**The Botanical Dance of Death: Programmed cell death in plants.**

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**ABSTRACT**

Programmed cell death (PCD) describes a small number of processes that result in a highly controlled, and organised, form of cellular destruction, activated in every part of the plant, throughout its entire life cycle. For example, PCD is a critical component of many vegetative and reproductive developmental processes, senescence programmes, pathogen defence mechanisms and stress responses. Cell destruction can manifest as apoptotic-like, necrotic or autophagic cell death and these processes are likely to overlap extensively, sharing several regulatory mechanisms. Several of the key PCD regulators and signals have now been revealed, for example, many cell organelles, including mitochondria, chloroplasts, Golgi apparatus, endoplasmic reticulum and vacuoles have been shown to have a role in controlling PCD activation. Following activation the actual dismantling of the cell appears to involve cell death proteases including those with caspase-like, or metacaspase, activity. This review will examine the current state of knowledge about the regulation of events during plant PCD. We will describe numerous examples of developmental or environmentally-induced deaths and outline their potential as models systems for use in PCD research programmes. Similarly, a range of techniques and *in vitro* model systems and that can be used to identify, and quantify, rates of plant PCD are reviewed. These model systems and techniques can be used to identify the underlying signals and events that drive and regulate PCD and ultimately reveal the steps necessary for the botanical dance of death.

**I. Introduction**

Lockshin and Zakeri (2004) defined PCD as the sequence of (potentially interruptible) events that lead to the controlled death of the cell. PCD generally describes apoptotic (Type I) or autophagic (Type II) cell death, in contrast to necrotic (Type III) cell death (Bras *et al.*, 2005, Lockshin and Zakeri, 2004). Apoptosis in animal cells is phenotypically characterised by cell shrinkage, nuclear condensation and fragmentation, plasma membrane blebbing and finally, collapse of the cell into small fragments – apoptotic bodies, which are subsequently removed by phagocytosis (Lennon *et al.*, 1991). Molecular mechanisms of mammalian apoptosis are well understood: cellular dismantling is executed by caspase (*C*ysteine dependent *ASP*artate-directed prote*ASES*) activation (Adrain and Martin, 2001). Caspase activation may be initiated either via an extrinsic pathway which is death receptor mediated, or an intrinsic pathway, which is controlled by the release of pro-apoptotic proteins from mitochondria. In plants, most elements of the PCD machinery remain unknown and moreover, truly apoptotic morphology (formation of apoptotic bodies) is not universally observed (McCabe *et al.*, 1997a). This is not surprising due to presence of the plant’s cell wall, preventing final clearance by phagocytosis by adjacent cells. Consequently, in order to acknowledge similarities between plant PCD and apoptosis while at the same time recognising differences between them, the term ‘apoptotic-like PCD’ (AL-PCD) was introduced (Danon *et al.*, 2000). AL‑PCD describes a type of plant cell death pathway which is characterised by DNA degradation and condensation of the protoplast away from the cell wall (Fig. 1), similar to the apoptotic morphology seen in animal cells (McCabe and Leaver, 2000, McCabe *et al.*, 1997a, Reape and McCabe, 2010, Reape and McCabe, 2008). Autophagic cell death on the other hand occurs without chromatin condensation and is accompanied by massive autophagic vacuolisation of the cytoplasm (Kroemer *et al.*, 2008) while necrosis is often described as unorganised cell destruction process which occurs following overwhelming stress. During necrosis the cell loses its ability to osmoregulate which results in water and ion influx and swelling of the cell membrane and organelles (Lennon *et al.*, 1991, Lockshin and Zakeri, 2004). Until recently, necrosis has been considered a passive and accidental cellular event, but recent data suggests that in certain cases this process can be programmed and controlled to a certain extent (Festjens *et al.*, 2006, Golstein and Kroemer, 2007). Similarities between the cell death programmes seen in animal and plant cells such as conservation of autophagic genes or apoptotic cell shrinkage, chromatin condensation, DNA fragmentation and mitochondrial release of cytochrome *c* (cyt *c*) suggest that at least some death mechanisms are conserved throughout the plant and animal kingdoms, having been derived from ancestral unicellular death programmes.

*<Fig. 1 (A photo of AL-PCD) near here>*

* + 1. **Recognizing, and reporting, different modes of cell death in plants**

PCD is a broad term describing multiple, possibly overlapping death pathways operating in eukaryotic cells. New types of organised cell death are being described and the terminology referring to PCD is constantly expanding (Kroemer *et al.*, 2008). It seems unlikely that a definite and unconditional distinction between different forms of cell death, based only morphological criteria, can be established, as dying cells often display mixed cell death morphologies (Martin and Baehrecke, 2004, Nicotera and Melino, 2004). The contribution to a particular cell death pathway by specific cellular death machinery is still being defined. It is therefore important to introduce a non-rigid, but uniform nomenclature and if possible give details (e.g. in terms of time, morphology, and presence of different markers) defining the specific type of cell death under investigation, rather than simply referring to the process as PCD (Reape and McCabe, 2008). In the case of plant cells, clear descriptions of the processes examined is particularly important, especially as the mechanisms of plant PCD are far less understood compared to the animal kingdom. Nomenclature and definitions created and used traditionally for description of cell death in animal cells may not always be adequate for plant-focused research and their misuse may result in confusion and incorrect interpretation of data. Therefore it is advisable that experimental data is carefully analysed with special focus put on the methodology and experimental design used by the researchers. For example, the presence of hallmark features of AL-PCD, such as cyt *c* release or DNA laddering should be monitored throughout the course of the cell death process rather than at one particular time point. Furthermore, if cell death is induced by application of external stimuli, the magnitude of the stress applied has to be carefully selected to ensure that it is sufficient to induce PCD, but not so high that it is overwhelming and results in necrosis (McCabe *et al.,* (1997a). One should also critically consider the assays used to investigate different instances of PCD and be aware of both the advantages and drawbacks of each particular assay. For example, monodansylcadaverine (MDC) has been considered an autophagy-specific marker but its specificity is now being questioned (see Section IV) and sample preparation procedures have occasionally been shown to affect the outcome of a PCD biochemical assay, such as TUNEL (Wang *et al.*, 1996).

The optimal means of communicating results concerning cell death events in plants is to provide the scientific audience with comprehensive descriptions of morphology, biochemistry and timing-related data, rather than using PCD as the general term describing the whole death process. In this review, while examining diverse examples of plant PCD, we have endeavoured to describe the characteristic features and events accompanying the type of cell death under consideration.

1. **Regulators of plant PCD**
2. **Mitochondria and chloroplasts**

Although the regulation of plant PCD has been a subject of intensive research, the sequence of events leading to organised cell death has only begun to emerge. Due to the assumed evolutionary conservation of at least some elements of the PCD machinery, significant research efforts have focused on examining the similarities between PCD programmes in animal and plant cells. In animal cells, apoptosis can be activated either through the intrinsic or extrinsic pathway. The intrinsic pathway is triggered by increased cellular stress (for example, DNA damage caused by different factors). When a stress signal is received, cytoplasm-residing proapoptotic proteins bind to the outer membrane of the mitochondria, inducing mitochondrial permeability transition pore formation and release of cyt *c* (Yang *et al.*, 1997) and other apoptotic-related proteins such as, endonuclease G (endo G) (Li *et al.*, 2001), apoptosis inducing factor (AIF) (Susin *et al.*, 1996), high temperature requirement A2 (HtrA2/Omi) (Suzuki *et al.*, 2001), and second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (SMAC/Diablo) (Du *et al.*, 2000). Upon release, cyt *c* induces assembly of a complex termed the apoptosome, which activates caspase-9, promoting further caspase activation events and subsequent cellular demolition (Adrain and Martin, 2001). The extrinsic pathway is activated when signalling molecules (ligands) bind to transmembrane death receptors of the cell which induce signal cascades leading to caspases activation and subsequent permeabilisation of the mitochondrial outer membrane.

Experimental data also points to involvement of the mitochondria, and molecules expressing caspase-like activity, during PCD activation in plant cells. Moreover, recently the potential role of the chloroplast in regulating AL-PCD has been suggested (Doyle *et al.*, 2010, Seo *et al.*, 2000, Wright *et al.*, 2009). Blackstone and Green (1999) have hypothesised that the release of cyt *c* and upregulation of mitochondrial reactive oxygen species (ROS) production during PCD, are vestiges of ancient events that arose during proto-mitochondrion – host cell conflict. As suggested by Reape and McCabe (2010), an apoptotic role for the chloroplast may also relate to the endosymbiotic origin of this organelle, as it too is a significant producer of ROS within the cell. During normal plant life cycles, ROS produced during processes such as photosynthesis or respiration are normally scavenged by the plant’s antioxidant defence system. However, this delicate balance can be distorted by an array of stresses, such as drought and desiccation, salt stress, chilling, heat shock, heavy metals, ultraviolet radiation, air pollutants such as ozone and SO2, mechanical stress, nutrient deprivation and pathogen attack, which result in enhanced reactive oxygen intermediates production (Mittler, 2002 and references therein). Although ROS can cause physicochemical damage, they are also thought to play an important role as signalling molecules for the activation of stress defence pathways (Dat *et al.*, 2000). Indeed, PCD can be triggered by accumulation of ROS (Chen and Dickman, 2004, Laloi *et al.*, 2004, Pennell and Lamb, 1997, Wagner *et al.*, 2004) and evidence suggests that this occurs by activation of genetically programmed pathways of gene expression, which lead to controlled cell suicide events (Foyer and Noctor, 2005a, Foyer and Noctor, 2005b).

* + - * 1. **Mitochondria – key regulators of plant PCD?**

The mitochondrion is a central regulator of apoptosis in animal cells and a similar regulatory role has been suggested by several plant PCD studies (Balk *et al.*, 2003, Scott and Logan, 2008). Balk *et al.* (2003) used an *Arabidopsis* cell-free system to monitor PCD-associated changes in isolated nuclei when they where incubated with mitochondria and/or cytosolic extract. They observed that adding broken mitochondria resulted in DNA degradation by two mechanisms. One mechanism required the addition of cytosol and resulted in DNA fragmentation after 12 hours. The second mechanism did not require the cytosol and led to the induction of high-molecular-weight fragmentation of DNA and chromatin condensation. They found a Mg2+ dependent nuclease contained in the IMS was involved in the high molecular weight DNA cleavage and chromatin condensation (Balk *et al*., 2003). Scott and Logan (2008) used an *Arabidopsis* protoplast system expressing mitochondrial targeted GFP to investigate the role of mitochondria in plant cell death. They observed that very shortly after chemical (ROS) or physical (moderate heat treatment) stress, mitochondria undergo a so-called mitochondrial morphology transition (MMT), gaining a swollen appearance and this preceded cell death by many hours. MMT was eliminated by pre-incubation with lanthanum chloride (calcium channel blocker), cyclosporin A (inhibitor of permeability transition pore formation) or superoxide dismutase analogue TEMPOL (ROS scavenger), and as a result cell death was blocked. Changes in mitochondrial shape has also been reported in other studies on UV-C (Gao *et al.*, 2008a) or protoporphyrin IX - PPIX (Yao and Greenberg, 2006) induced PCD in *Arabidopsis* protoplasts and also prior to the final stages of senescence in *Medicago truncatula* (barrel clover) cell suspension cultures (Zottini *et al.*, 2006). The aforementioned studies suggest that the mitochondrial events constitute a relatively early and significant component of plant cell PCD.

Consistent with animal cell death studies, the release of cyt *c* has been reported during PCD events in plant systems, for example, during developmental PCD in the tapetum of CMS sunflower (Balk and Leaver, 2001), death of pollen tubes during self-incompatibility in *Papaver* (poppy) pollen (Thomas and Franklin-Tong, 2004), or in cell culture models after application of death inducing stimuli such as, heat shock, D-mannose, menadione, harpin or ceramide treatment (Balk *et al.*, 2003, Balk *et al.*, 1999, Krause and Durner, 2004, Stein and Hansen, 1999, Sun *et al.*, 1999, Vacca *et al.*, 2006, Yao *et al.*, 2004). Cyt *c* release has also been observed during differentiation of TEs in *Zinnia* cultures (Yu *et al.*, 2002) and following activation of the HR (Curtis and Wolpert, 2002, Kiba *et al.*, 2006). Nevertheless, purified cyt *c* itself was not sufficient to induce PCD in an *Arabidopsis* cell free system (Balk *et al.*, 2003) and the death of TEs in *Zinnia elegans* culture could be blocked with cyclosporine A, without blocking cyt *c* release, suggesting that cyt *c* relocation is insufficient to trigger death in these cells (Yu *et al.*, 2002). Therefore, unlike in animal cells, plant cyt *c* may not be a direct protease activator, but participates in the cell death process in other ways. It has been suggested that cyt *c* can activate or amplify the cell death process by disrupting electron transport, which would lead to generation of lethal levels of ROS, creating a feedback loop leading to augmentation of the initial PCD-inducing cellular stress signal (Reape and McCabe, 2010).

During animal apoptosis, release of cyt *c* is an indicator of mitochondrial membrane permeabilisation (MMP), and is considered ‘the point of no return’ (Kroemer *et al.*, 2007). In mammalian cells, MMP can occur through a Bax/Bcl-2 controlled pore, when the balance between pro (Bcl-2, Bcl-xL) and anti (Bax, Bid, Bad, Bak) –apoptotic proteins is disturbed (Youle and Strasser, 2008). Although an evolutionarily conserved death suppressor Bax Inhibitor-1 (BI-1) exists in plants ((Hückelhoven, 2004) and references therein), to date there is no evidence for the existence of plant homologues of the Bcl-2 family proteins. MMP in animal cells can also be achieved by formation of the permeability transition pore (PTP) and subsequent release of mitochondrial proapoptotic proteins. PTP is formed as a protein complex at apposition sites between the inner and outer mitochondrial membranes, and is composed of the voltage dependent anion channel (VDAC), adenine nucleotide translocator (ANT), cylophilin D and the benzodiazepine receptor (Jones, 2000). As a consequence of PTP formation, the following sequence of events occurs; depolarisation of the inner mitochondrial membrane, rapid water influx, osmotic swelling of the mitochondria, rupture of outer mitochondrial membrane and, finally, the release of mitochondrial IMS proteins including cyt *c*. PTP formation can be triggered by increase in [Ca2+], especially at conditions of low ATP (Crompton, 1999) or ROS induced stress (Petronilli *et al.*, 1994). Indeed calcium influx seems an important event in plant PCD and application of calcium binding agents, calcium channel inhibitors or agents inhibiting calcium release from internal stores significantly affected numerous PCD events, for example, causing HR inhibition in soybean leaves (Levine *et al.*, 1996), reducing developmental PCD in lace plant (Elliott and Gunawardena, 2010) or during aerenchyma formation (Drew *et al.*, 2000, He *et al.*, 1996b), or by preventing salt stress induced PCD in rice root tip cells (Li *et al.*, 2007a). Therefore, it seems likely Ca2+ is involved in PCD related signalling and indeed, it has been proposed to mediate the mitochondrial permeability transition (MPT) events (Lin *et al.*, 2005, Wang *et al.*, 2006). Another mechanism by which Ca2+ controls PCD in mammalian cells is the activation of the calcium/magnesium-dependent endonuclease responsible for DNA fragmentation (Wyllie, 1980). HR-associated nuclease activities in tobacco were also stimulated by Ca2+ (Mittler and Lam, 1995) and interestingly, they were inhibited not only by calcium chelators, but also by Zn2+ ions. This life-promoting function of Zn2+ was confirmed by Helmersson *et al.* (2008), who found that a decrease in free intracellular [Zn2+] induced cell death in *Picea abies* (Norway Spruce) embryos. In the same study, plant metacaspases were found to be suppressed by increasing levels of Zn2+ and cell death levels decreased accordingly. It has been postulated that Zn2+ may interfere with calcium by acting as a calcium dependant endonuclease blocker (Lohmann and Beyersmann, 1993).

There is evidence of PTP involvement in plant PCD processes. For example, oxidative burst and breakdown of mitochondrial membrane potential was noted early in victorin-induced PCD in oat cells (Yao *et al.*, 2002), Moreover, application of CsA, which blocks PTP formation, was shown to inhibit calcium induced swelling of isolated potato mitochondria (Arpagaus *et al.*, 2002), oxidative stress induced PCD in *Arabidopsis* cell suspension cultures (Tiwari *et al.*, 2002), betulinic acid triggered PCD of tracheary elements (Yu *et al.*, 2002) and death induced by nitric oxide in *Citrus sinensis* cells (Saviani *et al.*, 2002). CsA has been also shown to inhibit the loss of mitochondrial membrane potential and cyt *c* release from *Arabidopsis* protoplasts treated with PPIX and C2 ceramide (Yao *et al.*, 2004).

Hexokinases are enzymes that participate in a variety of cellular processes. The mitochondria-associated hexokinasehas been shown to play an important role in control of mammalian apoptosis (Birnbaum, 2004, Downward, 2003, Majewski *et al.*, 2004). It binds to the VDAC and interferes with the opening of the PTP, thereby inhibiting cyt *c* release and consequently preventing apoptosis (Azoulay-Zohar *et al.*, 2004, Pastorino *et al.*, 2002). Kim *et al.* (2006) have shown that plant hexokinases participate in the regulation of PCD in *Nicotiana benthamiana*. Tobacco rattle virus (TRV)–basedvirus-induced gene silencing (VIGS) of the hexokinase gene *Hxk1* was shown toinduce the spontaneous formation of lesions in leaves. Cells within these lesions exhibited AL-PCD characteristic features such as, nuclear condensation, DNA fragmentation, loss of mitochondrial membrane potential, cyt *c* release, activation of caspase-9-like and caspase-3-like proteases. *Hxk1* was shown to be associated with the mitochondria, its expressionwas stimulated by various cell death–inducing stresses and moreover overexpression of mitochondria-associated *Arabidopsis* hexokinases Hxk1 and Hxk2, increased the plants resistance to oxidative stress induced cell death. Other studies have shown mitochondria-associated hexokinases have an antioxidant role in potato tubers (Camacho-Pereira *et al.*, 2009). Studies suggesting that mitochondria-associated hexokinase activity could be involved in the regulation of both mitochondrial respiration, and ROS production, in plants was recently reviewed by Bolouri-Moghaddam *et al.* (2010). It is worth noting that chloroplast hexokinases could also have a role as antioxidants in plants (Giese *et al.*, 2005, Wiese *et al.*, 1999).

* + - * 1. **Putative role of the chloroplast in regulating PCD**

Elevation in ROS is often associated with PCD and the chloroplast is one of the main sources of ROS in leaf cells (Asada, 2006, Zapata *et al.*, 2005). Indeed data is emerging that suggests the chloroplast does influence rates of PCD in some cells, although its specific role is still to be elucidated. For example, transgenic tobacco plants defective in the plastid ndhF gene, and subsequently therefore characterised by altered ROS production, exhibit delayed senescence (Zapata *et al.*, 2005). Samuilov *et al.*, (2003) have shown that cyanide induced PCD was promoted by chloroplasts while the HR response in tobacco was accelerated by loss of chloroplast function (Seo *et al.*, 2000). Recently, light and dark grown suspension cultures of *Arabidopsis* were used to gain insight into the influence of the chloroplast on AL-PCD (Doyle *et al.*, 2010). The level of heat induced AL-PCD was found to be significantly higher in dark grown cultures (cells lacking developed chloroplasts) than in light grown cultures (cells containing functional chloroplasts). Cells in light grown cultures were more prone to necrosis, suggesting an increase in cellular stress (thought to be due to increased chloroplast produced ROS) due to the presence of functional chloroplasts. Antioxidant treatment of light grown *Arabidopsis* suspension cultures did not protect cells from death but increased levels of AL-PCD (Doyle *et al.*, 2010), reducing stress from an overwhelming (necrosis inducing) to a moderate (AL-PCD inducing) level.

