7

Cell Death and Organ Development in Plants

Hilary J. Rogers
School of Biosciences, Cardiff University, Cardiff
United Kingdom CF10 3TL

Programmed cell death (PCD) is an important feature of plant development; however, the mechanisms responsible for its regulation in plants are far less well understood than those operating in animals. In this review data from a wide variety of plant PCD systems is analyzed to compare what is known about the underlying mechanisms. Although senescence is clearly an important part of plant development, only what is known about PCD during senescence is dealt with here. In each PCD system the extracellular and intracellular signals triggering PCD are considered and both cytological and molecular data are discussed to determine whether a unique model for plant PCD can be derived. In the majority of cases reviewed, PCD is accompanied by the formation of a large vacuole, which ruptures to release hydrolytic enzymes that degrade the cell contents, although this model is clearly not universal. DNA degradation and the activation of proteases is also common to most plant PCD systems, where

Current Topics in Developmental Biology, Vol. 71

0070-2153/05 $35.00
DOI: 10.1016/S0070-2153(05)71007-3

they have been studied; however, breakdown of DNA into nucleosomal units (DNA laddering) is not observed in all systems. Caspase-like activity has also been reported in several systems, but the extent to which it is a necessary feature of all plant PCD has not yet been established. The trigger for tonoplast rupture is not fully understood, although active oxygen species (AOS) have been implicated in several systems. In two systems, self incompatibility and tapetal breakdown as a result of cytoplasmic male sterility, there is convincing evidence for the involvement of mitochondria including release of cytochrome c. However, in other systems, the role of the mitochondrion is not clear-cut. How cells surrounding the cell undergoing PCD protect themselves against death is also discussed as well as whether there is a link between the eventual fate of the cell corpse and the mechanism of its death.

I. Introduction

Programmed cell death (PCD) is now recognized as an important event in shaping plant organs (Jones and Dangl, 1996; Pennell and Lamb, 1997). In animal cells PCD has been defined as cell death that is part of the normal life cycle of the organism, is triggered by specific physiological signals, and involves *de novo* gene transcription (Ellis *et al.*, 1991). This distinction is important in differentiating developmental PCD from necrotic cell death that may occur as a result of injury or exposure to toxic substances. In plants an important response to pathogenic attack is the hypersensitive response (HR) in which selected cells around the site of infection undergo PCD to prevent spread of the pathogen. Although the cellular features of HR-PCD show many similarities to developmental PCD, this area has been reviewed (Greenberg and Yao, 2004) and will not be dealt with in detail here. In plants it is also important to distinguish between PCD and senescence, terms that have led to much controversy in this field and have been the focus of review (van Doorn and Woltering, 2004). In this review, PCD will be used to indicate cellular death as opposed to the death of whole organs or individuals.

In some plant organs the effect of PCD is particularly evident. Thus, although in animals, sculpting of the fingers is often quoted as the classic example of the action of PCD in organ development, in plants an obvious example is the prominent holes in the leaves of the house plant *Monstera* (Melville and Wrigley, 1969). However, the phenomenon is much more widespread, and examples are found at all stages of plant development in which the targeted removal of specific cells or cell types is a requirement for normal development.

In animal cells at least two forms of PCD have been described: apoptosis and autophagy. A number of cytological features describe apoptosis. These include nuclear condensation and marginalization, chromatin condensation, followed by fragmentation of DNA into nucleosomal units known as DNA laddering, and the formation of membrane inclusions known as apoptotic bodies. In animals the apoptosed cell remains are finally engulfed by neighboring cells through phagocytosis and the cell corpse disappears (Cohen, 1993). In a few cases PCD may be defined as truly autonomous as, for example, in the very tightly regulated cell death in *Caenorhabditis elegans* (Yuan and Horvitz, 1990). However, in many cases, signals external to the cell, such as changes in hormone levels, trigger PCD (Cohen, 1993). Once PCD has been triggered (for an overview of animal PCD, see Krishnamurthy *et al*., 2000), a complex network of regulators is switched on involving increases in cytosolic calcium concentrations, an oxidative burst, and release of pro-PCD factors such as cytochrome c from the mitochondrion. Release of cytochrome c is regulated by a growing family of Bcl-2 proteins that interact with the mitochondrial membrane, facilitating or inhibiting its release. These intracellular events activate a family of *c*ysteine *asp*artate specific prote*ases* known as caspases, which are both regulators and the effectors of cell death. Caspases act on a plethora of targets initiating cell condensation, nuclear fragmentation, and DNA breakdown. The characteristic DNA laddering occurs as a result of cleavage at the nucleosome-linker sites, by DNAases, which are activated both by the caspases and more directly by increases in cytosolic calcium levels (Peitsch *et al*., 1993).

