / or activity of pathway components - including receptors, ligands, adaptors, caspases and substrates - contribute to several neurodegenerative diseases, some

types of autoimmune disorder and cancer. Alzheimer's disease, Parkinson's disease and Huntington's disease are associated with excessive cell death that involves defective regulation of caspase activity (Ho & Hawkins 2005). Increased activities of caspases-8 and -9 have been observed in brain tissues (Rohn et al. 2001; Yew et al. 2004; Rohn et al. 2002) and in peripheral blood mononuclear cells of Alzheimer's disease patients (Tacconi et al. 2004). The activities of caspases-8 and -9 were reported to be elevated in brain tissue from Parkinson's disease patients (Hartmann et al. 2001; Viswanath et al. 2001). Huntington's disease, another neurodegenerative disorder, is caused by the abnormal expansion of polyglutamine repeats in the Huntington protein, which recruits and activates caspase-8 (Sanchez et al. 1999). Caspase-10 has also been proposed to play a role in the pathological development of Huntington's disease, in a fashion similar to that of caspase-8 (U et al. 2001). The Bcl-2 gene has been implicated in a number of cancers, including melanoma, breast, prostate and lung carcinomas. It is also thought to confer cellular resistance to chemotherapy (Pusztai et al. 2004). Carcinogenesis has been linked to abnormalities in the apoptotic pathway, and many drugs that are targeted at different parts of this pathway are being developed.

Many promising drugs target the extrinsic death receptor pathway as well as the intrinsic mitochondrial apoptotic pathway. There are also developments in targeting initiator and effector caspases, as well as the death domains that are involved in transducing the apoptotic signals (Dlamini et al. 2005).

Caspase-like proteases and programmed cell death in plants

Observation of plant PCD

In plants, PCD plays an important role in development and in the responses to pathogens and abiotic stress (Wang et al. 1996a, 1998, 1999; Pennell & Lamb 1997; Beers & McDowell 2001; Jones 2001; Danon et al. 2004; Kuriyama & Fukuda 2002). Several examples demonstrate that PCD is an important process occurring in plants during gamete and embryo formation or during plant development or in response to pathogen attack.

The tapetum plays a crucial role in pollen development. This secretory tissue produces numerous nutritive proteins that are necessary for pollen maturation. The tapetum also produces exine that is the main structural component of the pollen wall (Ku et al. 2003; Kawanabe et al. 2006). The tapetum undergoes PCD during the later stages of pollen development (Wu & Cheung 2000; Papini et al. 1999; Wang et al. 1999). Premature PCD of

the tapetum causes failure in pollen development and is associated with male sterility (Ku et al. 2003).

During pollination, plants enforce self-incompatibility (SI) as an important means to prevent self-fertilization. Research on the corn poppy (*Papaver rhoeas*) has revealed that proteins in the pistil on which the pollen lands interact with pollen, and triggers PCD in incompatible (self) pollen. Thomas & Franklin-Tong (2004) found that the response involves rapid inhibition of pollen-tube growth, followed by PCD.

PCD is involved in the elimination of certain cells during plant embryogenesis, as was shown in Norway spruce somatic embryogenesis. Two successive waves of PCD are implicated in the transition from proliferating proembryogenic masses (PEMs) to somatic embryos and in correct embryonic pattern formation, respectively (Filonova et al. 2000). The first wave of PCD triggers the degradation of PEMs when they give rise to somatic embryos. The second wave of PCD eliminates terminally differentiated embryo-suspensor cells during early embryogenesis.

In barley androgenesis, PCD takes place during the transition from multicellular structures to globular embryos (Maraschin et al. 2005, chapter 3). PCD takes place in the small cell domain of the multicellular structures. The cell death of this domain marked the site of exine wall rupture. Chromatin condensation and DNA degradation preceded cell detachment and cytoplasm dismantling. This PCD was characterized by the formation of vesicles and vacuoles that contained cytoplasmic material. PCD was accompanied by an increase of activity of caspase-3-like proteases.

The endosperm of cereals functions as a storage tissue in which the majority of starch and seed storage proteins are synthesized (Young & Gallie 2000). During seed maturation, the endosperm degradation process is accompanied by typical PCD-related changes of cell morphology and internucleosomal DNA cleavage (Wojciechowska & Olszewska 2003).

The perforations in the leaf blades of *Monstera obliqua* (Araceae) arise through PCD early in leaf development because a subpopulation of cells undergoes PCD simultaneously (Gunawardena et al. 2005). DNA cleavage was demonstrated by TUNEL but was found to be randomly degraded but not as multiples of internucleosomal units. Cells were found to be misshapen with densely stained nuclei with condensed chromatin, disrupted vacuoles, and condensed cytoplasm.

During post-germinative seedling growth, the white spruce (*Picea glauca*) megagametophyte undergoes PCD (He & Kermode 2003a and 2003b). The death process is accompanied by internucleosomal DNA cleavage, activation of several nucleases and proteases.

During germination of barley grains, DNA fragmentation was observed in the aleurone by TUNEL staining accompanied by activation of hydrolytic enzymes such as alpha-amylase (Wang et al. 1996a).