*Arabidopsis* MDARs (monodehydroascorbate reductases) are close homologues of Apoptosis Inducing Factor (Lisenbee *et al.*, 2005), which during cell death, hav a pro-apoptotic function in animal cells. Plant MDARs are involved in the ascorbate-glutathione antioxidant cycle and seem to play an important role in plants’ protection against ROS damage and oxidative stress (Mittler, 2002). MDAR 1 and dehydroascorbate reductase (DHAR) are downregulated during senescence of broccoli florets (Nishikawa *et al.*, 2003) and a decrease in MDAR transcript was observed during *Ipomoea nil* (morning glory) petal senescence (Yamada *et al.*, 2009). Taking into account that PCD is the terminal event in the senescence process, and ROS are believed to be involved in PCD-related signalling, this decrease in antioxidant protection may be significant. MDARs are dual targeted to both the mitochondria and chloroplast (Chew *et al.*, 2003, Obara *et al.*, 2002) which suggests that both organelles may be important in PCD regulation.

Research by Yao and Greenberg (2006) also suggests that both chloroplast and mitochondrial events play a role in PCD. They showed thatthe *Arabidopsis* chloroplast localized protein ACD2 (accelerated cell death 2) shifts upon pathogen infection from being largely in chloroplasts, to partitioning between chloroplasts, mitochondria, and to a small extent, the cytosol. ACD2 was shown to shield root protoplasts from light and PPIX induced PCD and overexpression of both ACD2 (localised in mitochondria and chloroplast) and ascorbate peroxidise (localised in chloroplast) reduced *Pseudomonas syringae* induced PCD. Porphyrin-related molecules can generate reactive oxygen species, cause altered organelle behaviour, and can activate a cascade of PCD-inducing events. The authors hypothesised that during infection ACD2 may bind/reduce porphyrin-related molecules in the mitochondria, and possibly in the chloroplasts, thereby protecting cells from PCD activation (Yao and Greenberg, 2006). Indeed, *acd2* mutant has been previously demonstrated to undergo excessive cell death during infection and displays spontaneous spreading cell death (Greenberg *et al.*, 1994). Wright *et al.* (2009) observed an interesting chloroplast phenomenon during developmental PCD in lace plant. At the later stages of PCD, they observed the cells’ chloroplasts gathering around the nucleus and forming indents in the nuclear periphery. Similarly, in the case of tobacco cells under osmotic stress, chloroplasts also clustered around the nucleus, where the tonoplast forms a cavity (Reisen *et al.*, 2005). This chloroplast distribution may be irrelevant, but the closeness of two organelles suggests that the chloroplast may indeed be involved in the regulation of PCD.

Leaf epidermal peels are an interesting system for studying the role of chloroplasts in PCD regulation (Samuilov *et al.*, 2003). The epidermal peel represents a monolayer of cells and this tissue is convenient to use with light microscopy. Moreover, it is highly useful tissue for studying the role of the chloroplast because the peel consists of two types of cells: guard cells (chloroplast containing phototrophic cells) and epidermal cells (chemotrophic and without chloroplasts). To date, leaf epidermal peels have been used for PCD related observations in several studies which focused on guard cells only or on both guard cells and epidermal cells (Ali *et al.*, 2007, Kiselevsky *et al.*, 2010, Samuilov *et al.*, 2003, Vasil’ev *et al.*, 2009). For example, Samuilov *et al.* (2003) used peels from pea (*Pisum sativum* L.) leaves and treated them with cyanide to induce PCD. They observed that illumination of chloroplast-containing guard cells enhanced PCD rates, while rates of death in the epidermal cells were unchanged.

1. **Metacaspase and caspase-like activities**

*Arabidopsis* lacks canonical caspases which can be activated in animal cells as a result of cyt *c* release. However, a small family of phylogenetically distant genes, named metacaspases, have been identified in the *Arabidopsis* genome (Uren *et al.*, 2000). Nine metacaspases have been identified in *Arabidopsis* (Watanabe and Lam, 2004); however they fail to cleave caspase substrates (Bonneau *et al.*, 2008). Nevertheless, several studies reported metacaspase involvement in PCD during embryogenesis of *P. abies* (Bozhkov *et al.*, 2005b, Suarez *et al.*, 2004) and during death induced by UV-C exposure, H2O2, or methyl viologen in *Arabidopsis* (He *et al.*, 2008). Metacaspase mcII-Pa initiates the burst of caspase activity that is responsible for PCD of the proembryogenic masses (PEMs) and the suspensor in somatic embryos of *P. abies* (Suarez *et al.*, 2004). Metacaspases localise to the nucleus and cytoplasm and execute nuclear and cytoplasmic degradation upstream of a vacuolar collapse (Bozhkov *et al.*, 2005b). Recently, the first natural metacaspase substrate was identified. Sundstrom *et al.* (2009) showed that a metacaspase mcII-Pa cleaves a phylogenetically conserved protein, TSN (Tudor staphylococcal nuclease), during both developmental and stress-induced PCD. Moreover, TSN knockdown was demonstrated to result in activation of ectopic cell death during reproduction, impairing plant fertility. Interestingly, human TSN, which is a multifunctional gene expression regulator, is cleaved by caspase-3 during apoptosis and therefore, despite different properties, caspases and metacaspases can cleave common molecules in the cell, which suggests possible evolutionary conservation between animal and plant PCD pathways (Sundstrom *et al.*, 2009). Recently, an experimental evidence for the role of two type I metacaspases, AtMC1 and AtMC2, in control of PCD in *Arabidopsis* was published by Coll *et al.* (2010). They established AtMC1 as a pro-death caspase-like protein required for both superoxide-dependent cell death in a reactive oxygen-sensitized state and for hypersensitive response mediated by nucleotide-binding site leucine-rich repeat immune receptor proteins, whereas AtMC2 was shown to act antagonistically. AtMC1 function requires conserved caspase-like putative catalytic residues, but negative regulation of cell death by AtMC2 is independent of the putative catalytic residues.

Caspase-like activity has been detected numerous times in plant cells undergoing PCD, and application of caspase inhibitors have been shown to suppress cell death (Chichkova *et al.*, 2004, D'Silva *et al.*, 1998, del Pozo and Lam, 1998, Dickman *et al.*, 2001, Iakimova and Woltering, 2009). VEIDase caspase-like activity, which is similar to both caspase-6 in mammalian cells and the YCA1 metacaspase in yeast, was shown to be the only caspase-like activity induced during embryogenesis of *P. abies* and was necessary for correct differentiation of the suspensor cells (Bozhkov *et al.*, 2003). Caspase-like activity was also demonstrated to be active during development of the barley caryopsis (Boren *et al.*, 2006). A functional analogue of animal caspases was identified in tobacco by Chichkova et *al.* (2004), who described a protease exhibiting high selectivity towards the *Agrobacterium tumefaciens* VirD2 protein, cleaving it in a caspase-like manner. This caspase-like protease (CLP) was dormant in healthy tissues but was activated in the course of TMV-induced HR and when its activity was suppressed by a peptide aldehyde matching its cleavage site, PCD mediated by TMV was inhibited in tobacco leaves (Chichkova *et al.*, 2004). Moreover, CLPs with specificity and biochemical properties similar to the tobacco enzyme, were found to be ubiquitous in plants and sensitive to a range of peptide aldehyde inhibitors of animal caspases (Chichkova *et al.*, 2008). Recently, Chichkova *et al.*, (2010) reported the isolation and identification of this protease from rice and tobacco, which they named phytaspase (plant aspartate-specific protease). Phytaspase was found to be synthesised as a proenzyme and autocatalytically processed to generate a mature enzyme. Overexpression/silencing studies showed that phytaspase is essential for PCD-related responses to biotic (TMV) and abiotic stresses. The authors also discovered that this enzyme is constitutively secreted into the apoplast before PCD, but surprisingly it is partially reimported into the cell during PCD. Another plant protease exhibiting caspase-like activity analogous to that of caspase-1 is vacuolar-processing enzyme (VPE), required for PCD induced by TMV (Hatsugai *et al.*, 2004), fumonisin (Kuroyanagi *et al.*, 2005) or developmental cell death in seeds (Nakaune *et al.*, 2005). Further examples of caspase-like molecules involved in plant PCD have been reviewed by Reape and McCabe (2008) and Bonneau *et al.* (2008). Future work aiming to identify the location, mode of action and natural substrates of plant proteases involved in the execution of PCD, will provide insights into plant PCD pathways. Table 1 summarises evidence for the possible role of metacaspases and caspase-like activities during plant PCD.

*<Table. 1 near here>*

1. **Endomembrane system mediated control of PCD**

Cacas (2010) discussed the possible regulation of PCD events mediated by the cell endomembrane system (ES). The author proposed that the components of the ES (endoplasmic reticulum (ER), Golgi apparatus (GA), and the vacuole) may orchestrate cellular death, as various PCD mediators are present along the ES or can travel via the secretory pathway en route to specific destinations (Cacas, 2010).

1. **ER stress**

Stress can cause accumulation of unfolded proteins in the ER, triggering an evolutionarily conserved response, termed unfolded protein response (UPR) (Xu *et al.*, 2005). In plants, UPR is thought to be involved in seed development and pathogen response (Vitale and Ceriotti, 2004). In animal cells, UPR can lead either to restoration of proper protein folding and ER homeostasis or, if the protein folding defect cannot be resolved, PCD may be triggered as the result of chronic activation of UPR signalling (Malhotra and Kaufman, 2007). Although plant UPR signalling pathways still require deciphering, counterparts of several yeasts and animal UPR effectors have been identified in plants (Koizumi *et al.*, 2001, Okushima *et al.*, 2002) and plant-specific UPR regulators have been discovered (Oh *et al.*, 2003). Studies connecting abiotic/biotic stress tolerance with ER resident proteins (for example; Alvim *et al.*, 2001, Danon *et al.*, 2004, Gao *et al.*, 2008b, Jelitto-Van Dooren *et al.*, 1999) imply a stress-sensing role of the ER in plant cells (Cacas, 2010). Indeed, chemically-induced ER stress has been shown to induce apoptotic-like PCD in sycamore cell cultures where accumulation of hydrogen peroxide, shrinkage of cytoplasm and detection of a DNA ladder occurred (Crosti *et al.*, 2001). While in soybean cells, the accumulation of hydrogen peroxide, cyt *c* release from mitochondria, caspase 9- and caspase 3-like protease activation, cytoplasmic shrinkage and chromatin condensation were highlighted (Zuppini *et al.*, 2004), and in *Arabidopsis*, the accumulation of H2O2, chromatin condensation, and oligonucleosomal fragmentation of nuclear DNA was recorded (Watanabe and Lam, 2008). Genes known to be upregulated by ER stress, for instance molecular chaperone binding protein (BiP), protein disulfide isomerase (PDI), carleticulin (CRT1 and CRT2) and calnexin (CNX1) (Kamauchi *et al.*, 2005, Koizumi *et al.*, 2001, Noh *et al.*, 2003, Urade, 2009) can be treated as UPR markers. In *Arabidopsis*, the ER is an intracellular calcium store, which can be accessed and mobilised for signalling purposes during stress response and possibly during activation of PCD (Wyatt *et al.*, 2002). Furthermore, the ER residing protein, At-BI1 (Bax Inhibitor-1) has been proposed to be involved in protection against ER stress by mediating calcium fluxes, as its overexpression decreases the cytosolic calcium peak that occurs in response to cyclopiazonic acid, and increases tolerance to this drug in *Arabidopsis* (Ihara-Ohori *et al.*, 2007). It has also been proposed that BI1 can indirectly control the intracellular concentration of known PCD regulators like sphingolipids (Nagano *et al.*, 2009). Therefore, as concluded by Cacas (2010), there is strong evidence linking ER stress with PCD pathway(s), however, whether ER stress is essential for all PCD events or is specific for certain types of PCD has not yet been resolved. Also, the details of the pathway(s) by which ER stress induces death have still to be elucidated.

1. **Peroxisomes**

Peroxisomes are single membrane bound, subcellular respiratory organelles, that contain catalase and H2O2 producing flavin oxidases as basic enzymatic constituents (del Río *et al.*, 2002). Peroxisomes were traditionally regarded as semiautonomous, static, and homogenous subcellular compartments whose assembly, as organelles outside the secretory and endocytic pathways of vesicular flow, does not involve intercompartmental vesicular trafficking (Lazarow, 2003). However, it was recently postulated that peroxisomes derive from the ER and form a multicompartmental endomembrane system similar to the secretory endomembrane system of vesicular flow (reviewed by Titorenko and Mullen, 2006, Titorenko *et al.,* 2008). Peroxisomes, which are characterised by an essentially oxidative type of metabolism, are a source of signalling molecules such as ROS and nitric oxide, releasing these messenger molecules to the cytosol where they play a role in PCD activation (Corpas *et al.*, 2001, Nyathi and Baker, 2006). Moreover, research also suggests that they play a role in senescence (del Rio *et al.*, 1998).

1. **Golgi apparatus**

Recently, research suggests a putative role for the Golgi Apparatus in PCD signalling. Gubser *et al.* (2007) recently identified a Golgi apparatus-resident apoptosis inhibitor (GAAP) which has homologues in all sequenced plant genomes (Cacas, 2010). However, a functional investigation of *Arabidopsis* GAAP multigenic family is required to elucidate the possible contribution of Golgi apparatus to PCD (Cacas, 2010).

1. **Vacuole**

The vacuole is a degradative organelle and is thought to participate in a variety of processes including PCD. Vacuolar processing enzymes (VPEs) have been proposed to be caspase-like executioners of PCD during HR (Hatsugai *et al.*, 2004) and they have also been found to be necessary for mycotoxin-induced cell death in *Arabidopsis* (Kuroyanagi *et al.*, 2005). Another vacuolar protease, δVPE is involved in cell death associated with early seed development (Nakaune *et al.*, 2005).

1. **Transport mechanism of degradative molecules through secretory pathway**

A variety of PCD regulators reside in the minimal secretory pathway (encompassing the nuclear envelope and ER, the GA, the vacuole and small vesicular carriers that shuttle between these different compartments (Foresti and Denecke, 2008) supporting a role for the ES in the initiation/transduction of cell death signals (Cacas, 2010). However, it is unclear how the degradative molecules (like VPE) travel through the secretory pathway without causing any damage (Cacas, 2010). A potential explanation may be provided by the mechanism described by Andeme Ondzighi (2008), who studied AL-PCD in the endothelium of developing seeds. It was revealed that protein disulfide isomerise 5 (PDI5), which oxidizes, reduces, and isomerizes disulfide bonds of proteins, accumulates in protein storage vacuoles prior to tissue self-destruction and a subsequent decrease in its concentration was correlated with the progress of cell death. Moreover, PDI5 was shown to interact with three cysteine proteases, one of which, together with PDI5, trafficked from the ER through the Golgi apparatus to vacuoles and its recombinant form, was functionally inhibited by recombinant PDI5 *in vitro*. Loss of PDI5 function led to premature initiation of PCD during embryogenesis, suggesting a role for this co-chaperone in the regulation of PCD timing and converting the protein storage vacuole into a lytic vacuole (Andeme Ondzighi *et al.*, 2008). PDI were also detected in ricinosomes (protease precursor vesicles) in senescing plant tissue (Schmid *et al.*, 2001).

1. **Sphingolipids and plant PCD**

Sphingolipids are ubiquitous constituents of eukaryotic cells and in their complex forms they constitute major components of cell membranes. They are thought to protect the cell surface by forming part of the chemically resistant and mechanically strong outer shell of the lipid bilayer (Takabe *et al.*, 2008). In plants, sphingolipids account for as much as 40% of the plasma membrane and tonoplast lipids (Markham *et al.*, 2006, Sperling *et al.*, 2005) and altering sphingolipid metabolism often leads to embryo lethality in *Arabidopsis* (Chen *et al.*, 2006).

Sphingolipids are integral players in many processes in eukaryotic organisms such as in the response to heat stress, cell proliferation, cell growth, apoptosis and PCD (Chalfant and Spiegel, 2005, Jenkins, 2003, Spiegel and Milstien, 2003, Wallis and Browse, 2010). In animals, their involvement in so many wide-ranging processes is due to their functionality both in and outside the cell, where amongst other targets, they are ligands of five specific G protein-coupled cell surface receptors (GPCRs) (Spiegel and Milstien, 2003).

Several comprehensive reviews have been published recently detailing the structure, metabolism and function of sphingolipids in plants (Lynch and Dunn, 2004, Pata *et al.*, 2010, Sperling and Heinz, 2003). Plant sphingolipids are composed of a relatively simple long base chain (LCB) of 18 carbons (a sphingoid base) bound to a fatty acid via an amide link. LCBs or fatty acids can differ due to the addition or subtraction of hydroxyl groups and the degree of unsaturation. In addition, the fatty acid can contain between 16 to 26 carbon atoms and sphingolipids can also have phosphate groups attached. Taking this wealth of possible structural modifications into account and allowing for stereoisomers of some of these molecules, the number of probable individual sphingolipids is vast.

Early research into sphingolipid-mediated PCD, identified the sphingolipid metabolites S1P and ceramide as important signalling molecules in cell fate decisions (Taha *et al.*, 2006b). In mammalian cells, it has been suggested that ceramide is a pro apoptotic signal while S1P protects against death. Cuvillier *et al*. (1996) found that ceramide-induced apoptosis was attenuated by the application of S1P, in addition, they found that inhibition of sphingosine kinase induces apoptosis but death is attenuated by the application of exogenous S1P. Furthermore, Cuvillier *et al.* (1998) found that S1P protected against apoptosis in serum deprived hippocampal neuronal cells and Colombaioni *et al.* (2002) used thin layer chromatography to identify an endogenous rise in ceramide after the same treatment. It is now hypothesized that in animal cells a sphingolipid rheostat exists which determines cell fate, with the relative levels of intercellular ceramide and S1P being the key players (Hait *et al.*, 2006).

Evidence exists to suggest that sphingolipids also modulate cell fate in plant cells. For example, numerous plant-based studies suggest that fumonisin B1 (FB1) and *Alternaria alternate lycopersici* (AAL) toxin cause apoptotic cell death in various species by inhibiting ceramide synthase activity. Asai *et al.* (2000) found that the fungal toxin FB1 induces PCD in *Arabidopsis* protoplasts and this death depends on the jasmonate, ethylene, and salicylic acid signalling pathways indicating that in concert with an array of plant growth regulator (PGR) signalling pathways, sphingolipids are integral in the HR. Further investigation of SPT subunits used a mutant of LCB1 named *fumonisin B1 resistant11-1* (*fbr11-1*). This mutant was unable to initiate PCD when treated with FB1 but direct feeding experiments showed that the LCBs dihydrosphingosine, phytosphingosine and sphingosine induced ROS which induced cell death. Later research which focused on examining internal levels of ceramide, found that FB1 and AAL toxin also inhibited ceramide synthase, causing higher levels of PCD in tomato leaves. In these experiments, analysis of sphingolipid metabolism changes in the dying cells showed that free LCBs accrued and disruption of sphingolipid synthesis occurred (Spassieva *et al.*, 2002).