Autophagy in mammalian cells (reviewed by Stromhaug and Klionsky, 2001) was originally associated with a response to starvation. It is characterized by the formation of vesicles containing proteins and organelles, which are transported to the lysosome. Known as autophagosomes, these vesicles have a short half-life (in the range of a few minutes) and their contents are then digested by the hydrolase-packed lysosomes to generate monomeric building blocks. The signaling pathways leading to autophagy are much less well-defined than those involved in apoptosis; however, heterotrimeric G proteins (Ogier-Denis *et al*., 2000) and type III phosphoinositide 3-phosphates (Petiot *et al*., 2000) have been implicated. Interestingly, autophagy and apoptosis may not be completely independent mechanisms. Beclin 1, which interacts with Bcl-2 family proteins, is associated with type III phosphoinositide 3-phosphates and may have a role in sorting proteins destined for the autophagosome (Kihara *et al*., 2001). Also, sphingosine, known to activate apoptosis, increases the activity of lysosomal proteases such as cathepsin B, which in turn are caspase activators (Ferri and Kroemer, 2001). Notably the lysosomes appear to act upstream of the mitochondria, suggesting a regulatory role in apoptosis (Yuan *et al*., 2002).

Few genes with sequence homology to those involved in animal PCD have been identified in plants despite the availability of genome sequences from representatives of the major taxonomic divisions of flowering plants, namely *Arabidopsis* representing dicotyledonous plants (The *Arabidopsis* Genome Initiative, 2000) and rice representing the monocotyledons (Goff *et al.*, 2002). Putative homologues include those for Bax-Inhibitor-1, an inhibitor of the proapoptotic Bcl-2 family member Bax (Kawai *et al.*, 1999) involved in apoptosis, Beclin (Laporte *et al.*, 2004; C. Wagstaff, unpublished results) involved in autophagy, and *dad-1* (Orzáez and Granell, 1997), although doubt has been cast on the regulatory role of *dad-1* (Kelleher and Gilmore, 1997). Although homologues of caspases have not been identified from the genome sequences (Lam and Del Pozo, 2000), plant metacaspases members of a related superfamily are found in plant genomes (Lam, 2004) and can trigger apoptosis-like cell death in yeast (Watanabe and Lam, 2005). In addition, caspase inhibitors inhibit plant PCD in several systems (see following). Thus, whether homologues to the other components of the apoptotic or autophagic machinery in animal cells are present in plants but with insufficient homology to be identifiable through database searching, or whether the whole mechanism of cell death is different in plants remains as yet unresolved. A number of features differentiate plant and animal cells and these may account for the divergence in cell death mechanisms, one major difference being the cell wall. However, parallels to apoptosis are evident at a cytological level in some types of plant PCD and proteases are prominently upregulated in both plants and animals. Hence, a mechanism may emerge for plant PCD that is divergent from that in animals but shares some common features. As examples of PCD are examined from diverse developmental processes questions about the input signals triggering PCD, the process of cell death, and the consequences of the death of the cell can be asked.

II. Seed and Embryo Development

Several seed tissues undergo PCD as part of their normal development, and best studied of these is the endosperm. The endosperm is a transient nutritive triploid tissue formed from the secondary fertilization event characteristic of higher plants. It is essential for embryo development, but endosperm life span is limited and cell death progresses until in some species all the cells in the tissue are dead as the seed reaches maturity. The life span of the endosperm is species specific; in dicotyledonous seeds such as beans, it is barely detectable and the nutrients are transferred to the embryo before the seed reaches maturity. In cereal seeds, however, the endosperm is a prominent component and a layer of the endosperm, the aleurone layer, is still

viable at seed maturity. Aleurone cells, however, are nondividing, and the whole tissue disappears following seed germination. The aleurone has a secretory role, producing the hydrolytic enzymes required to break down the reserves stored in the endosperm, and is tightly regulated through hormonal control (Huttly and Phillips, 1995). Both have been intensely studied, due to their importance for seed development and also as models for plant PCD (Young and Gallie, 2000a).