During vascular development, xylem precursor cells give rise to tracheary elements (TE), xylem parenchyma cells and xylem fibres, which together form xylem. For maturition TE are emptied by the loss of all cell contents, including the nucleus, to form hollow xylem tubes. Several enzymes - such as cysteine proteases, serine proteases, RNases, S1-type nucleases, acid phosphatases and lipases (Demura et al. 2002) - are synthesized and then transported to the vacuole where they are activated (Funk et al. 2002). The autolysis of TE starts with the rupture of the vacuole (Groover et al. 1997; Kuriyama 1999; Obara et al. 2001).

Plants can recognize certain pathogens and activate defenses, called the resistance response, that result in the limitation of pathogen growth at the site of infection. One hallmark of the resistance response is the induction of a localized cell death response, called the hypersensitive response or HR, at the site of the infection. HR limits the pathogen's nutrient supply, since the dying tissue rapidly becomes dehydrated. The induction of HR by some pathogens and elicitors (molecules secreted by pathogens) is similar to apoptosis in animals, since apoptotic features - such as DNA breaks with 3'OH ends, blebbing of the plasma membrane as well as nuclear and cytoplasmic condensation - are present in some cells undergoing HR (Levine et al. 1996; Wang et al. 1996b; Ryerson & Heath 1996, Heath 2000). Many researchers have identified genes involved in the control and execution of HR. They, for example, identified mutant lines in which cell death is deregulated. These mutants (*acd2* for accelerated cell death 2 and *lsd* lesions simulating disease) are called lesion mimics because their phenotypes resemble pathogen-inducible HR cell death. They were classified into two groups: initiation mutants and feedback or propagation mutants (Pontier et al. 1998; Lorrain et al. 2003). This classification is based upon the assumption that two different mechanisms are involved in controlling cell death: a pathway to initiate PCD and a pathway to suppress PCD.

The examples given so far concern PCD during gamete, embryo or post embryo development. In natural environments plants are subjected to many environmental stresses - such as cold stress, light stress, mechanical stress and also stresses caused by bacterial and fungal pathogens - which in turn can cause secondary oxidative stress (Hippeli et al 1999). The excessive formation of reactive oxygen species (ROS) in response to the primary, environmental stress activates a signal transduction pathway that may be independent of or additive to the signals induced by the primary stress (Levine et al. 1996). The intensity of the secondary oxidative stress may vary from very mild to extreme, depending on the severity of

the primary stress. During mild stress, plants activate the antioxidant responses; more severe stress activates the PCD pathway, while extreme stress causes necrosis (Levine et al. 1994; Willekens et al. 1997).

Many examples demonstrate that PCD occurs in plant cells following abiotic stimuli. Heat-shock treatment triggers PCD as demonstrated in tobacco (Vacca et al. 2004), and in rice (chapter 4). DNA laddering was found in cucumber (Balk et al. 1999), nuclear condensation and cytoplasm shrinkage were found in carrot (Mc Cabe et al. 1997), and caspase-like activity was detected in tobacco (Tian et al. 2000) and in oat (Coffeen & Wolpert 2004) after heat-shock induction.

The apoptotic pathway

Concerning animals, the term apoptosis (Kerr et al. 1972) refers to a morphological type, often observed in PCD, that involves nuclear shrinkage and fragmentation, cellular shrinkage, DNA fragmentation, membrane blebbing, formation of apoptotic bodies, and digestion by macrophages (Wyllie 1980). Some elements of the same cell suicide mechanisms used in animal cells may be functionally conserved in plants. Though the biochemical mechanisms responsible for cell suicide in plants are largely unknown, a variety of reports suggest similarities to apoptosis that occurs in animal species. The morphological characteristics of plant cells undergoing PCD also bear some striking similarities to apoptosis in animals, though the presence of a cell wall around plant cells imposes certain differences.

Akin to animal cells, PCD in plants is associated with cytochrome c release, cell shrinkage, cytoplasmic condensation, chromatin condensation, and internucleosomal DNA fragmentation (DNA ladders). DNA fragmentation is a PCD hallmark. Salt stress, for instance, induces nuclear fragmentation and DNA degradation into oligonucleosomal fragments in barley roots (Katsuhara & Kawasaki 1996; Katsuhara 1997). Another example is DNA laddering in Arabidopsis roots and maize cultured cells induced by mannose (Stein & Hansen 1999). Ito and Fukuda (2002) reported nuclear degradation triggered by the collapse of the vacuole in tracheary elements of the xylem.

In various plant systems, the release of cytochrome c from mitochondria into the cytosol precedes cell death (Sun et al. 1999; Hansen 2000; Balk et al. 1999; Balk & Leaver 2001; Curtis & Wolpert 2002; Krause & Durner 2004; Tiwari et al. 2002). *In vivo*, cytochrome c release into the cytosol was observed as an early event during PCD in cucumber (Balk et al., 1999), maize suspension culture cells (Stein & Hansen 1999), tobacco protoplasts (Sun et al., 1999), and Arabidopsis suspension cells (Krause & Durner 2004).

In addition, evidence of the existence of a mitochondrial permeability transition (MPT) during PCD is accumulating in isolated plant mitochondria *in vitro* and plant tissue *in vivo* (Arpagaus et al. 2002; Curtis & Wolpert 2002; Tiwari et al. 2002; Yao et al. 2004). These findings indicate that mitochondrial function may be shared in a very similar way during PCD in both animals and plants.