More recent research shows that SphK1 and CerK may both be key determinants of the balance between cell death and cell survival. SphKs lipid kinases, responsible for the phosphorylation of sphingosine to S1P, are evolutionary highly conserved and have been identified in amoeba, yeast, *C. elegans,* *Drosophila melanogaster* and *A. thaliana* (Alemany *et al.*, 2007). A recent addition to the family of bioactive sphingolipids that might play a role in plant PCD is ceramide-1-phosphate (C1P). Ceramide can be phosphorylated by ceramidases kinase (CerK) to form C1P (Chalfant and Spiegel, 2005, Liang *et al.*, 2003). In mammalian cells, SphKs have been shown to have differing substrate specificity and be differentially expressed at various stages of development (Alemany *et al.*, 2007). Two isoforms of SphKs have been identified in mammals; SphK1 and SphK2 (Maceyka *et al.*, 2005). SphKs are activated by five specific GPCRs and three other putative GPCRs located on the cell surface (Takabe *et al.*, 2008). The regulation of SphK1and SphK2 is poorly understood but SphK1 appears to promote cell growth while SphK2 promotes PCD. Although they appear to have opposing roles, Alemany *et al.* (2007) report that they can substitute for each other. Subsequently, Coursol *et al.* (2003) demonstrated the presence of SphK activity in *Arabidopsis* and found that the enzyme activity is stimulated by ABA in mesophyll and guard cell protoplasts. Furthermore, Coursol’s group found that phytosphingosine can act as a substrate for *Arabidopsis* SphK. Phytosphingosine is the most abundant LCB in plants and once phosphorylated, it can act as a regulator of guard cell aperture (Coursol *et al.*, 2005). Further investigations showed that knockdown of SphK in breast cancer cells causes a decrease in the internal level of S1P while at the same time increases the levels of ceramide (Taha *et al.*, 2006a). Interestingly, ceramide levels have also been shown to increase when yeast cells are heat treated (Mao *et al.*, 1999). This large body of work adds convincing evidence for the existence of the sphingolipid rheostat that we mentioned previously. Research by Alden *et al.* (2011) attempted to test the sphingolipid rheostat hypothesis in plant cells by exogenously treating suspension cultures of *A. thaliana* with S1P, heat-stressing the cultures to induce the heat shock response and then examining levels of PCD using characteristic protoplast retraction morphology. Results showed that augmenting exogenous levels of S1P infers a protective effect against PCD at temperatures that without the addition of S1P, would normally induce PCD. Conversely, when exogenous ceramide was added to the cultures, it induced PCD in a concentration dependent manner.

Ceramide and S1P have emerged as key signalling molecules in the control of cell survival and death in plant cells. It has been proposed that in healthy unstressed cells, the levels of ceramide and S1P balance each other but when a stress such as heat is applied, the level of ceramide increases and PCD is induced (Takabe *et al.*, 2008). As previously mentioned, ceramide can be phosphorylated by ceramidases kinase (CerK) to form C1P. BLAST searches have identified putative homologs of the human CerK enzyme in plants, nematodes and insects but not in yeast (Sugiura *et al.*, 2002). Mammalian research reviewed by Arana *et al.* (2010) has suggested that C1P has the opposite effect to ceramide in that it has prosurvival properties. Modifications in the level of sphingolipids have been shown to be directly associated with PCD. Research by Brodersen *et al. (*2002) found that the unusual cell death in the *Arabidopsis* mutant *acd11* was most likely caused by perturbation of sphingolipid metabolism. They came to this conclusion by examining the *in vitro* enzymatic activities of ACD11 as a sphingosine transfer protein.

Liang *et al.* (2003) examined the CerK mutant *Arabidopsis* *accelerated cell death 5 (acd5)*. ACD5 encodes a protein with ceramide kinase activity which causes an accumulation of nonphosphorylated ceramides in the plant. They found that *acd5* exhibited extreme PCD when *Arabidopsis* protoplasts were treated with the bacterial pathogen *Pseudomonas syringae*. When they tested the effect of ceramide on *acd*5 and wild type protoplasts, they found that ceramide treatment induced more PCD in *acd*5 protoplasts than the wild type. In addition, the application of C1P partially abrogated the effect of ceramide providing evidence that a balance between ceramide and C1P may control PCD in plants.

In plants there is evidence that suggests sphingolipids may act in concert with PGR pathways, ROS and Ca2+ to modulate developmental pathways including PCD. A number of sphingolipids have been shown to regulate cellular Ca2+ homeostasis and other Ca2+ dependent pathways. Work by Ng *et al.* (2001) suggest that S1P acts as a Ca2+ mobilising molecule in plants. It was shown that guard cells subjected to drought accumulated S1P and that in this case, S1P mediates guard cell response to drought and ABA. Subsequent research by Townley *et al.* (2005) linked ROS, sphingolipids and Ca2+ pathways when they showed a transient change in Ca2+ and ROS in *Arabidopsis* cell suspension cultures that have been treated with synthetic ceramide. Recently, Lachaud *et al.* (2010) have shown that when *D-erythro*-sphinganine (DHS) was exogenously applied, endogenous levels of LCBs rose in tobacco BY-2 cells. This was followed by dosage-dependent increases in free cellular Ca2+ and subsequent PCD. When lanthanum chloride, a Ca2+ channel blocker was used, cellular fluctuations of Ca2+ were attenuated and PCD levels were reduced.

1. **Role of PCD in developmental, defence and stress responses.**

PCD is an essential component of complex multicellular body plan formation. The following sections serve to illustrate how developmentally regulated, and environmentally induced, changes in plant structure cannot be accomplished without PCD mediating the remodelling of cells and tissues.

1. **PCD in vegetative tissue development**

PCD events associated with plant development occur at specified points and locations throughout the plant life-cycle. Cell death plays a crucial role in the correct development of many vegetative plant tissues, for example as the final step of xylogenesis, during root cap sloughing, leaf morphogenesis, trichome differentiation and in late senescence.

1. **Xylogenesis**

Vascular plants form xylem – a system of rigid, hollow tubes for water transport from the roots to the rest of plant. Differentiating tracheary elements (TEs), which are the distinctive xylem cells, are characterised by the formation of a secondary cell wall with annular, spiral, reticulate, or pitted thickenings (Fukuda, 1997). A maturing TE loses its contents forming a functional corpse consisting of a hollow tube, and this process is broadly recognised as the example of developmental PCD in plants (Pennell and Lamb, 1997). During differentiation of TEs, hydrolytic enzymes including proteases, DNases and RNases (Fukuda, 1997) accumulate in the large, central vacuole. Once the secondary cell wall is formed, the tonoplast ruptures and releases its hydrolytic load to the cytoplasm (Groover *et al.*, 1997), resulting in the degradation of cell contents, including organelles and part of the cell wall (Fukuda, 2000).

The sequence of events during xylogenesis has been widely studied with the use of an *in vitro* system based on cell cultures derived from isolated *Z. elegans* mesophyll cells (Fukuda, 1997, Fukuda and Komamine, 1980, Fukuda *et al.*, 1998). In this model system, single isolated mesophyll cells transdifferentiate directly (without division) into TEs when cultured in the presence of the phytohormones auxin and cytokinin (Fukuda and Komamine, 1980). The high frequency of differentiation and the possibility of being able to observe single cells make this an attractive system with which to study TE differentiation (Chasan, 1994, Fukuda, 1994, Fukuda, 1996). Twumasi *et al.* (2009) recently reported an improved method of establishing *Z. elegans* suspension cultures characterised by increased TE yields as high as 76%. *Arabidopsis* cells cultures with induced TE differentiation have been established (Oda *et al.*, 2005) and TE differentiation has been investigated in tree species such as poplar (Hertzberg *et al.*, 2001, Schrader *et al.*, 2004). TE differentiation has also been investigated in *Arabidopsis* mutants exhibiting alternations in vascular tissue (Turner and Somerville, 1997).

Several studies have shown that cell death during xylogenesis exhibits features characteristic of PCD. First of all, TE differentiation is an active process, accompanied by upregulation of genes triggering cell destruction (Turner *et al.*, 2007). The fact that cyclohexamide treatment was shown to suppress TE death in *Zinnia* cultures indicates that protein synthesis is a prerequisite for the process (Kuriyama, 1999). Extracellular calcium uptake is also required for completion of TE maturation (Roberts and Haigler, 1990). Cyt *c* release prior to TE PCD has been detected, although this release was not sufficient to trigger PCD and therefore the causative role of cyt *c* in this death is questionable (Yu *et al.*, 2002). Depolarisation of mitochondrial membranes and changes in mitochondrial ultrastructure prior to vacuolar collapse suggests involvement of mitochondria in TE PCD (Yu *et al.*, 2002). Suppression of TE differentiation in the presence of human caspase inhibitors has also been reported, which suggests a role for caspase-like proteases in the process (Iakimova and Woltering, 2009). However, apoptotic hallmarks such as prominent chromatin condensation and nuclear fragmentation are not generally observed during TE differentiation (Groover *et al.*, 1997, Obara *et al.*, 2001) and only one study reported appearance of a weak DNA ladder in developing secondary xylem of *Eucommia ulmoides* (Cao *et al.*, 2003). DNA degradation has been shown in developing TE *in vitro* (Groover *et al.*, 1997) and *in vivo* (Mittler and Lam, 1995) and this degradation was presumed to occur after the release of endonucleases previously contained within the vacuole (Obara *et al.*, 2001).

1. **Root cap**

The tip of the root is covered by the cap, which consists of living parenchyma cells derived continuously from the apical meristem (Esau, 1977). The root cap protects the root apical meristem during germination and seedling growth (Pennell and Lamb, 1997) and root cap cells are continually displaced to the root periphery by new cells (Schiefelbein *et al.*, 1997), where they may continue to live for several days, after which they die (Harkes, 1973, Pennell and Lamb, 1997, Schiefelbein *et al.*, 1997). This death is a normal part of development rather than an effect of mechanical stress during soil penetration, as it also occurs when roots are grown in water (Pennell and Lamb, 1997). Wang *et al.* (1996) noted that dying onion root cap cells shrink to form distinct bodies containing fragmented DNA and formation of these bodies appeared to be the final step before a cell was shed from the root cap. DNA staining showed condensation of nuclei in dying onion root cap cells and TUNEL assay confirmed accumulation of free 3’-OH ends in the DNA (Wang *et al.*, 1996). These characteristic PCD features, together with the fact that dying cells are exposed on the root surface, make root cap cells potentially interesting targets of cell death-related research. Isolation of mutants exhibiting altered PCD in root cap cells would facilitate elucidation of this death process regulation. Moreover, maize root cap cells can be cultured in the form of a cell suspension culture in order to obtain large number of isolated cap cells (Caporali, 1983).

1. **Leaf morphogenesis**

Formation of complex leaf shape during leaf morphogenesis is another example of developmentally controlled PCD. Swiss cheese plant (*Monstera obliqua*, *Monstera deliciosa*) or Madagascar lace plant (*Aponogeton madagascariensis*) both exhibit remodelling of leaf blades occurring through the death of discretesubpopulations of cells, a process which is rarely encountered in leaf shape development (Gunawardena *et al.*, 2004). *Monstera* leaves are characterised by small, distinctive perforations composed of dead cells formed early in development, which as the leaf expands, extend their area to fissures of significant size (Gunawardena *et al.*, 2005). Indeed, dying cells of *Monstera* exhibit several characteristic features such as chromatin and cytoplasm condensation, DNA degradation (although internucleosomal cleavage was not observed), disrupted vacuole and finally, organelles remaining intact until late in the process (Gunawardena *et al.*, 2005). PCD occurs simultaneously throughout the perforation site and the boundary between dying and healthy cells is sharply delineated, suggesting that all target cells receive a death signal in unison (Gunawardena *et al.*, 2005). This is not the case of lace plant leaves, where a complex and a unique lattice-like pattern of equidistantly positioned perforations is formed at a specific developmental point (Gunawardena *et al.*, 2004, Serguéeff, 1907). PCD in lace plant leaves (Fig. 2) is initiated by centrally located cells within each perforation and cell death extends sequentially as the perforation expands, stopping approximately five cell layers from the vascular tissue. Occurrence of PCD in lace plant leaf perforations has been confirmed by features such as DNA degradation (without DNA ladder), invagination of membranes, abundantvesicles, shrinkage of the cytoplasm, and the late degradationof organelles (Gunawardena *et al.*, 2004). Moreover, treatment of aquatic lace plants with a calcium channel blocker, resulted in the reduction of perforation formation but without detrimental effects on leaf growth, therefore providing indirect evidence of the possible role of calcium in this developmental PCD (Elliott and Gunawardena, 2010). Lace plant is considered an attractive system for PCD studies due to accessibility of dying cells (significantly higher than for *Monstera*) and the predictability of the length of time it takes for perforations to develop (developmental stage). Other advantages include the cells location (in relation to the vein system) and the fact that this plant can be propagated in sterile conditions relatively easily (Gunawardena, 2008).

*<Fig. 2 near here>*

1. **Trichome differentiation**

Trichomes are shoot epidermal hairs, found on the majority of plants and are composed of either single or several cells (Esau, 1977). They play various protective roles, such as, being a mechanical barrier to insect herbivores, filtering UV light and reducing respiration (Fordyce and Agrawal, 2001, Karabourniotis *et al.*, 1992, Levin, 1973, Ripley *et al.*, 1999, Van Dam and Hare, 1998). Plants belonging to the genus *Tillandsia* are able to survive independent of soil in the neotropical epiphytic environment, as their adventitious roots function only to adhere to substratum (Papini *et al.*, 2009). Epiphytism of *Tillandsia* plants is possible due to a large number of peltate trichomes on their leaf epidermis, which are specialised in the absorption of solutions (Brighigna, 1974). The absorbing trichome in *Tillandsia* has a nail-like shape, formed by an axis (stem) connected to the internal tissues of the leaf and by an external shield. At the last stage of trichome development, the shield cells die and this death event is characterised by chromatin condensation, nuclear fragmentation and ER dilation (Papini *et al.*, 2009).

As trichomes are present in large numbers on the surface of the leaf/stem and are composed of single/few cells they provide an easily observable, identifiable and *in vivo* target for PCD related research. For instance, Schnittger and colleagues (2003) investigated the function of CDK inhibitor proteins (ICK/KRPs) on cell growth and differentiation by expressing ICK/KRP under a trichome specific promoter in *Arabidopsis.* ICK/KRPs shares some sequence homology to animal p27Kip1(De Veylder *et al.*, 2001), a cell-cycle inhibitor which can induce apoptosis (Katayose *et al.*, 1997).They demonstrated that *Arabidopsis* trichomes where ICK/KRPs were overexpressed died earlier than wild type (WT) trichomes and it was observed that their nuclear structure started to change (the chromocenters and the nucleolus disappeared) suggesting the programmed nature of this death (Schnittger *et al.*, 2003). Reina-Pinto *et al.* (2009) reported that the epidermal mis-expression of fatty acid elongase1 (FAE1) in *Arabidopsis,* induced a cell-type specific cell death programme in trichome cells, with death characterised by loss of viabilityand membrane integrity, generation of ROS, and DNA fragmentation. Therefore, it may be concluded that trichomes are potentially an attractive model system for investigating PCD, especially since the characteristic AL-PCD ‘corpse’ morphology can be induced in trichomes by stress treatment and easily examined under the microscope (Fig. 1E).

1. **Senescence**

Senescence can be considered the last stage of plant vegetative and reproductivedevelopment and is followed by the death of cells and organs. It is an ordered process characterised by complex changes in cell structure, metabolism and gene expression resulting in degradation and remobilisation activities aimed at retrieving valuable nutrients and their allocation to viable parts of the plant (e.g. seeds). Senescence culminates in organ death, but can often be reversed quite late in the process (Thomas *et al.*, 2003). However, the relationship between PCD and senescence is much debated amongst research groups. Attitudes vary from implying a total overlap and synchronous progress of both processes (van Doorn and Woltering, 2008, van Doorn and Woltering, 2004), via assumed, but not defined overlap, up to the belief that senescence and PCD are separate processes (Delorme *et al.*, 2000, Thomas *et al.*, 2003). Certainly the death finalising the senescence process exhibits apoptotic like features such as a condensed nucleus, shrinkage of the cytoplasm away from the cell wall (Delorme *et al.*, 2000, Swidzinski *et al.*, 2002) and oligonucleosomal DNA cleavage (Delorme *et al.*, 2000, Kawai and Uchimiya, 2000, Yamada *et al.*, 2004). The PCD associated gene, Bax inhibitor-1 (Bl-1) is upregulated during flower senescence in oilseed rape and tobacco (Bolduc *et al.*, 2003) and also during post-harvest senescence in broccoli (Coupe *et al.*, 2004) while defender against apoptotic cell death (DAD1) is upregulated during senescence of leaves, fruits and relatively long-lasting petals in apple (Dong *et al.*, 1998). It is also notable, that during the initial stages of leaf senescence, an increase in low-molecular weight antioxidants such as like α-tocopherol is observed (Munne-Bosch and Penuelas, 2003) possibly to protect the cellular functions required for progression and completion of cellular recycling. However, in the latter stages of senescence, antioxidants level decreases and a concomitant increase in lipid peroxidation and protein oxidation takes place (Munne-Bosch and Penuelas, 2003). Moreover, AL-PCD hallmarks (morphology and laddering) during senescence of cucumber cotyledons were not observed until as late as 12 h before organ death (Delorme *et al.*, 2000). It is therefore possible that as long as the recycling of nutrients takes place during senescence, AL-PCD is actively suppressed (Reape and McCabe, 2008).

1. **PCD in reproductive tissue development**

PCD functions in many natural plant processes as a facet of reproductive growth and development. Embryo formation in angiosperms and gymnosperms relies on PCD for organ formation. During embryo formation, the suspensor and the nucellus are eliminated by PCD, as are supernumerary embryos. As has been discussed earlier, PCD has been clearly implicated in xylogenesis in maturing plants but new research suggests that xylogenesis, aided by PCD, may start as early as the mature embryonic stage. During embryo germination, the central endosperm and the aleurone layer are degraded by PCD. In evolutionary terms, plant diversity has been driven to some extent by the PCD-dependent process by which plants force outbreeding by making their own pollen incompatible with their stigma. In the following sections, each of these reproductive PCDs will be discussed in detail and the cellular and molecular features of particular types of cell death will become apparent.

1. **Embryo formation and germination**

During both plant and mammalian life cycles, the first occurrences of developmental PCD occur during embryogenesis (Brill *et al.*, 1999). Angiosperms and gymnosperms diverged from a common ancestor 300 million years ago (Bowe *et al.*, 2000) and separate evolutionary paths have resulted in distinct developmental processes leading to seed. For instance, gymnosperm embryos originate from a single fertilisation event resulting in a diploid embryo with the notable absence of an endosperm tissue (Singh, 1978), whereas regeneration of angiosperms is instigated with a double fertilisation event that produces a diploid single-celled zygote and an endosperm precursor cell (Cairney and Pullman, 2007). The all-encompassing term ‘embryogenesis’ describes the subsequent stages of development. PCD plays an integral role during embryogenesis (Bozhkov *et al.*, 2005a). Firstly, a group of cells known collectively as the suspensor are eliminated by PCD. The suspensor anchors the embryo in a growing seed but also acts as a ‘lifeline’ along which nutrients and PGRs travel to and from the seed during development (Friml *et al.*, 2003, Yeung and Meinke, 1993). Also, PCD occurs to facilitate the elimination of all but one dominant embryo during monozygotic polyembryony (Bell, 1996). This phenomenon occurs when many embryos arise from the same zygote and usually, only one dominant embryo survives while the remainder die by PCD. Finally, the nucellus is degraded by PCD to create space for the expanding endosperm.

Due to its inaccessible location*,* the embryo is a difficult life-stage to study *in vivo* as it is buried under numerous cell layers and as is the case with angiosperms, often embedded within the endosperm (Maheshwari, 1950). In addition, ovules generally develop asynchronously (Owens, 1995) resulting in embryos at varying stages of development. Somatic embryogenesis of gymnosperms to mass-produce genetic clones of elite conifer genotypes for use in forestry has emerged as a novel system for the examination of embryos in all stages of development. Embryogenic cultures of many angiosperms have been established successfully, including carrot and *Arabidopsis* (Mordhorst *et al.*, 1998, Nomura and Komamine, 1986) and single carrot-cells in suspension cultures can develop into somatic embryos (McCabe *et al.*, 1997b). Interestingly, a novel method of producing *Brassica napus* (rapeseed) embryos from a megaspore culture results in individual embryos with the suspensor attached and may be useful in the future for studying embryogenesis and PCD of the suspensor (Supena *et al.*, 2008). Advantages of embryogenesis originating from *in vitro* cultured somatic cellsis the possibility of obtaining embryos, which are free of the ovule, synchronous, more numerous induction of embryos and ease of manipulation. Cell cultures capable of somatic embryogenesis can be induced to do so simply by varying or withdrawing PGRs (Bozhkov *et al.*, 2002, Kawashima and Goldberg, 2010, Larsson *et al.*, 2008, Nomura and Komamine, 1986), resulting in a culture of numerous embryos all at the same stage of development.

* 1. **Suspensor elimination by PCD**

Embryogenesis in dicotyledon plants has been defined as having three overlapping phases (West and Harada, 1993). The first phase, the latter of which is most reliant on cell death, culminates in extensive morphological changes during which the polar axis of the plant body is delineated and the axis of the shoot and root meristems become apparent. The second phase involves the storage of reserves such as carbohydrate, lipids and proteins, followed by embryo maturation. During the third and final stage, the embryo becomes desiccated and enters a period of dormancy.

Although there are significant differences in the cell division patterns of developing conifer and flowering plant seeds (Cairney and Pullman, 2007), the first phase of development of the embryo in seed plants can be generalised as follows; the zygote divides into a small group of cells and subsequent differentiation results in an embryo and a short stalk-like structure called the suspensor. Suspensor morphology differs remarkably between species (Kawashima and Goldberg, 2010, Maheshwari, 1950, Yeung and Meinke, 1993). In *Arabidopsis* the suspensor is composed of a row of seven cells, in the orchid it can be a single cell and in runner bean, the suspensor can be composed of over 200 cells (Kawashima and Goldberg, 2010, Lombardi *et al.*, 2007c). As highlighted in the Leguminosae**,** morphological differences can occur between members of the same family; some members have a one-celled suspensor while others have structures comprised of large numbers of cells (Lersten, 1983). Crucially, most seed plant embryos possess suspensors (Yeung and Meinke, 1993) and in all cases, they are eliminated by PCD and do not contribute to the next generation (Bozhkov *et al.*, 2005a).

During somatic embryogenesis, the dicotyledonous gymnosperm Norway spruce produces proembryogenic masses (PEMs) from which individual somatic embryos arise. Two waves of PCD occur during somatic embryogenesis; the first eliminates the PEMs and the second eliminates the embryo suspensor cells. The suspensors exhibit AL-PCD hallmarks such as nuclear lobbing, degradation of nuclear DNA into 50 kb and multiples of 180bp fragments. Interestingly, the death pathway appears to follow an autophagic type PCD route where firstly the organelles and nucleus are dismantled, followed by lysis of the tonoplast, resulting in a large empty cell (Filonova *et al.*, 2000). This study used the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to demonstrate that DNA had been degraded. The TUNEL assay has also been used to successfully identify DNA degradation during the PCD-mediated elimination of the suspensor in *Zea mays* (maize), *Phaseolus coccineus* (runner bean) and Abies alba (European Silver Fir) embryos (Giuliani *et al.*, 2002, Lombardi *et al.*, 2007c, Petrussa *et al.*, 2009). Lombardi *et al*. (2007b) have shown that cyt *c* is released from the mitochondria and metacaspases are induced just prior to PCD in runner bean suspensors. Moreover, research using somatic embryos of A. alba (Petrussa *et al.*, 2009) has shown that increased activity of K+ channels on the mitochondrial membrane could increase the flow of K+ ions into the mitochondrial matrix. In doing so, the researchers suggest that the resulting mitochondrial swelling may induce cyt *c* release.

PCD elimination of suspensor cells has also been studied in relation to factors such as concentration of PGRs, cytoskeleton arrangement and arabinogalactan protein expression (Gao and Showalter, 1999, Larsson *et al.*, 2008, Majewska-Sawka and Nothnagel, 2000, Smertenko *et al.*, 2003). The addition and subtraction of PGRs have been show to influence PCD rates during embryogenesis. It has been shown that conifer PEMs produce somatic embryos once the PGRs cytokinin and auxin have been withdrawn (Larsson *et al.*, 2008). Larsson *et al*., (2008) used the auxin transport inhibitor 1-N-naphtylphthalamic acid (NPA), to disrupt the polar auxin dynamics of *P. abies*. This resulted in the normal cell number in the embryo and suspensor being altered and endogenous levels of the auxin indole acetic acid (IAA) doubled. Furthermore, if normal auxin movement was blocked during the period of critical cell division leading up to growth and elongation of the suspensor, the suspensor cells fail to undergo PCD. Smertenko (2003) investigated the structure of microtubule and F-action filaments in normal embryo and suspensor cells and found that total degradation of the microtubules and disorganisation of the F-action in the suspensor cells but normal organisation of the microtubules and F-action in embryo cells. Upon treatment with F-action depolymerisation drugs, normal cell patterning in the embryo was disrupted resulting in an embryo double the normal size. In addition, elongation of the suspensor cells was hindered and PCD did not occur in the suspensor cells.

Arabinogalactan proteins (AGPs) are hydroxyproline-rich glycoproteinspresent at the plasma membrane and extracellular matrices of most plant cells (Du *et al.*, 1996, Mashiguchi *et al.*, 2008). Secondary monoclonal antibodies can be raised against AGPs and in the past have been successfully used to identify and sort somatic embryo carrot cells into specific AGP expression and non-expressing cultures (McCabe *et al.*, 1997b). Filonova *et al*., (2000) identified a disparity in the expression of the monoclonal antibody JIM13-reactive epitopes of AGPs in the cell walls of PEMs and somatic embryos. They found that the majority of PEM cells expressed the epitope, but expression was absent in the early somatic embryos. As reviewed by Majewska-Sawka and Nothnagel (2000), there is evidence that AGPs may play a role in plant PCD. For example, Gao and Showalter (1999) used (β-D-Glc)3 to bind AGPs of suspension cultures of *Arabidopsis* and found that PCD was induced. Previously, immunocytochemical detection studies suggested that particular AGPs mark cells destined for PCD. In fact, it is specifically the JIM13-reactive and JIM-14 reactive epitopes of AGPs which mark the cells destined for PCD. For instance, JIM13 and JIM14 reactive epitopes in maturing sclerenchyma cells of maize coleoptiles have been identified (Schindler *et al.*, 1995), while a JIM13-reactive epitope in *Z.* *elegans* is localised to the secondary thickenings of mature TEs but not in their surrounding primary walls (Stacey *et al.*, 1995).

Undoubtedly, future work aimed at clarifying the mechanisms that influence embryogenesis and PCD will involve the use of embryo-specific mutants. Many embryo-specific mutants have been identified in *Arabidopsis* but the majority result in embryo lethality (Bozhkov *et al.*, 2005a, Marsden and Meinke, 1985). Fortunately, thus far two embryo-specific mutants useful for studying suspensor death have been identified. Firstly, the *yda* embryo-specific mutant results in a loss-of-function mutation of YODA, a gene encoding a mitogen-activated protease kinase kinase kinase (MAPKK kinase) (Lukowitz *et al.*, 2004) and the involvement of MAP kinase signalling cascades in eukaryotic PCD have been well documented (Chang and Karin, 2001). During normal development, the *Arabidopsis* zygote divides into two daughter cells of unequal size. The basal cell gives rise to the suspensor while the apical cell proceeds to form the embryo proper. YODA suppresses elongation and alters division of the zygote resulting in a basal and apical cell of equal size. Consequently, the crucial role the basal cell plays in determining polarity is lost. Instead of being incorporated into the suspensor, the basal cell is incorporated into the embryo resulting in a hyper-elongated suspensor and no recognisable embryo formation. *Vacuoleless1 (vcl1),* another *Arabidopsis* embryo-specific mutant that lacks vacuoles is also useful for studying suspensor death (Rojo *et al.*, 2001). The cells of the suspensor are usually highly vacuolated (Bozhkov *et al.*, 2005a) but in this mutant, the vacuole is completely absent. In addition the embryo progressively accumulates autophagosomes until most of its internal organisation is lost. Consequently, the *vacl1* mutant may be useful for investigating the apparent overlapping of AL-PCD and autophagic PCD in the elimination of embryo suspensor cells (Sanmartin *et al.*, 2005).

* 1. **Embryo abortion**

For at least twenty genera of gymnosperms, it is a normal strategy of plant reproduction for many embryos to arise from the same zygote in a process termed monozygotic polyembryony (Singh, 1978, Sorensen, 1982, Zhivotovsky, 2002). This too is apparent in the animal kingdom where thus far, polyembryony has been identified in 18 taxa in at least six different phyla (Craig *et al.*, 1997, Zhurov *et al.*, 2007). Interestingly, polyembryony is rare in angiosperms (Sorensen, 1982), where abortion of the non-dominant embryos by PCD is common. Peter Bell argued that the organised death of three of the four megaspores in a seed plant tetrad was a result of PCD (Bell, 1996). Bell also suggests that a single gene may be sufficient to start PCD in the megaspores of the fern *Marsilea* and that the genetic mechanism controlling this death may be related to that controlling self-incompatibility. Embryo abortion has also been identified in *Pinus* *sylvestris* (Scots Pine), a species which makes a good model system for the study of embryo abortion due to the fact that multiple equal-sized embryos arise from the same zygote, all of which are all in the same location and in close proximity to one another (Filonova *et al.*, 2002). This particular study clarified the steps that lead to the formation of multiple embryos and the subsequent abortion of all but one. Firstly the product of the zygote, a single embryo, cleaves into many embryos of the same size. All of the embryos start their development at the same rate until one out-competes the rest and becomes dominant. After dominance is established, the remaining embryos suffer a decline in growth rate. Once the dominant embryo reaches its cotyledon stage, the rest of the embryos are eliminated by PCD. This schedule of events was elucidated by examining levels of DNA integrity using the TUNEL assay over the entire time course of these events. The researchers found that DNA was already being fragmented in the suspensor cells of the non-dominant embryos while the dominant embryo was achieving dominance. The embryos themselves did not stain positive for DNA fragmentation until the cotyledon stage was attained by the dominant embryo after which ultra-structural examination of the embryos uncovered many enlarged vacuoles engulfing and degraded cell organelles, therefore suggesting a possible autophagic cell death process (Filonova *et al.*, 2002).

* 1. **Nucellus PCD**

In angiosperms, the nucellus contains the embryo sac and is surrounded by the integuments. As occurs in the majority of known examples, the nucellus begins to degenerate immediately after fertilisation and in doing so, provides nutrients to the young embryo and the growing endosperm, leaving a cavity into which the embryo and endosperm grow. Greenwood *et al.* (2005) used electron micrography to identify labyrinthine ingrowths on the thin external walls of expanding endosperm cells, these ingrowths being morphologically characteristic of cells that carry out a transfer function. These transfer cells were situated on the outmost layer of the endosperm, which suggests the catabolites released by thedying nucellar cells are taken up via these morphologically differentiated cells. Although the nucellar cells take on a crushed appearance in line with the expanding endosperm, it has been frequently confirmed that nucellar cells are programmed to die rather than suffering gross mechanical damage which may induce a necrotic type death.

The degradation of the nucellus seems to occur in all flowering plants with some differences in the rate and pattern of breakdown between species. In studies of *Sechium edule* (chayote), *Ricinus communis* (castor oil plant)and *Triticium aestivum* (wheat) researchers used the TUNEL assay to highlight DNA fragmentation during PCD of the nucellus. Interestingly, strong TUNEL staining was evident in only the first two to three layers of nucellar cells along the side of the nucellus closest to the expanding endosperm (Domínguez *et al.*, 2001, Greenwood *et al.*, 2005, Lombardi *et al.*, 2007a). Lombardi *et al.,* (2007b) also report the lack of a nucleolus in the outermost cells of the nucellar cells undergoing PCD in *Sechium edule*. Although not characterised as PCD at the time, similar DNA alterations were identified in the dying nucellar cells of *Oenothera biennis* (Evening Primrose) and *Hordeum vulgare* (barley) (de Halac, 1980, Norstog, 1974).What initiates PCD in the nucellus? Ricinosomes are organelles which originate from the lumen of the endoplasmic reticulum and have been identified as PCD elicitors during nucellus death in *Ricinus* seeds. Previously, they have been labelled as cellular ‘bombers’ after it was documented that ricinosomes bud from the ER at the same time as extensive DNA breakdown is instigated during PCD (Greenwood *et al.*, 2005). Ricinosomes harbour a pro-cysteine endopeptidase (CysEP) enzyme with a wide-ranging affinity for different proteins targeted for breakdown during PCD (Than *et al.*, 2004)**.** Ricinosomes, and the role they play in senescing plant tissues, is extensively reviewed by Gietl and Schmid (2001). Genomic studies aimed at examining the activity of proteinases, and studies investigating the induction of plant metacaspases sensitive to specific inhibitors, have added to our knowledge of nucellus death. Chen and Foolad (1997) identified the nucellin gene in nucellar cells of barley that functions as a protease during PCD and is upregulated in dying nucellar cells after fertilisation. Further work by Linnestad (1998) localised the nucellin gene expression to the cell walls of barley nucellar cells, indicating that this particular protease may play a role in cell wall turnover. Furthermore, a rice ortholog of nucellin (named asparticprotease1 (OsAsp1)) has been identified as a protease active during PCD in the nucellus of rice embryos (Bi *et al.*, 2005). A cysteine protease has also been localised to the nucellar cells during PCD in *Solanum melongena* (Brinjal) (Xu and Chye, 1999).Lombardi *et al.*(2007a) highlighted increased activity of serine, aspartic, and cysteine proteases in the dying nucellar cells of *Sechium edule*. Of these proteases, the cysteine proteases are most closely related to the caspases responsible for PCD in animals. Lombardi *et al.* (2007b) further narrowed their search for a plant-like caspase, by confirming caspase-1, 3, and 6-like protease activity in the nucellar cells undergoing PCD.

Recent studies have investigated possible links between nucellus death and the growth of the pollen tube. Gymnosperm pollen tube growth progresses much more slowly than that of angiosperms making it an excellent model system for the study of nucellus breakdown. Researchers examined *Pinus densiflora* (Japanese Red Pine ) pollen tube growth through the nucellus, and the level of TUNEL staining in the cells adjacent to it, and identified a layer of cells around the growing pollen tube that were dying by PCD. The nucellar cells nearest the pollen tube exhibited strong staining compared to the cells further away. In addition, as the tube grew further into the nucellus, pollen tube branching extended the range of TUNEL positive cells (Hiratsuka *et al.*, 2002). Furthermore, ultrastructural examination of the nucellus has shown the death of nucellar cells next to the growing pollen tube tip of *Pseudotsuga menziesii* (Douglas fir) (Owens and Morris, 1990). However, these studies cannot be taken as definitive evidence of pollen tube induced PCD, as in cotton a column of nucellar cells undergoes degeneration before the arrival of the pollen tube. The pattern of degeneration is from the embryo sac towards the micropyle (Jensen, 1969) indicating that fertilisation is not always necessary to trigger PCD in the nucellus and perhaps, the death signal emanates from the embryo sac instead.

* 1. **Endosperm and aleurone cell PCD**

The endosperm of angiosperms functions as a nutrient storage tissue for the seedling during germination (Olsen, 1998). There is diversity among flowering plant species in the nature of the food reserves stored in the endosperm, for example, starch-laden amyloplasts and protein bodies, lipids, a variety of carbohydrates and a miscellaneous array of other organic and inorganic molecules (Lopes and Larkins, 1993). The endosperm is usually comprised of two different tissues. The aleurone layer, usually only one cell thick in most cereals but morphologically diverse between species, surrounds the endosperm (Fath *et al.*, 2000, Mrva *et al.*, 2006). The endosperm cells begin to undergo PCD until eventually, all but the outermost cells die. The outermost layer of cells becomes the aleurone layer and in contrast to the endosperm cells, this layer remains viable even in the mature seed stage with death delayed until just after germination (Fath *et al.*, 2000). Gymnosperms lack an endosperm but alternatively, similar to the function of the endosperm in angiosperms, it is proposed that the gymnosperm gametophyte mobilises nutrients to the gymnosperm seedling (Baroux *et al.*, 2002). Seed development ends in a maturation step that leads to a decline in the rate of accumulation of reserves and to dehydration and dormancy. A large body of work aimed at investigating hormonal interactions during the regulation of seed dormancy release and germination of seeds is reviewed comprehensively by Finch-Savage and Leubner-Metzger (2006) and Koornneef *et al.,* (2002) but to remain within the scope of this review, we can presume that a fine balance between the endogenous levels of the PGRs, gibberellic acid (GA) and abscisic acid (ABA) regulate seed germination and subsequent PCD in the aleurone layer. As cereal seeds mature, the levels of ABA rise which serves to halt germination of an immature embryo (Lopes and Larkins, 1993). Soon after germination begins, the embryo increases its production of GA and the aleurone cells begin synthesising and releasing a battery of hydrolytic enzymes such asα-amylase (Bethke *et al.*, 1999). These enzymes breakdown lipids, proteins, nucleic acids and the cell wall polysaccharides of the endosperm during germination therefore mobilising nutrients to the growing plantlet (Mrva *et al.*, 2006). Once it has completed its task and the endosperm is degraded, the aleurone layer is programmed to die by PCD ((Mrva *et al.*, 2006) and references therein.)

Until 1997, the role of the PGR ethylene in the onset of PCD in endosperm had not been elucidated. Previously, ethylene had been implicated in the formation of aerenchyma in maize roots (Campbell and Drew, 1983) and subsequently as a mediator in the signal transduction pathway which leads to PCD after the hypersensitive response in *Arabidopsis* is triggered by pathogen attack (Greenberg and Ausubel, 1993). Beltrano *et al.,* (1994) investigated wheat ear maturation and found that ethylene positively regulated grain maturation. In light of these studies, a research team lead by Daniel Gallie, investigated whether ethylene signalling was required for the onset of PCD in the central endosperm of *Z. mays* and wheat (Young and Gallie, 2000b, Young *et al.*, 1997). They found that when exogenous ethylene was applied to maize kernels and wheat ears throughout seed development, a greater amount of DNA fragmentation occurred and cell death manifested earlier and was more extensive. Additionally, they found that PCD patterning differs between maize and wheat. For instance in maize, PCD starts with the upper central endosperm and expands laterally, whereas PCD in the wheat endosperm is initiated and continues randomly. Is it now known that ethylene peaks during two distinct developmental phases. In maize the first peak corresponds with the morphological manifestation of PCD in the endosperm and the latter coincides with an upregulation of nuclease activity and subsequent DNA fragmentation (Young and Gallie, 2000a). Further work by Young and Gallie (2000b) tested an inhibitor of ABA biosynthesis on ethylene production in maize and found that inhibition of ABA caused an increase in ethylene production which accelerated PCD in the endosperm. Overall, these results suggest that an imbalance in ABA and ethylene levels affects the initiation and subsequent rate of execution of PCD during maize endosperm development. More recent studies using wheat confirmed the findings reported in maize. Li *et al.* (2004) found that DNA fragmentation in wheat endosperm cells undergoing PCD could be accelerated by ethylene while treatment with ABA did not delay the onset of DNA fragmentation but did reduce the extent of DNA fragmentation. PCD in endosperm cells is not solely influenced by PGR fluctuations but is also accompanied by the accumulation of a spectrum of nuclease and protease activities. As was shown in nucellus PCD, an antibody against a cysteine endoprotease located ricinosomes in the cells of the endosperm of castor bean seeds (Schmid *et al.*, 1999). TUNEL staining revealed extensive DNA fragmentation which corresponded to release of ricinosomes into the cytoplasm. VEIDase, a caspase-like protease, homologous to mammalian caspase-6, was shown to be localised *in vivo* to vesicles in randomly distributed cells of the endosperm in developing barley caryopsis (Boren *et al.*, 2006). Interestingly, these vesicles were identified as autophagosomes which could be indicative of autophagic cell death.