Calcium is an almost universal intracellular messenger, controlling a broad range of cellular processes, including animal apoptosis. In plant PCD, Ca^{2+} has also been recognized as a ubiquitous signal. Elevated Ca^{2+} levels have been observed during tracheary element differentiation (Yu et al. 2002), aerenchyma formation (Bouranis et al. 2006), wheat aleurone differentiation (Kuo et al. 1996), HR (Mittler et al. 1999), and leaf senescence (Oh et al. 1996).

Ectopic expression of certain animal anti-apoptosis genes in transgenic plants has been demonstrated to provide protection from pathogens and other insults as a result of cell death suppression (Dickman et al. 2001; Mitsuhara et al. 1999). Conversely, expression of animal pro-apoptotic proteins such as Bax in plants can induce cell death mechanisms similar to endogenous programs for cell suicide (Lacomme & Santa Cruz 1999; Yoshinaga et al. 2005). Heterologous Bax induces PCD in a predominantly ROS-dependent manner and localizes to Arabidopsis mitochondria *in vivo* (Baek et al., 2004). Bax Inhibitor-1 (BI-1) is an anti-apoptotic protein which is conserved in both animal and plant species - including rice, Arabidopsis, barley, oilseed rape and tobacco - (Bolduc et al. 2003; Huckelhoven et al. 2003; Kawai et al. 1999; Kawai-Yamada et al. 2001; Lam et al. 2001; Sanchez et al. 2000; Watanabe & Lam 2006). Arabidopsis BI-1 has been shown to protect transgenic plants against cell death induced by ectopic expression of mammalian Bax (Kawai-Yamada et al. 2001; Baek et al. 2004), indicating an *in vivo* role for BI-1 in cytoprotective pathways in planta and suggesting that the biochemical mechanism regulated by BI-1 is evolutionary conserved. BI-1 overexpression also regulates resistance to fungal pathogens in barley, probably due to its cell death-suppressive effects (Huckelhoven et al. 2003). Antisense-mediated downregulation of BI-1 in tobacco BY-2 cells results in accelerated cell death upon carbon starvation (Bolduc & Brisson 2002). Endogenous expression of BI-1 is induced during wound-healing responses and upon exposure to certain pathogens in plants (Huckelhoven et al., 2001; Sanchez et al., 2000), suggesting that BI-1 may play a role in host defense mechanisms during times of stress. Thus, BI-1 represents the first endogenous gene to be identified that regulates cell death in both plant and animal cells.

As described previously, some common features of type I cell death or apoptosis are conserved in both plants and metazoa (Danon & Gallois 1998; Yao et al. 2004). Apoptosis, as it is in animals, apparently does not occur in plants. Plants do not have an immune system

with phagocytosis. For example, engulfment of apoptotic bodies and degradation in another cell is not found during plant PCD and the rigid and thick cell walls of plant cells do not help engulfment and degradation. PCD in plant cells mostly resembles that of type II or autophagic cell death in animals (van Doorn & Woltering 2005; Liu et al. 2005). Vacuolization of the cytoplasm through autophagy and vacuole disruption are observed in most cases of plant PCD: somatic embryogenesis, xylem differentiation, aleurone layer elimination during germination, and HR. The vacuole contains many of the hydrolytic activities that carry out PCD - including proteases, RNases and DNases - physically separated from their targets (Lam 2004; Ito & Fukuda 2002). The tonoplast (the vacuolar membrane) collapses by a mechanism that is still not understood, initiating the massive degradation of cellular contents.

The enzymes involved in plant PCD

In view of the strong conservation of the regulators and the executioners in animals, it was initially expected that the same actors would be involved in plant PCD. Indeed, caspase-like enzymatic activities were detected in plant tissues undergoing PCD. Different proteases were found to be associated with plant PCD, including senescence (Delorme et al. 2000; Schmid et al. 2001), oxidative stress (Solomon et al. 1999), seed development (Schmid et al. 1998, 1999; Wan et al. 2002), tracheary element development (Runeberg-Roos & Saarma 1998; Groover & Jones 1999), and the HR (Vera & Conejero 1988; D'Silva et al. 1998; Kruger et al. 2002). These proteases may be involved in the terminal decomposition of the dying cell, but not in the initiation or progression of PCD (Rotari et al. 2005).

Caspase-like activity associated with PCD was mentioned in several studies (del Pozo & Lam 1998; Lam & del Pozo 2000; Sun et al. 1999; Tian et al. 2000; de Jong et al. 2000; Mlejnek & Prochazka 2002; Danon et al. 2004; Belenghi et al. 2004; Maraschin et al. 2005; see also chapters 2, 3 and 4). Table 3 lists the various caspase-like activities detected in plants. Researchers who used synthetic caspase substrates and inhibitors have reported on different caspase-like proteases, such as -1, -3 and -6 (Chen et al. 2000; Bozhkov et al. 2004). Caspase-like activity was found during HR induced by a pathogen attack of tobacco leaves (del Pozo & Lam 1998), and during chemical and stress induced cell death of tobacco suspension cells and protoplasts (de Jong et al. 2000).