The characteristic hallmarks of apoptosis such as DNA fragmentation, plasma membrane and nuclear blebbing and formation of apoptotic bodies are absent in dying aleurone cells. Aleurone cells become highly vacuolarised prior to death (Bethke *et al.*, 1999) and death is triggered by a sudden loss of plasma membrane integrity resulting in cellular autolysis (Fath *et al.*, 2000, Fath *et al.*, 1999). PCD of the aleurone layer commences once germination is initiated which suggests that PCD in aleurone tissue could be influenced by environmental stimuli normally associated with germination. Germination is a stressful developmental stage for a plant therefore may have to have evolved methods of dealing with challenges such as hypoxia and osmotic stress. Consequently, it is possible that many interacting transduction pathways are present in aleurone cells making the elucidation of transduction pathways complicated. For example, ABA, GA and oxygen stress induce their own exclusive responses while it has been shown that a simultaneous lack of oxygen and ABA, alters the GA transduction pathway (Kuo *et al.*, 1996).

PCD in barley aleurone cells is accompanied by activation of nuclease and protease activity and the release of a range of hydrolases including, serine, aspartic and cysteine proteases. Domínguez and Cejudo (1998) report that a serine-protease and a cysteine protease are expressed during PCD of the aleurone layer in wheat. Despite the evidence to suggest that proteases play a central role in cell death, a study by Bethke *et al.* (1999) cautioned against earmarking these proteases for consideration in the cellular autolysis death seen in aleurone death. This study treated barley aleurone protoplasts with media conditioned with the aforementioned hydrolases and PCD levels stayed unchanged. This suggests that it is not a toxic accumulation of hydrolytic enzymes that induce PCD in the aleurone cells. Subsequent examination of proteases which are synthesised but not secreted from the aleurone cells prompted the discovery of a candidate which may play a role in effecting cell death in the aleurone. RunbergRoos and Saarma (1998) found accumulations of phytepsin during autolysis of developing TE and sieve cells in barley roots. Phytepsin is a plant homologue to cathepsin D, a mammalian lysosomal aspartic proteinase which has been shown to mediate PCD in HeLa cells. Thus protease mediated death may be an important facet of aleurone PCD.

In recent years, the aleurone layer has been used as a model system to examine the role of ROS in plant PCD. There are at least two proposed functions of ROS during plant PCD. Firstly, ROS in the form of hydrogen peroxide (H2O2), have been show to induce a cascade culminating in death (Lamb and Dixon, 1997). Secondly, application of exogenous ROS can kill directly, as was the case when barley aleurone protoplasts were incubated with GA and media containing H2O2 (Bethke and Jones, 2001). The aleurone cells are rich in mitochondria and glyoxysomes (Jones, 1969). Glyoxysomes harbour peroxisomal β-oxidation enzymes which hydrolyse fatty acids to acetyl-CoA during endosperm and aleurone degradation and in doing so, are natural producers of ROS in aleurone cells (del Rio *et al.*, 1998). Mitochondria are abundant in aleurone cells and are also potent producers of ROS via the electron transport chain during ATP production (Maxwell *et al.*, 1999). Research has shown that altered electron transport in transgenic *Nicotiana tabacum* (tobacco) without mitochondrialalternative oxidase (AOX), induces a change in ROS which corresponds to an increased susceptibility to cell death signalling molecules such as salicylic acid and nitric oxide (NO) (Amirsadeghi *et al.*, 2006).Interestingly, Beligni *et al.* (2002) used barley aleurone protoplasts to show that NO acts as a scavenger of ROS and in doing so delays cell death. A study by Fath *et al.* (2002) investigated the ROS generating ability of glyoxysomes and mitochondria in barley aleurone protoplasts and examined the influence these organelles exert on PCD levels when treated with varying levels of PGRs. The researchers found that H2O2 killed GA-treated but not ABA-treated aleurone protoplasts, while ABA allowed aleurone protoplasts to abrogate the negative effect of exogenously applied H2O2. When antioxidants were applied as scavengers of ROS, GA induced cell death was reduced which reiterates the role of ROS during hormonally regulated PCD of aleurone cells.

In section IIIB1a we reviewed literature that proposed a role for AGPs in embryo and xylem differentiation PCD. Suzuki *et al.* (2002) used a synthetic chemical, β-glucosyl Yariv reagent (β-GlcY) to bind specifically to AGPs and found that GA-induced α-amylase production was inhibited as a result of β-GlcY treatmentin barley aleurone protoplasts. Interestingly, as protoplasts are devoid of cell walls, this suggests that the AGPs that function in α-amylase induction are localised in the plasma membrane. In a subsequent study, the researchers discovered that β-GlcY also inhibited GA-induced PCD in aleurone cells (Mashiguchi *et al.*, 2008) and in doing so added further evidence suggesting a potential role for AGPs in PCD.

1. **Anther dehiscence**

Development and dehiscence of the anther and the subsequent release of pollen are dependent on PCD of the tapetum and other sporophytic tissues. Pollen release, is facilitated by the degeneration of anther cells in the stomium, a rib-like structure that runs through the epidermis along the lateral side of each anther half. Failed dehiscence leads to male sterility in *Arabidopsis* and tobacco plants (Beals and Goldberg, 1997). The endothecium, which is flanked by the tapetum and the epidermis, undergoes degeneration just before anther dehiscence. Additionally, a specialised cell cluster known as the circular cell cluster, is located next to the stomium and this also dies (Wu and Cheung, 2000, and references therein.)

Tapetal cell death coincides with the development of the microgametophytes and as is the case with nucellus and aleurone cell death (see Section IIIB1c and IIIB1d), the constituents resulting from tapetal PCD provide nutrients, play a role in pollen exine sculpting and are incorporated into the pollen wall (Varnier *et al.*, 2005, Vizcay-Barrena and Wilson, 2006, Wu and Cheung, 2000). The timing of tapetal cell death must be highly regulated as premature death can cause pollen abortion. Shi *et al.* (2009) examined tapetum cells of mutant rice and found that premature tapetum degeneration was a major cause of pollen abortion.

It is clear from aforementioned studies that anther-associated PCD is connected with nutrient recycling activities so it was not surprising that recently published work (Senatore *et al.*, 2009) localised ricinosomes in the septum, the connective tissue and the anther endothecium of *Solanum lycopersicum* (tomato). All these cell tissues types are destined to die by PCD during anther dehiscence and pollen maturation (Varnier *et al.*, 2005), indicating that ricinosomes and the proteases they harbour are likely to play a role in PCD in these tissues. Studies carried out by Cecchetti *et al.*, (2004) examined early stage anther dehiscence and pollen maturation in tobacco flowers and suggested that auxin may be integral in both of these processes. Furthermore, Cecchetti *et al.* (2008) examined anther dehiscence and pollen maturation in *Arabidopsis* using *in situ* hybridisation of the auxin biosynthetic genes YUC2 and YUC6 and auxin receptor-encoding genes TIR1, AFB1, AFB2, and AFB3. This study confirmed that auxin is synthesised in anthers and timing of auxin fluctuation is crucial to effect correct pollen maturation and cell dehiscence.

A study examining cytoplasmic male sterility (CMS) in sunflower plants has provided additional insight on a possible role for cyt *c* in plant PCD. CMS mutants occur in many plant species where mutations in the mitochondrial genome affect anther and/or pollen development. Balk and Leaver, (2001) identified characteristic hallmarks of apoptotic-like PCD demonstrating that a signal in the cytoplasm of the sunflower mutant PET1-CMS leads to premature PCD of the tapetal cells, whichthen expands to other anther tissues. Subsequent immunocytochemicalstudy showed that cyt *c* was partially released fromthe mitochondria into the cytosol of tapetal cells before PCD occurred.

1. **Pollen self-incompatibility**

Pollen self incompatibility (SI) has evolved independently several times in angiosperms and is thought to have contributed to the successful radiation of flowering plants (Charlesworth *et al.*, 2005, Rea and Nasrallah, 2008). During SI, the pollen and pistil interact to degrade invading pollen from an incompatible source. This could be pollen from a different species, or pollen from the plant’s own anther.

Three mechanisms controlling SI have emerged and not all of these mechanisms involve PCD; SI control of the gametophyte has been characterised in Solanaceae, Rosaceae and *Anthirrhinum* while *Brassica* SI is mediated by the sporophyte (Rea and Nasrallah, 2008). SI in the nightshade, rose and snapdragon family is mediated by non-specific S-RNases (*S*-locus ribonucleases) which degrade RNA in the pollen germ tube, thus pollen tube elongation is halted early, usually in the upper third section of the style (McClure and Franklin-Tong, 2006). Conversely, Brassica SI is achieved by the action of two *S-*locus genes: the *S-*locus receptor kinase (*SRK*) gene located in the stigma epidermal cells and the pollen-coat-localised *S-*locus cysteine-rich protein (*SCR*) gene, which encodes the ligand for the SRK receptor. Interactions between these two gene products prevent the germination of an incompatible pollen grain (Haasen and Goring, 2010). SI in *Papaver* is late acting, in that the pollen germinates, grows into the stigma and growth is subsequently terminated, culminating in PCD of the pollen germ tube.

SI in poppy is genetically controlled by a single multi-allelic S locus (Foote *et al.*, 1994). Genes on this locus have been earmarked as recognisers of non-compatible pollen; it has been proposed that the stigma secretes small S gene products which accumulate in the extracellular matrix and interact with the incompatible pollen carrying the corresponding S allele(Kakeda *et al.*, 1998). This induces a Ca2+-dependent signalling cascade which stops pollen tube growth and culminates in PCD of the pollen tube.

As discussed in section IIA1, Ca2+ signalling has been implicated in a wide variety of PCD responses. A series of studies by Vernonica Franklin-Tong’s research group*,* (Franklin-Tong *et al.*, 1997, Franklin-Tong *et al.*, 1995, Franklin-Tong *et al.*, 1993) demonstrated a swift and large increase in free Ca2+ in the cytoplasm of incompatible pollen tubes. A subsequent study highlighted an influx of extracellular Ca2+ targeted to just behind the tube tip (Franklin-Tong *et al.*, 2002). Normally, this site requires tightly controlled calcium gradients for normal tube growth but Franklin-Tong *et al.,* (2002) found that the normal calcium gradient dissipates within 1 minute of calcium influx.

Within 60 seconds, the calcium imbalance triggers depolymerisation and rapid rearrangement of the actin cytoskeleton (Geitmann *et al.*, 2000) which is persistent for at least 1 hour (Snowman *et al.*, 2002) and interestingly, such rapid and gross changes in the actin cytoskeleton is enough to cause PCD by itself (Thomas *et al.*, 2006). Furthermore, the rapid change in [Ca2+] also triggers a mitogen-activated protein kinase (MAPK) cascade. Li *et al.,* (2007b) and Li and Franklin-Tong (2008) identified p56 MAPK as the only MAPK activated during SI and in doing so, implicated MAPK signalling involvement during early PCD.

Rudd *et al.,* (1996) found that within 90 seconds of [Ca2+] change, two cytosol pollen proteins are hyperphosphorylated in a Ca2+-dependent manner thereby reducing their activity and resulting in incompatible pollen inhibition. Later, de Graaf *et al.,* (2006) identified these proteins as the soluble inorganic pyrophosphatases (sPPases), Pr-p26.1a and Pr-p26.1b.

Calcium influx, actin rearrangement, pollen protein phosphorylation and MAPK activation can be seen as the preliminary stages of SI in *Papaver* after which various events exhibiting the hallmarks of PCD occur. For instance, a study by Jordan *et al.* (2000) used a TUNEL assay to show characteristic PCD DNA fragmentation in *Papaver* pollen tubes of an incompatible source that had been treated with S proteins. Furthermore, similar results were obtained by Thomas & Franklin-Tong, (2004) when they used TUNEL to identify DNA fragmentation in *Papaver* and positively correlated this DNA fragmentation with caspase-3-like activity and the application of S proteins. Additionally, an increase in cytosolic cyt *c* was also identified and cyt *c* leakage was detected 1–2 hours after SI induction.

To date, the majority of the work investigating PCD during SI has focused on *Papaver* with its distinct SI mechanism. Recently, PCD hallmarks have been identified in incompatible pollen tubes growing through the styles of *Olea europaea* (common olive)(Irene *et al.*, 2010) and *Pyrus pyrifolia* (asian pear) (Wang *et al.*, 2009). SI in *P. pyrifolia* has been shown to be under the control of gametophytic S-RNases (Matsuura *et al.*, 2001) although S-RNase activity has not yet been identified in the olive pollen (Irene *et al.*, 2010) nevertheless, these recent studies suggest that PCD may play a role in S-RNase mediated SI. For instance, Irene *et al.,* (2010) identified DNA fragmentation and caspase-3-like activity, while Wang *et al.,* (2009) discovered DNA fragmentation and loss of mitochondrial potential with the release of cyt *c* in the incompatible pollen tubes of olive and pear respectively.

1. **Selective abortion of primordia in some unisexual plants**

In some unisexual plants, such as maize and *Silene latifolia* (white campion), cells in either the male or female primordial must die by PCD in order to limit the flower to one sex (Wu and Cheung, 2000). In maize, the gynoecial initials are abolished in the male flowers and stamen development ceases in the female flowers. In both cases, cell death in these aforementioned tissues is preceded by extensive vacuolisation and the loss of organelle and cytoplasmic integrity (Cheng *et al.*, 1983) indicating a PCD route to death.

1. **The PCD response to abiotic and biotic stress**
2. **PCD during plant–environment interactions**

In habitats throughout the world, abiotic stresses such as flooding, temperature fluctuations, salt and excessive exposure to UV light pose a constant challenge to plant survival. Due to plants’ lack of mobility they cannot escape environmental stresses or indeed pathogen attack. Consequently, plants have evolved stress and defence responses which often involve PCD. There is pressure to mitigate the effects of biotic and abiotic pressures by developing stress tolerant crops in order to achieve the high yields necessary to feed a burgeoning human population (Mahajan and Tuteja, 2005). To attain this goal, it is first important to understand the types of PCD induced by a wide range of pressures. Only then can we understand how plants can adapt to tolerate varying levels of stress and use this information to generate plants that can adapt to a changing environment.

* 1. **Hypoxia stress – aerenchyma formation**

Aerenchyma is a tissue composed of a network of interconnected gas conducting intercellular spaces which provide plant roots with oxygen under hypoxic conditions. There are two types of aerenchyma: lysigenous, formed by collapse and programmed death of certain cells in the cortical region of the root to form air-filled cavities (Kawai *et al.*, 1998), and schizogenous, which develops through breakdown of pectic substances in the middle lamellae resulting in cell separation (Laan *et al.*, 1989). In many wetland species, aerenchyma is formed in a constitutive manner (even in dry conditions), being a pre-adaptive mechanism which can be enhanced in case of flooding in species such as rice (Jackson *et al.*, 1985) or *Juncus effuses* (Visser and Bögemann, 2006). Aerenchyma can also be formed in many dryland species where it is induced by adverse environmental conditions like hypoxia, for instance in maize (Konings, 1982), wheat (Thomson *et al.*, 1990), sunflower (Kawase and Whitmoyer, 1980) and tomato (Kawase, 1981). Formation of lysigenous aerenchyma can be also stimulated by other abiotic stresses which decrease the amount of available oxygen for respiration or the level of available nutrients. This is because aerenchyma development leads to less root respiration/less root organic material input while the same surface area is used for nutrient uptake (Fagerstedt, 2010). Other inducers of lysigenous aerenchyma formation are high temperature, nitrogen (Konings and Verschuren, 1980), phosphorous (Fan *et al.*, 2003) or sulphur deficiencies (Bouranis *et al.*, 2003), or mechanical impedance (He *et al.*, 1996a).

Cell death during lysigenous aerenchyma formation has been investigated in maize (Gunawardena *et al.*, 2001) and hallmark features of PCD were observed during this process including cytoplasmic changes and plasma membrane invagination, DNA internucleosomal fragmentation and chromatin condensation, cellular condensation and the presence of intact organelles surrounded by membrane, which resemble apoptotic bodies. Interestingly, formation of aerenchyma can be blocked in the presence of calcium binding agent EGTA or application of Ruthenium Red - which inhibits calcium release from internal stores such as the ER and mitochondria (Drew *et al.*, 2000, He *et al.*, 1996b). Analogously, agents increasing cytosolic free calcium (caffeine, thapsigargin) were shown to promote cell death (He *et al.*, 1996b).

* 1. **Salt & drought stress**

Small increases in salinity can hinder root and shoot growth and plants growing in arid and semi–arid environments are constantly subjected to salt stress (Tuteja *et al.*, 2007). Increased salinity damages the plant in a variety of ways, by deregulating ion channels and inducing osmotic pressure which leads to a reduction in soil water uptake (Williams and Dickman, 2008). PCD is an important response to salt stress as it allows the plant enough time to acquire increased tolerance. It may be counterintuitive to consider that PCD of a primary root is necessary to adapt to saline conditions, as significant levels of death in the primary root may compromise whole plant survival. However, Huh *et al*., (2002) suggest that while near-lethal salt stress of the primary root will indeed lead to its elimination the plant is sustained by the growth of new roots. Consequently, the stress eradicates the roots most susceptible to PCD while presumably, any new roots will be more adapted to the stress conditions.

Zuppini *et al.,* (2010) identified characteristic hallmarks of PCD such as increased production of H2O2 and enhanced caspase 3-like activity when they examined salt-induced PCD in the unicellular algae *Chlorella saccharophila*. Similarly, Affenzeller *et al.,* (2009) studied the freshwater green algae *Micrasterias denticulata* and recognised an autophagic type PCD induced by NaCl but not the osmotic stressor sorbitol, indicating that it is the ionic and not the osmotic stress that induces PCD. Huh *et al.,* (2002) published the first study that identified a salt-induced PCD in fungi when they showed that salt stress inhibited growth in yeast and caused an increase in autophagic-type PCD characterised by DNA fragmentation, vacuolisation and cell lysis. The plant root tip of a range of species has emerged as a model system for the study of the PCD response to various environmental stimuli, including salinity stress. Salt-induced PCD has been confirmed by DNA fragmentation in the meristematic cells of barley roots and rice (Katsuhara and Kawasaki, 1996, Liu *et al.*, 2007).

A growing body of work has identified many putative mediators in the signalling pathway of salt-induced plant PCD, including ROS, mitochondria permeability transition and Ca2+ (Chen *et al.*, 2009, Lin *et al.*, 2006, Lin *et al.*, 2005, Rengel, 1992). For example, increased ROS was observed before the orderly DNA degradation characteristic of PCD had appeared in salt stressed tobacco protoplasts, and subsequent treatment with ascorbic acid routinely decreased ROS levels and the number of protoplasts undergoing PCD (Lin *et al.*, 2006). Lin *et al*., (2005) used salt-treated tobacco protoplasts to investigate the role of Ca2+ in PCD, and found that augmented cytosolic Ca2+, together with the opening of mitochondrial permeability transition pore (MPTP), may regulate PCD in salt-treated tobacco protoplasts. This was not the first study to suggest that ion imbalance might be responsible for salt-induced PCD, for example, Huh *et al*., (2002) compared wild type yeast and *Arabidopsis* with mutants defective for ion homeostasis. They were able to show that characteristic hallmarks of autophagic type PCD such as DNA fragmentation, vacuolisation and cell lysis manifested more profoundly when Na+ homeostasis was deregulated. Shabala *et al*., (2007) showed that salt stress tolerance was enhanced in tobacco mesophyll cells that expressed the anti-apoptotic CED-9 gene, and this was due to altered K+ and H+ movement across the plasma membrane. To clarify the movement of ions during salt-induced PCD, Shabala, (2009) collated previous research findings and suggested the following chain of ionic flux events: when under salt stress, Na+ entering the cell causes significant membrane depolarisation resulting in K+ efflux, simultaneously, cytosolic [Ca2+] increases, leading to a sudden rise in cellular ROS which promotes further K+ efflux. A subsequent decrease in cytosolic K+ may activate caspase-like proteases which will lead to PCD but if there is no decline in K+, PCD will be attenuated. This would explain why PCD is not observed in sorbitol-treated cells (Affenzeller *et al.*, 2009), or in cells where membrane depolarisation is prevented by zinc (Affenzeller *et al.*, 2009) or lanthanum treatment (Li *et al.*, 2007a). Additional studies using stable overexpression of the animal anti-apoptotic genes CED-9 and Bcl-2 in plants, found that these genes inferred tolerance to many abiotic stresses including salt and drought (Chen and Dickman, 2004, Dickman *et al.*, 2001).

Autophagic-like PCD has previously been observed in response to abiotic stresses. For example, the formation of aerenchyma in maize roots, induced by hypoxia, exhibits hallmarks of both apoptosis and autophagic PCD (Gunawardena *et al.*, 2001). A more recent study using autophagy-defective RNAi-AtATG18a plants confirmed that autophagy is essential for tolerance to drought and salt stress in *Arabidopsis* and loss of function results in increased sensitivity of the AtATG18a mutants to osmotic and salt stress (Liu *et al.*, 2009). Wang *et al*., (2010) investigated the PCD response to salt stress in suspension cultures of the halophyte *Thellungiella halophila.* They found that characteristics similar to apoptosis, such as retraction of the protoplast, nuclear condensation, DNA laddering, cyt *c* release and activation of caspase 3-like protease activity, were induced by treatment with 300mM NaCl. Interestingly, upon examination of transmission electron micrographs, cells exposed to 300nM NaCl appeared to be degrading their cytoplasmic contents using autophagic vesicles. Furthermore, no autophagic vacuoles were present in untreated cells or necrotic cells, suggesting that the autophagic vacuole is a fundamental component of salt stress induced PCD and that autophagic PCD and apoptotic-like PCD may act in concert depending on the stress applied.

Plants employ a number of strategies to acquire tolerance to high levels of salt. For instance, halophyte plants that have adapted to living in saline environments often accumulate solutes such as proline and betaine in their tissues which act as osmoprotectants. These molecules are highly water soluble and non-toxic at high concentrations (Park *et al.*, 2006). Banu *et al*., (2009) exogenously applied proline and betaine to BY-2 tobacco cells exposed to high salinity and although cell death levels were unaffected, they found that ROS accumulation and lipid peroxidation of cell membranes was reduced. Research by *Ling et al.,* (2009) suggests that carbon monoxide (CO) may be involved in plant tolerance against salinity stress. They used wheat seedling roots to examine the effects of endogenously synthesized and exogenously applied CO on salt tolerance. They showed that, when salt stressed, wheat primary root tips dose-dependently synthesize CO (which is then catalyzed by heme oxygenase (HO)) and display inhibition of primary root growth and PCD. Additionally, they showed that root tip cell PCD increased in a dose-dependent manner and CO was released in significant amounts but only at the lower 100-200 mM salt concentrations. Furthermore, they found that HO was able to delay PCD in those root cap cells exposed to 200 mM NaCl suggesting that CO may be involved in plant tolerance against salinity stress. Previous research by Tiwari *et al.,* (2002) suggested that a brief or continuous oxidative stress in *Arabidopsis* cells decreased ATP production in the mitochondria and caused an increase in H2O2 production and subsequent cell death. Chen *et al.,* (2009) examined changes in the mitochondria during PCD induction using salt-stress and found that 2 hours after exposure, mitochondrial integrity was compromised and a small amount of cyt *c* was released. Analyses of the mitochondrial proteome identified 8 PCD related proteins. Upon examination of the upregulated proteins, a mitochondrial heat shock protein emerged as a potential candidate for PCD regulation while two of the four down-regulated proteins were cyt *c* oxidase subunit 6b and ATP synthase beta subunit.

Drought is one of the more common abiotic stresses encountered by plants. As the root is the primary organ the plant uses to regulate water uptake, it is not surprising that root tissue is the first to experience water deficit. It has long been established that drought can induce PCD in plant cells and the first cells to die are root cells. Jupp and Newman (1987) recognised death in the root tips of *Lolium perenne* (perennial ryegrass) as PCD induced by drought.As rainfall is so unpredictable, root death due to water shortages is recognised widely in many agricultural crops and often older roots die by PCD, driving the growth of new roots which will expand to exploit different patches of damp soil (Goss and Watson, 2003). Often, these new lateral and adventitious roots are more tolerant to drought conditions (Duan *et al.*, 2010). Despite the importance of the effect of drought stress on plant roots, the influences exerted on PCD induction pathways are poorly elucidated. One study which aimed to clarify the processes involved in this type of death was carried out by Duan *et al.,* (2010). They found that the root apical meristems cells were the first cells to die by an autophagic type PCD characterised by highly vacuolated cells and degradation of the cell organelles. The team also detected a significant accumulation of ROS in the root tip cells and BAX inhibitor-1 (AtBI1) expression in the endoplasmic reticulum (ER) increased. Previously, AtBI1 has been shown to play a pivotal role in ER stress-mediated cell death (Watanabe and Lam, 2008). When Duan *et al.,* (2010) examined the AtBI1 mutant, they found that death was accelerated, suggesting that the ER stress response pathway may also influence drought-induced PCD.

* 1. **Temperature stress**

Fluctuations in temperature have a number of deleterious effects on the plant cell that lead to PCD activation in a variety of ways and therefore could have multiple influences on pathways that initiate and control PCD. For instance, cold stress slows down enzymatic activity and membrane fluidity, destabilises protein complexes, promotes a build up of ROS in the cell and can cause leakages across membranes (Ruelland *et al.*, 2009). Conversely, heat stress affects metabolic activity, alters protein folding, destabilises the cell cytoskeleton affecting membrane fluidity and impairs enzyme function via protein denaturation. Once the plant detects an increase in temperature, (reviewed by Ruelland and Zachowski, 2010), an array of response mechanisms are initiated. Amongst those are the activation of heat shock proteins (HSPs), release of cyt *c* and it is proposed that the level of internal ceramide also increases (Balk *et al.*, 1999, Ruelland and Zachowski, 2010, Takabe *et al.*, 2008).

To facilitate plant growth in hot climates, plants have adopted a variety of mechanisms which allow them to develop a tolerance to temperature fluctuations. For instance, at a cellular level, mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades are induced which then initiate the production of ROS scavengers and antioxidants. In addition, some species accumulate osmoprotectants in their tissues and utilise cellular chaperones such as heat shock proteins (Wahid *et al.*, 2007). Koukalova *et al.,* (1997) examined BY-2 tobacco cells exposed to cold stress of 5-6°C for a period of 5 weeks and found that DNA was first degraded into 50-100kb fragments after which late stage, internucleosomal cleavage occurred. Likewise, Ning et al., (2002) looked at PCD induced by cold treatment in maize root cells and highlighted features characteristic of PCD such as nuclear condensation and DNA fragmentation.

The heat-stress-induced PCD response (reviewed by Reape *et al.,* 2008) has emerged as a model system in order to study plant PCD and has been used effectively in carrot (McCabe *et al.*, 1997a), *Arabidopsis* (McCabe and Leaver, 2000), tobacco cells (Burbridge *et al.*, 2006) and wheat leaves (Fan and Xing, 2004). Consequently, several potential players in the execution and/or regulation of PCD in response to heat treatment have emerged. One such PCD response is the release of cyt *c*. Balk *et al.,*(1999) examined heat-induced PCD in cucumber plants and found that cyt *c* was released from the mitochondria into the cytosol. Vacca *et al*., (2006) obtained similar results when they heat treated tobacco BY-2 cells and found that cyt *c* was released in a ROS-dependent manner. The role of cyt *c* is discussed in section IIA1, of this review. Recent workby Malerba *et al.,* (2010) highlighted a possible role for ethylene in the heat stress response. They identified fragmentation of actin cytoskeleton filaments after heat stress (35, 45 and 50°C) and found that actin depolymerisation by heat stress was prevented by the ethylene production inhibitor Co2+. Interestingly, it was previously reported that reorganisation of actin filaments occurs during PCD in embryos of *P. abies* and actin depolymerisation is enough to cause PCD in self-incompatible pollen of *Papaver rhoeas* (Smertenko *et al.*, 2003, Thomas *et al.*, 2006).

Caspase-3-like proteases have been shown to play a role in PCD in *Chlorella* *saccharophila* (Zuppini *et al.*, 2007). Similarly, Tian *et al.,* (2000) highlighted a role for caspase-like proteases in heat stress induced PCD in plants. They found that poly (ADP-ribose) polymerase (PARP), is cleaved by a caspase-3-like protease in tobacco suspension cells, with PCD manifesting at a late stage and exhibiting DNA laddering. HSPs have been shown to play vital roles in the execution of PCD in animal cells (Beere and Green, 2001, Garrido and Solary, 2003). Tonsor, *et al*., (2008) reported that five families of HSPs including HSP60, HSP70, HSP90, HSP100 and small HSPs are essential in preventing protein aggregation and misfolding caused by heat treatment. HSPs prevent misfolding by chaperoning the refolding of damaged or denatured proteins. They are highly conserved and are induced in animals, plants, yeast and insects after exposure to various abiotic and physiological stresses, including heat (Wang *et al.*, 2004). After initial sub-lethal insult, the HSPs enhance protection and recovery and confer resistance to subsequent heat stress of the cell. It has been shown that HSPs can interact with apoptosis inducing factor thereby influencing caspase-independent PCD (Lanneau *et al.*, 2008) therefore, it is probable that HSPs infer a protective effect on the cells.

* 1. **UV light stress**

UV light is part of the electromagnetic spectrum and is divided into three classes according to wavelength, UV-C (<280nm), UV-B (between 280-320nm) and UV-A (between 320-390nm) (Stapleton, 1992). UV-A and UV-B penetrate the outer atmosphere to reach the earth’s surface however UV-C is filtered out in the upper atmosphere by ozone. Consequently, plants are not ordinarily exposed to UV-C radiation, but it is used widely as a mutagen as it elicits the same DNA photoproduct responses as UV-B (Brash, 1997) and UV-C light sources are relatively less expensive than their UV-B counterparts (Stapleton, 1992). Another advantage of using UV-C is that it has a much shorter wavelength, so highly excited photons affect plant cells more quickly. Solar UV radiation has been shown to harm many plant processes by damaging DNA, cell proteins and membranes (Jansen *et al.*, 1998) and UV has been used to induce apoptosis in animal cells (Kulms and Schwarz, 2002, Mammone *et al.*, 2000, Martin and Cotter, 1991). UV light can also induce death in plant cells making it useful for the study of PCD in plants. Danon and Gallois (1998) carried out one of the first studies of UV-induced death in plantswhen they documented DNA fragmentation and laddering after UV-C treatment of *Arabidopsis* protoplasts. Danon *et al.* (2004) used caspase inhibitors and transient expression of the p35 and *At-DAD* genes in *Arabidopsis* protoplasts to confirm that UV-induced death is a form of PCD and that metacaspases and that the ER- localised *At-DAD* may play a role in plant PCD. More recently, Lytvyn *et al*. (2010) observed typical apoptotic morphological features such as cell shrinkage, condensation of chromatin, DNA fragmentation and cell acidification in UV-B treated tobacco Bright-Yellow 2 cells.

Studies aimed at identifying regulators of UV-light-induced death have uncovered both metacaspases and ROS generation as key players (Bethke and Jones, 2001). For instance, studies of barley aleurone protoplasts have shown that illumination with UV light leads to rapid increases in the rate of ROS production, specifically H2O2 (Bethke and Jones, 2001). He *at al*. (2008), have shown that metacaspases play a role in H2O2 or UV-induced death. They examined the effect of oxidative stress on the expression of the plant metacaspases genes when they treated *Arabidopsis* protoplasts with UV-C and H2O2 and found that of nine *Arabidopsis* metacaspase genes, metacaspase-8 (AtMC8) was strongly up-regulated by these oxidative stresses. Interestingly, many studies have shown that once cells have been exposed to UV light, an additional period in continuous light is required for PCD to occur. For example, while He *et al.* (2008) were investigating the first genetic evidence that a plant metacaspase plays a role in PCD, they found that death was dependent on an additional exposure to white light after UV exposure, indicating that this type of PCD may be light dependent. This light-dependent death is not an unusual occurrence and has been shown as a requirement for some types of plant PCD, induced by a variety of stimuli. As mentioned previously, Danon *et al*. (2004) also found evidence that exposure to continuous light was necessary for death after UV-C overexposure. Similar observations were seen by Asai *et al.* (2000) and Chandra-Shekara *et al.* (2006) who found that death after treatment with the mycotoxin fumonisin B1 or triggering of the hypersensitive response was light-dependent.

Oxidative damage as a result of UV overexposure has previously been documented in plants (Babu *et al.*, 2003, He and Häder, 2002) and recent work by Gao *et al.* (2008a) found that ROS generated by mitochondrial and chloroplast dysfunction induced by UV-C overexposure, may be one of the principal sources of death-inducing ROS. In this study, death was also light-dependent and a burst of ROS activity was detected during light exposure after initial UV-C overexposure (Gao *et al.*, 2008a). Interestingly, Jenkins *et al.,* (1997) uncovered a putative link between the UV and heat stress PCD response when they observed the early activation of HSP21 in the *uvh6* mutant of *Arabidopsis* which has an increased sensitivity to UV radiation.

1. **PCD during plant-pathogen interactions**
   1. **Hypersensitive Response**

The hypersensitive response (HR) is a rapid cell death induced at a site of pathogen infection. It is believed to limit pathogen spread beyond the site of challenge and it has a putative involvement in systemic signalling (Heath, 2000).

In general, HR is characterised by the presence of dead, brown cells and where enough cells die, visible lesions develop. Death may be restricted to cells having direct contact with the pathogen, but it may also expand beyond the initial site of infection. In addition, timing of death induction and execution may vary depending upon plant-pathogen interaction. Different infection strategies adopted by diverse types of pathogen often give rise to variations in the phenotypical appearance and timing of the HR response (Mur *et al.*, 2008). Plant-pathogen interaction resulting in the triggering of the hypersensitive response may be host specific. Host specific resistance is based on ‘gene-for-gene’ interaction between plant and pathogen and requires a resistance (*R*) gene in the plant and a corresponding avirulence (*Avr*) gene in the pathogen, with R genes being presumed to enable recognition of pathogen specific molecules, initiate signal transduction to activate defence and have the capacity to rapidly evolve new R genes specificities (Hammond-Kosack and Jones, 1997). Interaction of R gene and *avr* gene products can be either direct (Catanzariti *et al.,* 2010, Jia *et al.,* 2000)or indirect (Axtell *et al.,* 2003, Shao *et al.,* 2003). Homology has been detected between several R gene products (RPM1, RPS2, RPP5, N, and L6) and the nematode cell death proteins CED-4 and APAF-1 (van der Biezen and Jones, 1998). Considering the importance of the two latter proteins in the execution of PCD in animal systems, the conservation of the regulatory core of cell death programmes between kingdoms is possible.

Nonhost resistance is another pathogen response mechanism which may result in HR and describes the diverse defence strategies used to protect plants against a wide range of pathogens. Consequently, nonhost resistance is of great interest to those researchers developing resistance crops. Induction of nonhost resistance is driven by general elicitors produced by pathogens. General elicitors include numerous microbial surface-derived compounds which are often referred to as pathogen-associated molecular patterns (PAMPs) (Nürnberger and Brunner, 2002). Examples of these include flagellin (bacterial flagella protein activating mitogen-activated protein kinase cascade) (Asai *et al.*, 2002) or oligomers of chitin (Boller, 1995) or glucans (Yamaguchi *et al.*, 2000) released from the cell walls of pathogens. A wide range of compounds has been identified to exhibit elicitor activity, for example, arachidonic acid (Boller, 1995), *Xanthomonas* derived harpin (Kim *et al.*, 2004) or Pep-13 peptide fragment from *Phytophthora sojae* (Brunner *et al.*, 2002) (reviewed by Boller and Felix, 2009). General elicitors activate production of antimicrobial proteins and phytoalexins, papilla (local cell wall fortification is formed on the inner side of plant cell walls), cell wall lignifications, accumulation of phenolics, production of saponins and other plant defence responses (reviewed by Mysore and Ryu, 2004). The aforementioned mechanisms can be sufficient to arrest pathogen growth (type I nonhost resistance), but in cases where the pathogen is able to overcome early defence mechanisms, type II nonhost resistance is activated via cellular defence surveillance mechanisms. Recognition of pathogen elicitors (in cytoplasm or plant cell membrane) triggers defence mechanisms often leading to HR (Mysore and Ryu, 2004, Thordal-Christensen, 2003).

Despite increasing understanding of HR mechanisms and regulation, the precise contribution of HR to plant resistance is still unknown. While cell death may be an efficient defence mechanism especially against biotrophic (requiring living host cells for survival) pathogens (although it is not always supported by experimental data (Richael *et al.*, 1999)), it does not seem to be a necessary component of resistance in general. Indeed, plants of the ‘defence, no death’ mutant line of *Arabidopsis* thaliana, despite being HR defective, exhibited effective gene-for-gene resistance against *Pseudomonas syringae* (Yu *et al.*, 1998). Also cell death inhibition studies revealed that uncoupling of PCD from defence genes activation is possible (del Pozo and Lam, 1998). This uncoupling is supported by results of Bendahmane *et al.* (1999) who demonstrated that cell death and pathogen arrest are separate disease responses in plants based on the example of Rx-mediated resistance against potato virus X. It seems therefore possible that HR is a resistance induction reinforcing/stimulating strategy.

It has also been postulated that HR participates in systemic acquired response (SAR), which is a form of resistance triggered on a systemic scale in healthy tissues following local infection. It has been proposed that cells adjacent to the site of infection become more responsive to pathogen elicitors as a result of signals released during HR (phenylpropanoid metabolites - genistein and salicylic acid were suggested as the potent signalling molecules) (Graham and Graham, 1999). Alvarez *et al.* (1998) reported the induction of secondary oxidative bursts in distant tissues and the formation of systemic micro-HRs following local infection, both of which they considered to be important for systemic immunity. HR may therefore function more as a signalling system than as a direct defence mechanism (Heath, 2000).

There exists increasing evidence that HR is a form of PCD, sharing some common features with animal apoptosis. Ion fluxes are frequently observed to be an early step in HR induction (Orlandi *et al.*, 1992). Cells challenged with pathogenic elicitors respond by calcium influx into the cell and this increase in cytosolic [Ca2+] seems to be necessary for development of HR. Indeed, cowpea rust fungus infection was followed by elevation of [Ca2+] in resistant cells (Xu and Heath, 1998). Similarly, increase in [Ca2+] level was observed in H2O2 induced AL-PCD in soybean cells and addition of calcium channel inhibitors resulted in delayed cell death in soybean leaves (Levine *et al.*, 1996). Formation of reactive oxygen species (ROS) following an oxidative burst following pathogen infection (Lamb and Dixon, 1997) are widely accepted to play a role in triggering and/or the execution of HR (Levine *et al.*, 1994). Salicylic acid (Alvarez, 2000) and nitric oxide (Delledonne *et al.*, 1998) were reported to be involved in HR regulation. Several early studies report protein synthesis (He, 1994, Heath *et al.*, 1997, Mould and Heath, 1999) and an intact actin cytoskeleton (Skalamera and Heath, 1998) to be necessary for HR cell death induction in certain cases, suggesting the programmed and organised nature of the process. AL-PCD morphology is commonly observed during HR events, for example in soybean (Aist and Bushnell, 1991, Levine *et al.*, 1996), tobacco (Mittler *et al.*, 1997, Yano *et al.*, 1998) and oat (Curtis and Wolpert, 2004) cells). DNA cleavage into oligonucleosomal fragments is also often detected (Levine *et al.*, 1996, Mittler *et al.*, 1997, Ryerson and Heath, 1996, Tanaka *et al.*, 2001, Wang *et al.*, 1996), and cyt *c* release has also been reported during HR (Curtis and Wolpert, 2002, Kiba *et al.*, 2006, Krause and Durner, 2004). Involvement of caspase-like molecules in HR, indicated by cleavage of caspase substrates and presence of caspase-like molecules, has been demonstrated several times. For example, application of caspase inhibitors inhibited HR induced by a bacterial pathogen, tobacco mosaic virus, in tobacco (del Pozo and Lam, 1998) and delayed HR caused by a rust fungus in cowpea (D'Silva *et al.*, 1998). In addition, caspase specific substrates were cleaved during HR induced by tobacco mosaic virus (Chichkova *et al.*, 2004, del Pozo and Lam, 1998).