Caspase-like activities were measured with the caspase-specific substrate (Ac-DEVD-AMC) in the megagametophyte before seed germination (He & Kermode 2003a). These activities gradually increased after germination, until a certain length of the seeds radicle, hypocotyl and cotyledons was obtained. The activities were inhibited by the specific caspase-3 inhibitor (Ac-DEVD-CHO). Camptothecin-induced PCD in tomato suspension cells

can be inhibited with caspase-specific peptide inhibitors, which suggests that caspase-like proteases are involved in the death process (de Jong et al. 2000). Caspase-3 like activity has been detected in tobacco suspension cells after heat shock-induced apoptosis (Tian et al. 2000), and in embryonic suspension cells of barley (*Hordeum vulgare*) (Korthout et al. 2000). Both caspase-1 and caspase-3 like activities were observed in tomato suspension cells after chemically induced apoptosis (de Jong et al. 2000). Caspase-1 like activity has been detected in Arabidopsis suspension cultured cells after nitric oxide-induced cell death (Clarke et al. 2000), and in tobacco BY-2 cells after isopentenyladenosine-induced apoptosis (Mlejnek & Prochazka 2002).

Table 3: List of caspase-like activities in plants cells, adapted from Rotari et al. (2005).

Activity	Tissue	Buffer	Literature
Caspase-1 like (YVADase)	Tobacco leaf tissue	50mM HEPES, pH 7,5, 20% glycerol, 1mM EDTA, 1mM DTT, 1% BSA, 1mM PMSF	del Pozo & Lam (1998)
	Barley embryonic suspension cells	25mM HEPES -KOH, pH7.5, 10% sucrose,1mM DTT, 0.1% Triton X-100, 1mM PMSF	Korthout et al. (2000)
	Arabidopsis thaliana seedlings	50mM sodium acetate, pH5.0, 20% glycerol, 0.1% Triton, 10mM EDTA, 3mM DTT, 2mM PMSF	Danon et al. (2004)
	Germination of white spruce seeds	50mM sodium acetate pH5.5, 20% glycerol, 1mM EDTA, 1mM DTT, 0.2% BSA and 1mM PMSF	He & Kermode (2003)a and b
	Tobacco (BY2) suspension cells	50mM HEPES, pH7.2, 1mM EDTA, 0.2% CHAPS, 5mM DTT, protease inhibitor cocktail (Roche)	Mlejnek & Prochazka (2002)
	Pisum sativum shoots	20 mM MES pH6.6, 0.25 mM DTT, 100 mM NaCl, 2.5 mM EDTA,	Belenghi et al. (2004)
Caspase-3 like (DEVDase)	Rice suspension cells and Arabidopsis *sns* plant tissue	100mM HEPES, 10% sucrose, 0.1% CHAPS, 5mM DTT, 10^{-6}% Nonidet P-40, pH7.0,	Chapters 2,3,4 and 5
	Barley microspores	100mM HEPES, 10% sucrose, 0.1% CHAPS, 5mM DTT, 10^{-6}% Nonidet P-40, pH7.0,	Maraschin et al. (2005, chapter 3)
	Barley embryonic suspension cells	25mM HEPES -KOH, pH7.5, 10% sucrose,1mM DTT, 0.1% Triton X-100, 1mM PMSF	Korthout et al. (2000)
	Arabidopsis thaliana seedlings	50mM sodium acetate, pH5.0, 20% glycerol, 0.1% Triton, 10mM EDTA, 3mM DTT, 2mM PMSF	Danon et al. (2004)

Table 3: continued

Caspase-3 like (DEVDase)	Germination of white spruce (*Picea glauca*) seeds	50mM sodium acetate pH5.5, 20% glycerol, 1mM EDTA, 1mM DTT, 0.2% BSA and 1mM PMSF	He & Kermode (2003)a and b
	Tobacco (BY2) suspension cells	50mM HEPES, pH7.2, 1mM EDTA, 0.2% CHAPS, 5mM DTT, protease inhibitor cocktail (Roche)	Mlejnek & Prochazka (2002)
	Avena sativa leaves	20mM MOPS, pH7, 1mM DTT	Coffeen & Wolpert (2004)
	Tobacco (BY2) suspension cells	50mM HEPES, pH7.4, 100mM NaCl, 0.1% CHAPS,1mM DTT, 0.1mM EDTA, 10% glycerol	Tian et al. (2000)
	Embryogenic cell line of Norway spruce	100mM HEPES, 10% sucrose, 0.1% CHAPS, 5mM DTT, 10^{-6}% Nonidet P-40, pH7.0.	Bozhkov et al. (2004) Suarez et al. (2004)
	Papaver pollen	50mM HEPES pH7.4, 10mM NaCl, 0.1% CHAPS, 10mM DTT, 1mM EDTA, 10% glycerol	Thomas & Franklin-Tong (2004)
Caspase-6 like (VEIDase)	Rice suspension cells and Arabidopsis *sns* plant tissue	100mM HEPES, 10% sucrose, 0.1% CHAPS, 5mM DTT, 10^{-6}% Nonidet P-40, pH7.0,	Chapters 2, 3, 4 and 5
	Embryogenic cell line of Norway spruce	100mM HEPES, 10% sucrose, 0.1% CHAPS, 5mM DTT, 10^{-6}% Nonidet P-40, pH7.0	Bozhkov et al. (2004)
Caspase-8 like (IETDase)	*Arabidopsis thaliana* seedlings	50mM sodium acetate, pH5, 3mM DTT	Rotari et al. (2005)
	Avena sativa leaves	20mM MOPS, pH7, 1mM DTT	Coffeen & Wolpert (2004)
Saspase (VKMDase)	*Avena sativa* leaves	20mM MOPS, pH7, 1mM DTT	Coffeen & Wolpert (2004)
TATDase	Tobacco Xanthi, leaves	Buffer (?) at PH6.8, 1mM PMSF	Chichkova et al. (2004)

YVADase and DEVDase, known from animal studies as caspase-1 and caspase-3, were present in most plant PCD pathways (Woltering et al. 2002). Now research focuses on other activities: VEIDase, IETDase and TATDase. The activation of a protease or a group of proteases with the preferential cleavage of caspase-6 substrate Ac-VEID-AMC is associated with the Norway spruce embryo development (Bozhkov et al. 2004). A TATDase proteolytic activity was detected during the N gene-mediated HR in tobacco plants infected with the tobacco mosaic virus (TMV). Here ectopically expressed *Agrobacterium tumefaciens* VirD2 protein was used as a target, because according to prediction it was a substrate for human

caspase-3. A tetrapeptide aldehyde (biotinyl-TATD-CHO) was designed on the basis of the VirD2 cleavage site for plant caspase. The tetrapeptide aldehyde prevented cleavage of VirD2 by plant caspase, and it counteracted TMV triggered HR *in vivo* (Chichkova et al. 2004). To conclude, several caspase-like activities were measured in plants. Most of these activities have been detected in several species, and in various tissues or cell types (as shown in table 3).

Plant targets and caspase inhibitors

Animal caspases have many identified targets or 'death substrates' that are either deactivated - such as PARP1, nuclear lamin, gelsolin, Bcl-2, p53, pRb, FAK, STAT-1 and β-catenin - or activated - such as MEKK1, p21-activated kinase, protein kinase C and the ICAD/CAD complex - (Lopez-Otin & Overall 2002). Some of these substrates have plant orthologues, but at present, the only possible substrate for plant caspase-like activities is PARP1. Tobacco PARP1 is cleaved during PCD in tobacco protoplasts after menadione treatment (Sun et al. 1999), and in tobacco suspension cells after heat shock treatment (Tian et al. 2000). This cleavage was blocked by caspase inhibitors. A bovine PARP was cleaved in Thomas and Franklin-Tong's 2004 study of papaver pollen PCD during self-incompatible pollination. A DSVD site was conserved with the Arabidopsis AtPARP1 that is orthologous to the animal PARP1 (Doucet-Chabeaud et al. 2001). This would confirm AtPARP1 as a possible substrate for a DEVDase activity.

To conclude, PARP1 has been shown to be one possible substrate. This has been demonstrated in only a few studies. By contrast, many substrates have been found in animal studies. The substrates of the caspase-like proteases are mostly not known for plants. The substrates of plant caspase-like proteases are perhaps not similar to the substrates of animal caspases (Rotari et al. 2005). This is important because knowledge of the substrates would help to identify the plant caspase-like proteases. If it cannot be established that plant caspase-like proteases and animal caspases have the same substrates, other similarities can be taken into consideration. Do plant caspase-like proteases and animal caspases have similar substrate- and site-specificities?

A number of cysteine and serine protease inhibitors (table 2) can partially suppress cell death in plants or have no effect (Lam & Del Pozo 2000). In some cases, they block cell death but this requires high concentrations (Sun et al.1999; de Jong et al. 2000; Woltering et al. 2002). Animal caspase inhibitors have been found to inhibit cell death effectively at low concentrations. Both caspase-1 and caspase-3 inhibitors prevent cell death in various plants (del Pozo & Lam 1998; Clarke et al. 2000; de Jong et al. 2000; Korthout et al. 2000). PARP