* 1. **Pathogens modulating host death response**

Pathogens which do not elicit HR may modulate cell death in cells of the susceptible host. For biotrophic pathogens, it is desirable to suppress PCD in plant tissue whereas necrotrophic pathogens, which feed from dead tissue, produce substances triggering PCD in their host. For example, in order to suppress PCD associated with plant immunity, the tomato pathogen *Pseudomonas syringae* injects the AvrPtoB type III effector protein into the plant cell (Abramovitch *et al.*, 2003). AvrPtoB was found to be ubiquitinated *in vitro* and exhibited E3 Ub ligase activity. This suggests that it disrupts normal proteasomal function of the plant cell and interferes with protein degradation associated with disease resistance. Alternatively, this protein may bind and ubiquitinate a positive regulator of plant cell death, effectively targeting it for degradation (Abramovitch *et al.*, 2006). Soluble glucans in spore germination fluids of *Phytophthora infestans* suppress the oxidative burst and the HR in potato (Doke, 1975). Another example of host cell death suppression during compatible biotrophic interactions is the occurrence of ‘green islands’ - leaf areas around successful infection sites, which display delayed senescence in comparison with the rest of the leaf tissue (Scholes and Rolfe, 1996). On the other hand, necrotrophic bacteria and fungi were shown to promote PCD in their plant host. For example, the necrotrophic fungus *Sclerotinia sclerotiorum*, secretes oxalic acid which acts as non-host specific toxin and induces increased ROS levels leading to PCD induction in host cells (Kim *et al.*, 2008). The endopolygalacturonases produced by fungal species degrade pectic components of the plant cell wall generating oligosaccharide fragments that can act as endogenous signalling molecules (Côté and Hahn, 1994). These signalling molecules can induce intracellular [Ca2+] increase, hydrogen peroxide accumulation and finally PCD in host cells (Zuppini *et al.*, 2005). Due to the huge economic implications of the topic, modulation of PCD mechanisms employed by pathogens is the subject of intensive research.

* 1. **Lesion Mimic Mutants**

While considering the HR in terms of PCD related research, it is worthwhile examining mutants exhibiting misregulation of cell death formation. These mutants are called lesion mimic mutants (LMMs) since they exhibit either constitutive, or unregulated, cell death formation which resembles the HR in the absence of pathogen infection (Moeder and Yoshioka, 2008). LMMs are categorised into two groups: initiation LMMs (characterised by constitutive formation of lesions) and propagation LMMs (not able to control cell death once it is started) (Lorrain *et al.*, 2003). Numerous LMMs have been isolated in several species, including *Arabidopsis*, maize, rice and barley (Lorrain *et al.*, 2003). While it is possible that a mutation resulting in a LMM phenotype does not directly regulate PCD, but rather induces cell death via non-specific cellular perturbation, many LMMs are considered valuable tools for identification of genes involved in the regulation and execution of PCD in plants (Moeder and Yoshioka, 2008). A growing number of LMM genes have been cloned and they identify potentially important players in plant PCD. For example, calcium involvement in HR regulation is supported by lesion mimic phenotype of mutants such as *dnd1* and *dnd2* (defense no death 1,2) (Clough *et al.*, 2000, Jurkowski *et al.*, 2004), which are characterised by mutations in cyclic nucleotide-gated ion channel genes, or *cpr22*, where a mutation results in production of a novel chimeric protein derived from homologous recombination of two tandemly repeated cyclic nucleotide-gated ion channel genes (Yoshioka *et al.*, 2006), or *cpn1/bon1* (Hua *et al.*, 2001, Jambunathan *et al.*, 2001), which are thought to exhibit alternations in Ca2+ signalling. Mutants *acd5* and *acd11* (Brodersen *et al.*, 2002, Greenberg *et al.*, 2000, Liang *et al.*, 2003) suggest that HR cell death formation is mediated by sphingolipids, as ACD5 encodes ceramide kinase and ACD11 codes for sphingosine transfer protein. LMMs can be useful in identifying the signalling molecules promoting/suppressing cell death during the HR. One of the common approaches to identify HR cell death genes is to cross LMM with mutants altered in signalling pathways such as R-gene mediated pathways, ethylene, salicylic and jasmonic acid signalling (Lorrain *et al.,* (2003) and references therein). Such a strategy can unravel the potential crosstalk between a LMM gene and a specific signalling pathway. However to date no cloned LMM genes have been definitively shown to code for a protein clearly responsible for plant PCD execution.

1. **Autophagy**

Autophagy is an evolutionary conserved catabolic pathway in eukaryotes in which cytoplasm, including long-lived proteins and obsolete organelles, are sequestered into double-membrane vesicles (autophagosome) and delivered to the degradative organelle (in plants, the vacuole) for breakdown and final recycling of the resulting macromolecules (Yorimitsu and Klionsky, 2005). This sequestration process can occur either away from the vacuole (in which case it is termed macroautophagy), or at the vacuole surface (termed microautophagy) (Klionsky *et al.*, 2003). Autophagic cell death has been defined as type II cell death (Lockshin and Zakeri, 2004), however it should be remembered that autophagy itself is a routine process of cellular component turnover and only in some cases is connected with terminal cellular dismantling. Autophagic cell death is morphologically defined as a type of cell death that occurs without chromatin condensation and is accompanied by massive autophagic vacuolisation of the cytoplasm (Kroemer *et al.*, 2008). The aforementioned vacuoles have double-membranes and contain degenerating cytoplasmic organelles or cytosol (Levine and Klionsky, 2004). It should be highlighted, that the term ‘autophagic cell death’ does not imply a causative role of autophagy in the cell death process, but only describes death occurring in cells undergoing autophagic processes (Kroemer *et al.*, 2005).

Early investigations of autophagy in plants used light and electron microscopy to report the presence of double membrane structures surrounding portions of cytoplasm (Villiers, 1967) and indeed numerous electron microscopy morphological studies of plant cells implied involvement of autophagy in a plethora of processes. As a routine recycling pathway, autophagy occurs at a basal level in all growing cells but it has been also reported to play a role in starvation, development and defence responses to pathogens (Bassham *et al.*, 2006). On the basis of morphological observations, autophagy was suggested to participate in PCD events in a variety of processes, such as aerenchyma formation, senescence, embryogenesis, germination of seeds, root formation, xylem and phloem differentiation or petal senescence (van Doorn and Woltering, (2005) and references therein). Genetic analysis of autophagic processes was not possible until recently, when more than 30 autophagy-related (ATG) genes were initially isolated in yeast (Bassham *et al.*, 2006, Klionsky *et al.*, 2003). Subsequent sequence comparisons resulted in identification of plant homologues for many of yeast ATG genes (Bassham, 2007, Bassham *et al.*, 2006, Hanaoka *et al.*, 2002). Indeed, *Arabidopsis* homologues of yeast proteins atg8 and atg4 are able to partially complement the yeast deletion strains indicating that the principle components of the autophagy pathway are conserved between plants and yeast (Ketelaar *et al.*, 2004). Recently, autophagy associated genes were also identified in rice and maize (Chung *et al.*, 2009, Su *et al.*, 2006), however despite this, the machinery and regulation of the autophagy process are still yet not well understood in plants.

The biological roles of autophagy in plants have been investigated by numerous studies of ATG mutant plant phenotypes. For instance, an early senescence phenotype and a sensitivity to nutrient-limited conditions was observed in case of AtATG7, AtATG8 and ATG9 mutant plants (Contento *et al.*, 2005, Doelling *et al.*, 2002, Hanaoka *et al.*, 2002, Phillips *et al.*, 2008, Yoshimoto *et al.*, 2004), suggesting that autophagy helps to maintain cellular viability during starvation and also to distribute nutrients efficiently. However, *Arabidopsis* autophagy defective mutants (e.g. AtATG7, Doelling *et al.*, 2002, AtATG9, Hanaoka *et al.*, 2002, AtATG10 Phillips *et al.*, 2008)) and silenced transgenic lines like AtATG6‑RNAi (Patel and Dinesh-Kumar, 2008) all lack major developmental phenotypes under optimal conditions, suggesting that autophagy is not a key player in basic developmental processes such as embryogenesis, germination, shoot and root formation and elongation and flowering and seed production. Disruption of the AtATG6 gene has been shown to inhibit pollen germination (Fujiki *et al.*, 2007, Qin *et al.*, 2007), however it has not been established if this effect is autophagy dependent or whether the mutation affects another cryptic pathway required for pollen germination. The AtATG9 mutant exhibits subtle growth alternations even in nutrient-rich conditions which include an early flowering phenotype and a reduction in the number of rosette leaves (Hanaoka *et al.*, 2002) suggesting a less obvious role for autophagy in plant development. In contrast to previously formulated morphological observation-based assumptions, the genetic evidence suggests that the ATG pathway is not essential for plants in optimal conditions. However, it cannot be excluded that another pathway functions in the absence of the ATG system. Additionally, there is debate over whether autophagy can be a cause of PCD itself, or rather an important, but not inevitable, mechanism for recycling cellular material preceding, or associated with, death.

Autophagy has been shown to be involved in plant abiotic stress responses. It has been proposed that autophagy is required in the degradation of oxidized proteins during oxidative stress in *Arabidopsis* and that disruption of autophagy pathway results in increased oxidative stress, which is supported by increased expression of ROS scavenging enzymes and also, ROS levels themselves in AtATG18a-RNAi plants compared to wild type (Xiong *et al.*, 2007a, Xiong *et al.*, 2007b).

Experimental results suggest that the physiological functions of autophagy in animals are of a dual nature: at the organismal level it can play both a pro-survival role (e.g. maintaining energy homeostasis during starvation) or a pro-death role, depending on the extent to which the process is activated (Kang *et al.*, 2007). It is possible this dual role also function in plant cells explaining why different studies sometimes report conflicting results (Hofius *et al.*, 2009, Liu *et al.*, 2005, Patel *et al.*, 2006) and diverse hypotheses of the role of autophagy in cell death have been proposed (Cacas and Diamond, 2009, Love *et al.*, 2009, Love *et al.*, 2008). Recently evidence of a pro-death function of autophagy was presented by Hofius *et al.*, (2009), who examined the receptor-mediated HR PCD responses in autophagy-deficient knockout mutants of *Arabidopsis*. The authors analysed the autophagic response provoked by infection with three avirulent *Pseudomonas syringae* strains (Pto DC3000: AvrRps4, AvrRpm1 and AvrRpt2) and compared the intensity of HR between mutants and WT plants. It was reported that the HR was suppressed in atg mutants upon infection with AvrRpm1 and AvrRps4, but AvrRpt2-triggered cell death proved to be independent of autophagy (Hofius *et al.*, 2009). While this evidence suggests a prodeath function of autophagy in plants (Hofius *et al.*, 2009), data about the extent of autophagy required to facilitate pro-death signal transduction or to directly effectuate cell death are still to be provided. The hypersensitive response is thought to prevent pathogen infection development but in order to play a protective, pro-survival role, PCD during the HR must be restricted to infection sites. A study by Liu *et al.* (2005) provided evidence that autophagy prevents unrestricted HR PCD in tobacco after infection with tobacco mosaic virus (TMV). Plants where ATG6/BECLIN1 or other autophagy related genes were silenced exhibited uncontrolled development of hypersensitive lesions. Moreover, the systemic activation of autophagy following TMV infection was observed only in the WT. These findings suggest that autophagy may alter the induction, movement or recognition of the pro-PCD signal (Patel *et al.*, 2006). Similarly, Patel and Dinesh-Kumar subsequently reported that *Arabidopsis* AtATG6 antisense plants underwent senescence earlier, exhibited a more environmental-sensitive phenotype and were unable to control the extent of HR upon infection with avirulent Pst DC3000::AvrRpm1 (Patel and Dinesh-Kumar, 2008). Nevertheless, it is still unclear if autophagy plays a role in the execution of HR or rather affects the propagation of cell death signals. The striking discrepancies between the aforementioned works (Hofius *et al.* (2009) versus Lu *et al.* (2005), Patel and Dinesh-Kumar (2008) may be explained by the diverse roles of silenced genes or differences between plant species, however, they may also suggest that multiple PCD pathways operate in the same plant cell. Hence, although there exists experimental evidence suggesting that autophagy and AL-PCD may overlap, the nature of cross-talk between them and the signalling involved remains to be explained and is now the subject of active debate (Cacas and Diamond, 2009, Love *et al.*, 2009, Love *et al.*, 2008). The possible machinery regulating switches between autophagy, PCD and necrosis needs to be elucidated in order to expand our understanding of the described processes and the relationships between them.

Recently, research in this field has benefited by the emergence of new experimental tools with which to study autophagy, such as monodansylcadaverine (MDC) labelling of autophagic vacuoles, LysoTracker marking acidic compartments or green fluorescent protein (GFP) fused with ATG8 protein (Contento *et al.*, 2005). However, although MDC and Lysotracker are often reported to be the autophagy indicators, the results of staining should be interpreted with caution. Lysotracker labels acidic organelles in living cells and is therefore are not autophagy specific. Indeed, Lysotracker was used to indicate presence of senescence-associated vacuoles in the autophagy deficient mutant AtATG7 (Otegui *et al.*, 2005). Therefore it is applicable in situations where autophagic activity is clearly distinguished from other events that increase lysosomal or vacuolar activity (Yoshimoto *et al.*, 2009). Some reports also indicate that MDC is not autophagy-specific, as MDC staining does not always co-localise with GFP-LC3 (AtATG8 homologous protein) fluorescence (Mizushima, 2004), hence it is not a reliable method for monitoring autophagy by itself (Klionsky *et al.*, 2008). Techniques commonly used to study autophagy have been comprehensively reviewed by Mitou *et al.* (2009) and guidelines for the use and interpretation of autophagy assays have been published by Klionsky *et al.* (2008). Further genetic characterisation of other genes in the autophagy pathway and the nature of autophagosome-like structures will be important to clearly delineate what form of type II PCD actually exists in plants.

The definition of autophagic PCD derived from animal studies categorises it as a cell death type characterised by autophagic vacuolisation of cytoplasm, without implying that it is the cause of death process itself. This classic definition is considered insufficient by some researchers. Van Doorn and Woltering (2005) proposed the term ‘mega-autophagy’ to mark a fatal autophagic process characterised by tonoplast collapse resulting in the release of hydrolytic enzymes from the vacuole and degradation of cellular contents including the plasma membrane. Recently, the same researchers also suggested that the terminology connected with the description of autophagic cell death should be re-defined for the use of plant science and rather than the classical definition of type II cell death, the tonoplast rupture followed by activity of lytic enzymes released from the vacuole should be treated as the markers of autophagic PCD and using this criteria, van Doorn and Woltering (2010) argue that most of examples of developmental PCD in plants would be classified as autophagic PCD. On the other hand, Levine and Kroemer (2009) argue that numerous recent studies suggest that autophagy is largely a cell death impostor which, in reality, functions primarily to promote cellular and organismal health. Instead, they proposed that autophagy observed abundantly in dying cells may represent a failed attempt of cytoprotection rather than a mechanistic contribution to cell death. As already mentioned, autophagy is commonly observed during developmental death in plants and if Levine and Kroemer (2009) are correct it may be, that in the cases of pre-programmed, predictable death events during the plant life-cycle, autophagy is activated upstream of the actual mechanism of PCD, in order to enable nutrient recycling and in this way it promotes survival at an organismal level and is not a type of PCD. However, this view remains contentious and many researchers believe that in some circumstances, autophagy is a bona fide mechanism of cell death (Levine and Kroemer, 2009).

1. **Studying PCD in plants**
2. **Methods**

PCD in plants can be measured by microscopic and biochemical assays. Microscopic examination of PCD includes application of viability stains such as fluorescein diacetate, Evans blue or tryptan blue and use of *in situ* markers of DNA degradation such as the terminal dUTP-biotin nick end labeling of DNA fragments (TUNEL), which provides a measure of 3’-OH group accumulation (Gavrieli *et al.*, 1992) and the 4',6-diamidino-2-phenylindole (DAPI) staining, which specifically targets DNA located in the nuclei. It is often useful to use more than one PCD detection method in order to avoid false positives (Wang *et al.*, 1996). Other methods which were reported to be useful for labelling DNA breaks in plant cells are ISEL (*in situ* end labelling) (Ning *et al.*, 1999) and ISNT (*in situ* nick translation) (Amor *et al.*, 1998, Wood *et al.*, 1995). DNA breakage detection stains can be applied on samples analysed by microscopy or alternatively, they can be applied to samples subjected to fast and high throughput fluorescence activated cell sorting analysis. In terms of morphology, condensation of the cytoplasm leaving a visible gap between the cell wall and plasma membrane (McCabe and Leaver, 2000) is a hallmark feature of AL-PCD in plant cells observed under the microscope.

A classical biochemical marker for PCD is DNA fragmentation detected by conventional or pulsed field gel electrophoresis (PFGE). As opposed to the simple DNA breakage detection assays described above, these methods are useful for visualisation of 180 – 200 bp multimers and large >50 kbp DNA fragments. Fragments 300-50 kb in size result from DNA ordering into chromatin loops of approx. 50 kb and six of these loops fold as a 300 kb rosette structure (Reape and McCabe, 2008). At the early stage of PCD before the actual internucleosomal cleavage takes place, these rosettes are often cleaved into their 50 kb components and can be visualised by PFGE and ethidium bromide staining (Balk *et al.*, 2003, Young and Gallie, 2000a).The DNA ‘ladder’ which appears after separation of fragmented DNA by agarose gel electrophoresis is a recurring feature of PCD. Multimers of 180-200 bp in size are the result of internucleosomal DNA cleavage by nucleases activated during PCD. Visualisation of the DNA ladder with ethidium bromide staining tends to be weak and in order to avoid false negative results the commercial kits for ladder detection can be used (e.g. ApoAlertTM LM-PCR Ladder Assay Kit by Clontech Laboratories Inc.) (Giuliani *et al.*, 2002). Another method of visualising internucleosomal DNA cleavage, is using a hybridisation of a Southern blot of the gel with a total DNA probe that has been randomly digested into fragments and labelled with radioactive probes (McCabe and Leaver, 2000).