cleavage and DNA fragmentation could be inhibited in tobacco suspension cells. Here PCD was induced by high concentrations of nicotinamide. PCD was inhibited by addition of the caspase-3 inhibitor Ac-DEVD-CHO (Zhang et al. 2003). A cell free system was used in another study. Here cytochrome c of animal origin was capable of activating plant caspase-like proteases in carrot cytoplasm. The cytoplasm could then degrade rat liver nuclei (Zhao et al. 1999). In this study, the formation of a DNA ladder was inhibited by the specific caspase-1 and caspase-3 inhibitors (Ac-YVAD-CHO and Ac-DEVD-CHO, respectively). Belenghi et al. (2004) found that caspase-3 like proteases play an important role in the elimination of secondary shoots of pea seedlings after removal of the epicotyl. The injection of the caspase-3 like inhibitor into the remainder of the epicotyl strongly inhibited the death of the secondary shoot, resulting in a seedling with two equal shoots. Bozhkov et al. (2004) found that during plant embryogenesis, caspase-6 like activity was the essential caspase-like activity involved in PCD. The study concerned embryonic pattern formation during shape remodeling. The use of caspase-6 inhibitor blocked the embryo-suspensor differentiation, disturbing embryo development. Sun et al (1999) found that with menadione-induced cell death in tobacco protoplasts, Ac-DEVD-CHO inhibits DNA laddering. In another study, the heat-shock induced cleavage of lamin-like proteins in tobacco protoplasts correlated with the proteolytic activity of caspase-6 like proteases (Chen et al. 2000). In this study the use of the animal caspase-6 inhibitor (Ac-VEID-CH0) inhibited the lamin cleavage. Del Pozo and Lam (1998) prevented tobacco mosaic virus–induced HR in *Nicotiana tabacum* leaves by a treatment with short peptide inhibitors. These inhibitors preferentially inhibit caspase-1 and caspase-3 proteases. De Jong et al. (2000) showed that PCD chemically induced by camptothecin in tomato suspension cells can be inhibited by caspase inhibitors. Elbaz et al. (2002) obtained similar results with caspase inhibitors that prevent or limit staurosporine-induced cell death. Mlejnek and Prochazka (2002) performed this study with isopentenyladenosine to induce cell death, with similar results. Korthout et al. (2000) demonstrated caspase-like activity in barley cell extracts. Here the activity could be inhibited by a caspase-3 inhibitor, but not by cysteine protease inhibitors. Most of the animal caspase inhibitors that were tested in plants are remarkably effective at suppressing cell death. There are some exceptions, though. Caspase-3 inhibitor had no effect on TMV-induced necrotic lesions in tobacco, whereas caspase-1 inhibitor did have an inhibition effect (Chichkova et al. 2004; Hatsugai et al. 2004). Caspase-1 inhibitor did not suppress cell death in papaver pollen tubes, in contrast to caspase-3 inhibitor (Thomas & Franklin-Tong 2004). In chapter 4, a study is described in which caspase-3 and caspase-6 like activities could be inhibited by animal caspase-3 and -6 inhibitors.

In these studies, it was shown that animal caspase inhibitors can inhibit PCD in plants. It can therefore be inferred that caspase-like proteases play a role in PCD in plants.

The other possible candidates

Although caspase-like activities seem to be involved in PCD in plants, it has become clear that orthologs of the animal genes for caspases are not present in plants. In the search for the proteases that are involved in plant PCD, other proteins have been found and described as playing a role in plant PCD. Those proteins are described here. If there is a main executioner of plant PCD with caspase-like activity, it could be one of those proteins.

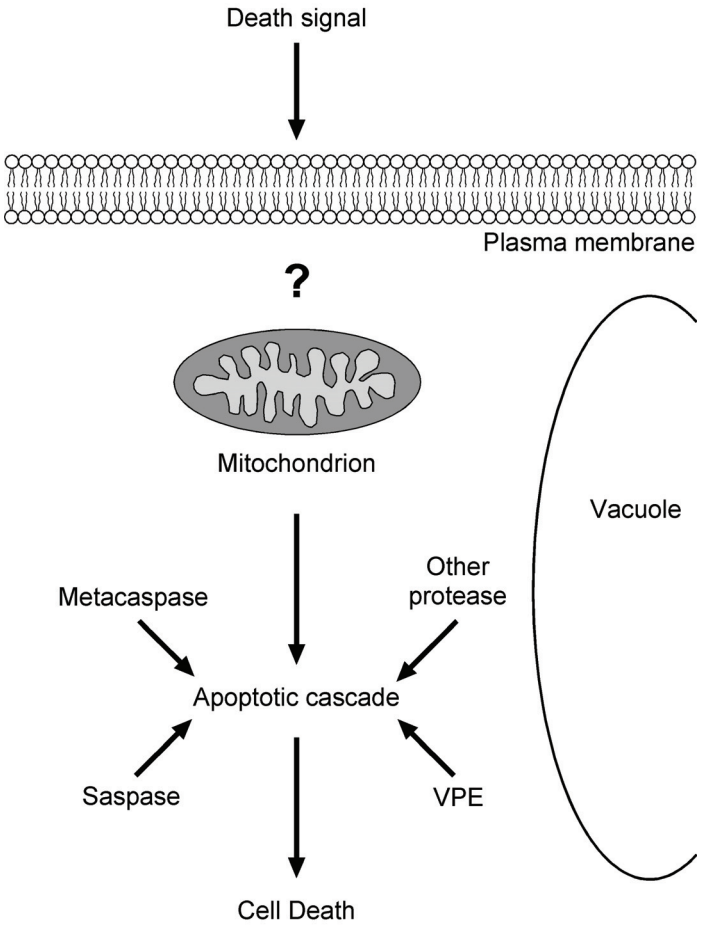

Figure 4: Caspase-like activities and PCD in plant cells. There are several proteases known to play a role during plant PCD. Those proteases are caspase-like candidates.

Metacaspases

Uren et al. (2000) found a family of distantly related caspase-like proteases - named metacaspases - in plants, fungi and Plasmodium. Depending on sequence similarities (within their caspase-like regions) and domain structure, the plant metacaspases can be divided in two classes (type I and II). Whether these metacaspases possess caspase-like proteolytic activity and are involved in plant PCD remains unknown (Woltering et al. 2002; Woltering 2004). Many investigations indicate that metacaspases can have a role in PCD.

Madeo et al. (2002) reported that the yeast metacaspase protein (YCA1) knockout is unable to complete PCD in the presence of H_2O_2. Hoeberichts et al. (2003b) have shown that mRNA levels of LeMCA1 - a tomato (*Lycopersicon esculentum*) type II metacaspase - increased upon infection of leaves with the fungal pathogen *Botrytis cinerea*. This increase correlates with the formation of primary necrotic lesions.