Electrolyte leakage is often used as the cell death indicator, as it is a crude measure of membrane integrity perturbations and cellular damage. Due to the presence of the cell wall, dying plant cells are not engulfed by neighbouring cells as is the case of animal apoptosis. Instead, at the late stages of cell death, the contents of the dying cell leak into the intercellular space and can be used to compare the relative amount of cell death in plant tissues (del Pozo and Lam, 1998). Leaf discs, segments, or even whole leaves are collected from plants following the death inducing treatment (biotic or abiotic) and incubated in water. The conductivity of the bathing solution is measured and related to the conductivity of the same sample after intense stress (e.g. incubation at 95 °C or autoclaving/boiling), which ensures complete release of electrolytes from cells. Results expressed as the percentage ion leakage is used to monitor hypersensitive cell death progress following pathogen infection, (for example, Katiyar-Agarwal *et al.*, 2006, Rudd *et al.*, 2008) and cell death caused by abiotic stress such as methyl viologen mediated oxidative stress (Sarowar *et al.*, 2008) or ozone treatment (Pasqualini *et al.*, 2003). However, it has to be highlighted that this assay does not presently distinguish between PCD and necrosis caused electrolyte leakage and therefore needs to be combined with other technique in order to differentiate between these different modes of death.

Another technique for biochemically investigating PCD in plants is by preparation of a protein extract from plant tissue and analysis of its nuclease/protease activity. Increase in nuclease activity was reported during the hypersensitive response following pathogen infection (Mittler and Lam, 1997, Mittler and Lam, 1995), in the aleurone layer of cereal grains undergoing PCD (Domínguez *et al.*, 2004) or during senescence of barley (Wood *et al.*, 1998), *Arabidopsis* (Perez-Amador *et al.*, 2000), parsley (Canetti *et al.*, 2002), petunia flower petals (Langston *et al.*, 2005) and also in other species. Nuclease activity of a plant protein extract may be assessed by running samples on SDS-PAGE gels containing herring sperm, followed by SDS removal and incubation of the gel in appropriate buffer to allow *in situ* DNA degradation. Afterwards, the gel is washed with the same buffer for diffusion of residual digested DNA fragments. Bands visible after staining with ethidium bromide represent nucleases (Chang and Gallie, 1997). The nuclear activity of plant protein extract may also be assayed by incubation with plasmid DNA (Young and Gallie, 1999). Due to endonucleolytic activity of the extract, the plasmid is initially converted from supercoiled (CCC) to open circular (OC) and linear form, which is lost with further DNA degradation.

No functional homologs of animal caspases have been identified in plants, but caspase-like activity has been observed during numerous PCD events in plants (del Pozo and Lam, 1998, Watanabe and Lam, 2004). Application of synthetic fluorogenic caspase substrates and caspase inhibitors is often employed in PCD studies in plant systems. For example, a synthetic tetrapeptide fluorogenic substrate of animal caspase-1 (Ac-YVAD-AMC) has been used to demonstrate caspase-like activity of extracts from TMV-infected tobacco leaves, which was inhibited by caspase-specific inhibitors (Ac-YVAD-CMK, Ac-DEVD-CHO and Ac-DEVD-FMK) but not significantly affected by the addition of other unrelated protease inhibitors (del Pozo and Lam, 1998). No activation of YVAD-specific protease activity was detected in mock-inoculated plants or plants unable to trigger the HR.

Fluorescence resonance energy transfer (FRET) microscopy is a powerful modern technique which can be used to monitor activation of caspase-like molecules in real time. FRET is the phenomenon whereby a fluorescent molecule (donor) transfers energy by a non-radiative (through space) mechanism to a neighbouring chromophore (acceptor) (Gadella Jr *et al.*, 1999). This energy transfer occurs when the absorption spectrum of the acceptor chromophore overlaps with the fluorescence emission spectrum of the donor and generally if the distance between two chromophores is < 100 Å. As a result the donor fluorophore’s fluorescence intensity decreases and if the acceptor chromophore is also a fluorophore, FRET increases its fluorescence. Useful for spatio-temporal caspase activity studies, FRET occurs when two proteins fluorescing at different wavelengths (donor and acceptor) are linked together and when they are separated by linker’s proteolytic cleavage, no FRET is detected. Indeed this technology was used to monitor dynamics and localisation of caspase activation in HeLa cells (Takemoto *et al.*, 2003). Recently, FRET was also employed to study PCD in plant cells (Zhang *et al.*, 2009). *Arabidopsis* protoplasts were transformed with recombinant substrate - enhanced cyan fluorescent protein (ECFP) linked by a peptide possessing the caspase-3 cleavage sequence, DEVD to enhanced yellow fluorescent protein (EYFP). PCD was induced by UV-C exposure and a decrease in FRET occurred within one hour in single living protoplasts. When a DEVD (caspase-specific) tag was mutated or a caspase-3 inhibitor was introduced to the system, no UV-C induced changes in FRET were observed, confirming that the method is a specific indicator for caspase-3-like protease activation.

1. **Model systems**

PCD is an area of intense focus for plant scientists. Studying the control mechanisms of PCD in whole plants is often difficult as death can occur in a small group of inaccessible cells buried in a bulk of surrounding healthy tissue. Therefore, it may be convenient to use *in vitro* cell suspension cultures which are often more amenable to investigation than their *in vivo* counterparts. Alternatively, *in vivo* plant PCD studies may be facilitated by focusing on particular types of cells (Fig. 3). Important criteria exist while assessing the suitability of a particular cell type for use as a model for PCD investigation. For instance, a researcher will require large numbers of these cells and it will be necessary to either easily visualise single cells, or use an alternate method of assessing the death rate. It is also important that potential PCD modulators can be introduced to the system easily.

*<Fig. 3 near here>*

Cell suspension cultures are a commonly used model systems for PCD investigation in plants. A cell culture is a homogeneous collection of cells and is easily maintained and propagated. Individual cells are easily accessible and it is feasible to monitor changes in viability and morphology. It is also useful that potential PCD modulators can be added or removed from the culture easily. Moreover, cell cultures can be used to study basal cell responses during a death programme (undifferentiated cultures) or during specialised death programmes such as embryogenesis, xylogenesis or the hypersensitive response (cultures treated with phytohormones inducing differentiation or challenged with pathogenic elicitor) (McCabe and Leaver, 2000). Sometimes, it is useful to further reduce complexity of cellular environment by establishing single cell or protoplast culture.

Cell cultures of a number of plant species have been used to investigate PCD in a range of studies (reviewed by McCabe and Leaver, 2000). For example, carrot cell suspension cultures have been used to investigate the presence of cell survival signals (McCabe *et al.*, 1997a), while the role of hydrogen peroxide on PCD induction has been studied in *Arabidopsis* (Desikan *et al.*, 2000), *Glycine max* (Levine *et al.*, 1996) and *N. tabacum* (O'brien *et al.*, 1998). A *Z. elegans* cell suspension culture was used in xylogenesis studies (Twumasi *et al.*, 2009) and hypersensitive response elicitors induced defence responses in tobacco cells (Wei *et al.*, 1992). However, although cell cultures are very useful *in vitro* systems for PCD investigation in plants, they can be labour intensive and time consuming to establish. Recently, a novel *in vivo* assay for determining rates of AL-PCD plants was described by Hogg *et al.* (in press). The assay is an easy and relatively rapid method for AL-PCD investigation based on observation of apoptotic-like morphological features of dying root hairs following stress treatment. Root hairs are single cells, present in large numbers, easily examined with light microscopy and indeed the AL-PCD phenotype in root hairs is readily identifiable. With the root hair assay, results may be obtained in a shorter time than with suspension cultures. The root hair assay was reported to be transferable between plant species (tested on *Arabidopsis*, *M. truncatula*, *Z. mays* and *Quercus*) and invaluable for determining modulation of AL‑PCD in mutant/transgenic plant lines.

Other models being used for cell death studies in plants or having the potential for studying plant PCD, which have already been described in this review and include root cap cells, lace plant, investigation of HR after infiltration with pathogen, trichomes, somatic embryogenesis and leaf epidermal peels. The important features of these systems are summarised in Table 2.

*<Table 2 near here>*

1. **Conclusions**

The decision of a cell to undergo PCD is the outcome of a complex signalling process. Although pathways governing this decision are less clear for plant than for animal cells, the sequence of events leading to plant PCD is starting to emerge. Through the study of PCD occurrences throughout the plant life-cycle, a diverse set of characteristics that specifically define plant PCD have been identified. However, several but not all of these features are shared with animal apoptosis. Unique characters exhibited by separate PCD events during plant development and abiotic interactions, suggest that several PCD pathways may operate in plants but extensive overlap between these pathways may exist.

To distinguish it from animal PCD, plant PCD characterised by DNA degradation and condensation of the protoplast away from the cell wall has been defined as AL-PCD (McCabe and Leaver, 2000, McCabe *et al.*, 1997a, Reape and McCabe, 2008, Reape and McCabe, 2010). Calcium influx has been identified as an early event in plant PCD and novel research suggests that zinc may also be involved. Undoubtedly, ROS and reactive nitrogen species are not only molecules that cause physicochemical damage, but are also important messengers in cell death signalling. MMP and release of apoptogenic proteins play a central role in plant PCD and metacaspases/caspase like activity were found to be the executors of cellular disassembly. Recently, the connection between sphingolipid metabolism and PCD regulation has become more evident and the chloroplast has also emerged as having a potential important role in plant cell death control. In addition, components of the endomembrane system have been suggested to create a complex regulatory network involved in plant PCD control. It is expected that future advances in our understanding of plant PCD regulation will be facilitated by the emergence of novel *in vivo* systems for studying cell death and also by further research investigating the role of both the mitochondria and the chloroplast in the regulation of the delicate cellular balance between death and survival signals.

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**Figures and Tables**

Figure 1.

AL-PCD morphology in various types of cells of *Arabidopsis thaliana*:A. cultured cells; B. root hair; C, D. root cap cells; E. trichome. Death was induced by temperature. Arrows indicate condensed cell content and protoplast retraction away from the cell wall. Cells exhibiting corpse morphology did not fluoresce after fluorescein diacetate viability staining. Bar is 10 µm.

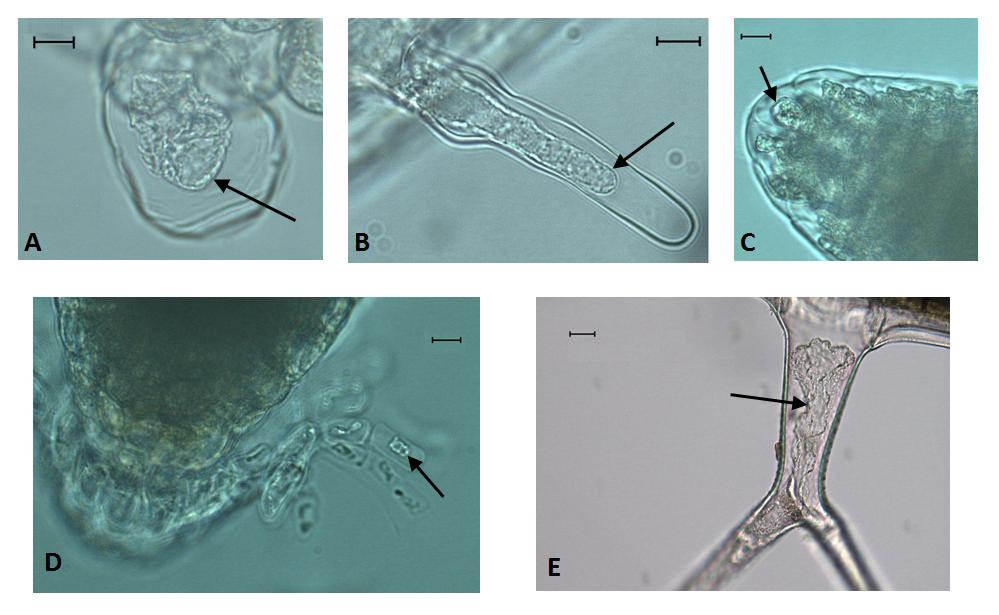


Table 1. Potential protease executors of plant PCD.

|  |  |  |  |
| --- | --- | --- | --- |
| **Putative cell death protease** |  | **Evidence for role in plant PCD** | |
| **METACASPASES**  Sequence and structural similarity to animal caspases (Uren et al. 2000), but do not appear functionally equivalent to caspases. Metacaspase degradome studies should shed more light on their role in PCD machinery. | | | Metacaspase mcII-Pa has been shown to be involved in PCD execution during embryogenesis of Norway Spruce (Suarez *et al.*, 2004, Bozhkov *et al.*, 2005b) |
| Metacaspase-8 is strongly up-regulated by UVC, H2O2, and methyl viologen and its involvement in PCD was suggested by knockout studies (He *et al.,* 2007) |
| LeMCA1, a type II metacaspase was upregulated in necrotrophic pathogen-infected tomato leaves suggesting involvement in the induction of plant cell death (Hoeberichts *et al.*, 2003) |
| Fail to cleave caspase substrates (Vercammen *et al.*, 2004, Watanabe and Lam, 2005) and are not suppressed by caspase inhibitors (Vercammen *et al.*, 2004). It is therefore possible that metacaspases expression results in activation of downstream proteases with caspase-like activities which execute PCD (Watanabe and Lam, 2005). |
| Tudor staphylococcal nuclease, first natural plant metacaspase substrate identified, it is also a substrate of animal caspase-3 (Sundstrom *et al.*, 2009). |
|  | | | Two type I metacaspases were shown to antagonistically control PCD in *Arabidopsis*: AtMC1 acting as a positive regulator and AtMC2 stalling cell death (Coll *et al.*, 2010). |
| **CASPASE-LIKE ACTIVITIES**  Putative cell death proteases with functional but not structural resembalance to animal caspases. Commonly investigated using synthetic fluorogenic tetrapeptide substrates designed to contain preferred cleavage sites of mammalian caspases and by inhibition studies with caspase inhibitors. | | | YVADase, DEVDase, LEHDase, LEVDase, TATDase, VEIDase activities were detected in correlation with PCD induction in various types of cells, tissues and species. Suppression of PCD induced by various treatments was commonly but not always observed after application of caspase inhibitors (Bonneau *et al.,* 2008 and references therein). |
| Phytaspase, protease expressing VEIDase activity shown to be necessary for PCD-related responses to tobacco mosaic virus and abiotic stresses (Chichkova *et al.*, 2010). |
| Saspases, plant proteases containing Ser-active site unlike caspases, which contain a Cys-active site were found to exhibit caspase-like activities (IETDase and VKMDase) and be involved in PCD in *Avena sativa* leaves (Coffeen and Wolpert, 2004). |
| Vacuolar processing enzyme (VPE) was found to have YVADase activity, and VPE deficiency rescued virus-induced HR death in tobacco (Hatsugai *et al*., 2004) and it was shown to be essential for mycotoxin-induced cell death in *Arabidopsis* (Kuroyanagi *et al*., 2005) and developmental cell death in seeds (Nakaune *et al.,* 2005). |

Figure 2. Lace plant: unique lattice-like pattern of perforations on lace plant leaves is formed by PCD of discrete subpopulations of cells (photo courtesy of A.H.L.A.N. Gunawardena).



Figure 3.

Examples of applied and potential model systems for studying PCD in plants.

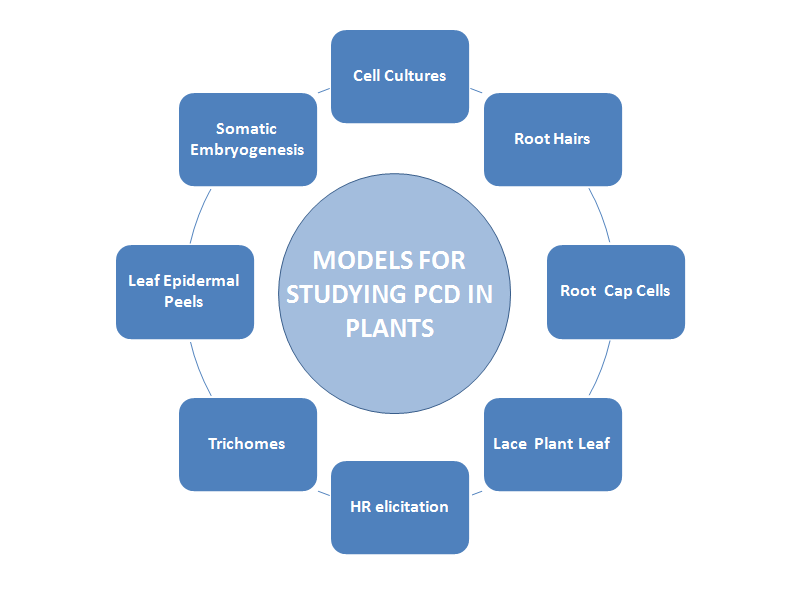


Table 2. Characterisation of potential model systems for studying plant PCD.

|  |  |
| --- | --- |
| **Model System** | **Characteristics** |
| **Cell Suspension Cultures**  reviewed by McCabe and Leaver (2000) | Homogeneous collection of cells routinely used for*in vitro* AL-PCD studies  Established for variety of species and easy to maintain and propagate, but can be labour intensive and time consuming to establish  Easy to add potential AL-PCD modulators, cells easily accessible  Useful for studying basal death responses, and differentiation may be induced in order to study developmental PCD - for example the *Zinna elegans* model system for xylogenesis studies (Fukuda and Komamine, 1980; Twumasi 2009) |
| **Root Hairs**  Presented by Hogg *et al.*  (in preparation) | Novel *in vivo* assay based on observation of AL- PCD morphology (protoplast condensed and retracted from the cell wall) of dying root hairs  Root hairs are single cells, readily observed by light microscopy, present in large numbers  Plants can be grown quickly and results can be obtained in shorter time than with cell suspension cultures, therefore the system is an invaluable tool to investigate modulation of AL-PCD in mutant/transgenic plant lines  Easy to add potential AL-PCD modulators and system is transferrable between species |
| **Root Cap Cells** | Dying root cap cells exhibit AL-PCD features (Wang *et al.*, 1996)  Maize root cap cells can be cultured in vitro (Caporali, 1983)  Cells exposed on the surface of the root create potential for application of PCD modulators  In Arabidopsis, root cap cells die before detachment. Mutants with altered PCD in root cap cells would provide an attractive tool for analysing cell death |
| **Lace Plant**  (Gunawerdena *et al.*, 2004; Gunawerdena *et al.*, 2008) | Numerous, developmental PCD events occur in lace plant leaves at predictable time and location  Different stages of PCD can be observed at the same time  High number and accessibility of cells undergoing PCD  Plant can be propagated in sterile conditions,  Easy to apply modulators, as this is an aquatic plant |
| **Hypersensitive Response** | Studies of HR elicited by injection/infiltration of Arabidopsis (or other species e.g. tobacco) leaves with high titres of bacteria (e.g. Liu *et al.*, 2005; Patel *et al.,* 2008; Hofius *et al.*, 2009, )  Large scale induction (naturally induced in few cells only) – one disadvantage is the artificiality of the method |
| **Trichomes** | Accessible (epidermal origin, composed of single or several cells) and present in large numbers  Arabidopsis functional trichomes are not essential for plant growth in laboratory conditions, which facilitates isolation of mutants (Haughn and Somerville, 1988)  AL-PCD hallmarks were observed in dying trichomes cells (Papini *et al*., 2009; Reina-Pinto *et al.*, 2009) and genes can be expressed under trichome specific promoter (Schnittger *et al.*, 2003), which makes it potentially attractive system for PCD studies  PCD modulators can be applied to the trichomes by spraying of leaf/stem surface |
| **Leaf Epidermal Peels** | Convenient for microscopic observations – peel is a monolayer of cells  Both phototrophic (guard) and chemotrophic (epidermal) cells present, and can therefore be used to investigate the role of the chloroplast in PCD (Samuilov *et al.*, 2003) |
| **Embryogenesis** | Somatic embryogenic cultures are suitable for studying PCD as numerous embryos at the same stage of development may be obtained (Bozhkov *et al.*, 2002; Kawashima and Goldberg, 2010)  Almost all seed plant embryos contain suspensors, which are eliminated by PCD (Yeung and Meinke, 1993)  Gymnosperm embryos are better for suspensor death studies, due to absence of endosperm |