The protein mcII-Pa (plant metacaspase type II) was expressed during PCD in somatic embryogenesis in Norway spruce. *In situ* hybridization analysis showed mRNA accumulation in the part of embryogenic tissues and structures committed to die (Suarez et al. 2004). The activation of proteases cleaving the caspase-6 substrate (VEIDase activity) is essential for PCD and embryogenesis in Norway spruce (*Picea abies*) (Bozhkov et al. 2004). Silencing of the *P. abies* metacaspase gene *mcII-Pa* inhibited VEIDase activity, suppressed PCD in the embryos and blocked suspensor differentiation (Suarez et al. 2004). Immunolocalization analyses and functional assays showed that mcII-Pa accumulates in the nuclei of the suspensor cells and that it is directly involved in the execution of nuclear disassembly (Bozhkov et al. 2005). Watanabe and Lam (2005) found that two type I metacaspases (At5g64240 and At1g02170) were up-regulated in Arabidopsis plants after infiltration with a bacterial pathogen, whereas type II metacaspases were not significantly affected.

The metacaspases could be involved in a plant PCD cascade. Such a cascade occurs with caspase activation in animals. Thus, the metacaspases might activate the plant caspase-like proteases, as was suggested by Rotari et al. (2005).

Vacuolar processing enzyme

The vacuolar processing enzyme (VPE) was originally identified as a processing enzyme that is involved in the maturation of seed storage proteins (Hara-Nishimura et al. 1991; 1998; 2005). VPEs - VPE α, β, γ and δ - also promote the maturation and the activation of various vacuolar proteins in plants (Yamada et al. 1999; Shimada et al. 2003; Rojo et al. 2003; Gruis et al. 2004). Their counterpart in animals similarly activates lysosomal proteins (Shirahama-

Noda et al. 2003). Recent data suggested that VPEs might be key factors in vacuolar collapse-triggered cell death.

In vegetative tissues, the isoform VPEγ is more expressed than the other three. In those tissues, this isoform plays a role in protein degradation during senescence, a PCD pathway in plants. VPEγ is localized in precursor protease vesicles (PPVs), which are hydrolase-containing organelles that are associated with PCD processes (Schmid et al. 1999). VPEγ is also found in the vacuole, an organelle essential for cell dismantling during plant PCD (Lam 2004; Rojo et al. 2004).

VPEs might convert the hydrolytic enzymes that are involved in the disintegration of vacuoles from an inactive to their active forms. The enzyme conversion would initiate the proteolytic cascade in plant PCD. Arabidopsis VPE genes are up-regulated in dying cells during development and senescence of tissues (Kinoshita et al. 1999). There is no sequence similarity between VPEs and caspases. However, VPEs have a proteolytic activity toward a caspase-1 substrate, and VPE activity is inhibited by a caspase-1 inhibitor. Tobacco VPE exhibits the caspase-1 like activity that is required for the completion of cell death during TMV-induced HR in tobacco (Hatsugai et al. 2004).

Saspases

Substilisin-like proteases, named saspase-A and -B, belong to the class of serine proteases. Subtilisin-like proteases were identified in several plant species (Beers et al. 2000). Saspase-A and -B have been found to be involved in victorin-induced PCD in oat (*Avena sativa*) (Coffeen & Wolpert 2004). They were found to cleave caspase specific substrates and they were found to be inhibited by caspase-specific inhibitors. Coffeen and Wolpert (2004) reported that caspase-specific inhibitors prevented rubisco proteolysis during victorin-induced PCD. They also found that a caspase-like activity toward the pan-caspase substrate z-VAD-AFC was present in extracts from victorin-treated *A. sativa* leaves. The two oat saspases show hydrolytic activities for caspase-6, -8 and -9 substrates (VKMD, IETD, and LEHD, respectively) but not for DEVD and VEID. It is important to determine how saspases contribute to plant PCD and to the caspase-like activities.

Other protease(s)

By studying PCD in plants, still some other proteases were also found to be associated with PCD. For many of them, their characterization was done on the basis of inhibitors. As described in table 1, the inhibitors can help to determine the class of the protease that is involved in a particular process. Some examples are here described.

In soybean cells, some cysteine proteases are activated during oxidative stress-induced PCD (Solomon et al. 1999) and the activation of these proteases is required for PCD. Ectopic expression of cystatin, an endogenous cysteine protease inhibitor gene, prevented the increase in cysteine protease activity and blocked PCD triggered either by an avirulent strain of *Pseudomonas syringae pv glycinea* or directly by oxidative stress.

Xylogenesis in *Zinnia elegans* cell cultures can be prevented by the addition of inhibitors such as E-64 and PMSF. This suggests the involvement of some cysteine or serine proteases in this process of PCD (Minami & Fukuda 1995; Ye & Varner 1996; Beers & Freeman 1997).

PCD of the megagametophyte during post-germinative seedling growth of white spruce (*Picea glauca*) is associated with the induction of serine and cysteine proteases and caspase-like activity (He & Kermode 2003a). When germinated seeds were treated with a caspase-3 inhibitor, both the peak of the caspase-like activities and the death of megagametophyte cells were delayed.

A matrix metalloproteinase Cs1-MMP was proposed as a candidate involved in cucumber extracellular cell matrix degradation, and in PCD (Delorme et al. 2000). Cs1-MMP activity was completely inhibited by a hydroxamate-based inhibitor that binds at the active site of MMPs in a stereospecific manner. PCD in the corolla of tobacco (*Nicotiana tabacum*) flowers was inhibited by leupeptin, but not by PMSF, suggesting that a cysteine protease was important for this process (Serafini-Fracassini et al. 2002).

A barley vacuolar aspartic proteinase (phytepsin), highly homologous to mammalian lysosomal cathepsin D, was expressed at a high level, both during formation of tracheary elements and during autolysis of sieve cells (Runeberg-Roos & Saarma 1998). The enzymatic activity was completely inhibited by pepstatin - a specific inhibitor of aspartic proteinases (Sarkkinen et al. 1992). The protease may play a role in the active autolysis of plant cells.

In the endosperm of castor bean (*Ricans communis*), PCD is associated with the accumulation of papain-like propeptidase (Cys-EP) in ricinosomes during germination (Schmid et al. 1999). This process of PCD is also associated with the release of the peptidase from ricinosomes during cell collapse. In *Brassica napus*, a papain-like cysteine protease is associated with PCD of the inner integument of seed coat during early stages of seed development (Wan et al. 2002).

The proteases discussed above do not seem to be the main executioners of plant PCD in general. They participate in PCD in specific tissues only. It may be possible that these proteases are involved in the progression of PCD, but not in the initiation of PCD (Rotari et al. 2005).

Outline of the thesis

The development of multicellular organisms involves an important balance between cell growth, cell division and cell death. Programmed cell death (PCD) plays a key role in all animals, by forming and deleting structures, controlling cell numbers and eliminating abnormal damaged cells (Baehrecke 2002). Apoptosis is a genetically and morphologically encoded form of PCD (Kerr et al. 1972). Caspases were found to be the executioners of the apoptotic pathway.

In plants, PCD plays normal physiological roles in a variety of processes. Some processes in which PCD plays a role are: (i) the deletion of cells with temporary functions, such as the aleurone cells in seeds and the suspensor cells in embryos; (ii) the removal of unwanted cells, such as the root cap cells found in the tips of elongating plant roots and the stamen primordia cells in unisexual flowers; (iii) the deletion of cells during sculpting of the plant body and formation of leaf lobes and perforations; (iv) the death of cells during plant specialization, such as the death of TE cells which creates channels for water transport in vascular plants; (vi) leaf senescence; (vii) responses to plant pathogens and abiotic stresses (Pennell & Lamb 1997; Dangl & Jones 2001; Kuriyama & Fukuda 2002; Danon et al. 2004).

Some common features of apoptosis were found to be conserved in both plants and animals (Danon & Gallois 1998; Yao et al. 2004). These include cytoplasm shrinkage, cytochrome c leakage out of mitochondria, chromatin condensation, altered nuclear morphology, DNA fragmentation and DNA laddering. After the elucidation of the complete Arabidopsis genome, it has become clear that no genes for the caspases or for the apoptotic regulators of the IAP and Bcl-2 families are present in plants. However, animal Bcl-2 members have been found to modify cell death processes in plants (Lam et al. 1999; Baek et al. 2004). This indicates that a similar apoptotic machinery may still be present in plants.

At the start of the work described in this thesis, caspase-like activities had been found to be involved in PCD processes in plants. These activities were measured with animal caspase substrates and inhibited by animal caspase inhibitors. This suggested that a core mechanism is conserved in both animal and plant PCD. However, orthologs of the caspase genes turned out not to be present in the plant genomes. Thus, the researchers at that time wondered which plant protease was responsible for the caspase-like activity in plant extracts.

The aim of the research described in this thesis was to describe PCD in different plant systems (see below), and to identify the proteases in plant cells that show caspase-like activities during PCD.

Chapter 2 describes the Arabidopsis spontaneous necrotic spot (*sns*) mutant. The necrotic spots were visible on the mutant leaves after two or three weeks of growth,

resembling the lesions that accompany the hypersensitive response after a pathogen attack. The phenotype of the *sns* mutant plants was analyzed and evidence for the occurrence of PCD in the necrotic spots was obtained.

Chapter 3 describes the transition from multicellular structures to globular embryos during androgenesis in barley. In this plant system, the transition is possible since some cells - part of the small generative domain - will die at the site of the exine wall rupture. Typical features of plant PCD, in the transition process, were found.

C**hapter 4** describes cell death in rice suspension cells after heat-shock. Evidence for the occurrence of cell death by necrosis and PCD was found.

For chapters 2, 3 and 4, caspase-like activities during PCD were measured. This confirmed that caspase-like proteases are involved in plant PCD. **Chapter 5** describes the development and implementation of the protocol to purify plant caspase-3 like activity. This protocol was based on the one used to purify human caspase-3 with biotin-DEVD-CHO.

Chapter 6 comprises a summarizing general discussion.

Acknowledgments

For valuable discussion and critical reading of the manuscript I am grateful to Prof. Dr. Paul Hooykaas (Institute of Biology Leiden, Leiden University) and to Dr. Wouter-Jan Oosten (Sociotext Foundation). Mr Peter Hock was so friendly to finalize the figures. This work was financed by the BVS Foundation (Binair Vector Systeem).

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