Another well-studied seed tissue with limited life span is the suspensor. This is composed of a string of cells connecting the embryo to the rest of the seed and acts as a conduit for the nutrients mobilized from the endosperm to reach the developing embryo (Yeung and Meinke, 1993). Once this function has been performed, its cells undergo PCD. Suspensor cell death has proven to be a particularly fruitful tissue for PCD studies in that it is possible to induce embryo formation from isolated cells in culture and to manipulate the formation and degeneration of the suspensor cells.

A. Cell Death in the Endosperm

In seeds with little or no endosperm, the lack of this tissue is due either to arrested development or to its early degeneration (Young and Gallie, 2000a). Thus, although the endosperm cells undergo cell death in all species, the timing of this event and the size of the tissue vary. Although the fate of the endosperm in noncereals is interesting, most studies have focused on cereal endosperm, especially in wheat and maize. Development and cell death in both species is not uniform, and the pattern of cellular change may be related to species-specific signaling events (Young and Gallie, 2000b).

1. The Aleurone

PCD in the aleurone is stimulated by Gibberellic acids (GAs), a class of plant growth regulators (Bethke *et al.*, 1999; Kuo *et al.*, 1996; Wang *et al.*, 1996), and the process can be simulated by the incubation of isolated aleurone layers, or protoplasts with GA (Fath *et al.*, 2000). *In vivo* GAs are produced by the embryo and stimulate the aleurone layer to release hydrolytic enzymes that make available nutrients stored in the starchy endosperm for embryo growth (Fincher, 1989). The hydrolases are synthesized *de novo* from the breakdown of storage proteins found in protein storage vacuoles (PSVs) within the aleurone cells. During GA stimulation, the PSVs coalesce to form a single large vacuole and change from storage to lytic organelles. This is accompanied by acidification of the PSVs (Swanson and Jones, 1996) and accumulation of hydrolytic enzymes. Although at first these are required for

storage protein mobilization, later they are implicated in death of the aleurone cell through autolysis and removal of the aleurone cell corpse. One possible mechanism for this autolysis is that the acidification activates specific proteases, which initiate a protease cascade within the PSVs (Fath *et al.*, 2000). The protease phytepsin is upregulated following GA treatment in aleurone cells (Bethke *et al.*, 1996) and was shown to process probarley lectin in roots (Sarkkinen *et al.*, 1992). Thus, protease activation by specific proteases may be an important mechanism in aleurone PCD. However, although protease cascades are reminiscent of the well-characterized caspase cascades in animal PCD (Salvesen and Dixit, 1999), caspases are unlikely to play an important role here. Caspases, which accumulate at the right time to be involved in PCD, have been identified in aleurone cells, but they are not GA-induced (Fath *et al.*, 2000). Furthermore, apoptotic bodies as seen in apoptotic animal cells have not been observed (Fath *et al.*, 2000), so GA-induced PCD in aleurone cells diverges significantly from animal cell apoptosis.

The final step of aleurone cell PCD is loss of integrity of the plasma membrane (Fath *et al.*, 2000, 2002) leading to loss of turgor and shrinkage of the cell corpse. AOS may play the final role in the execution of aleurone PCD by causing membrane lipid peroxidation resulting in loss of membrane integrity (Fath *et al.*, 2001). There is indeed a body of evidence implicating AOS in aleurone PCD. GA-treated protoplasts become more sensitive to exogenous hydrogen peroxide, and incubation of GA treated aleurone protoplasts with antioxidants such as butylated hydroxy toluene (BHT) reduced mortality (Fath *et al.*, 2002). The same study also revealed that both transcripts and activities of enzymes involved in AOS metabolism decrease on treatment of aleurone protoplasts with GA. The source of AOS was also investigated, and both an increase in glyoxylate activity and a change in the electron transport chain away from the alternative oxidase pathway were associated with GA treatment. Both of these are associated with increased AOS production. Thus, both an increase in AOS production and a reduction in AOS metabolism are associated with PCD following GA treatment, and membrane damage leading to PCD that may occur when AOS production exceeds removal of AOS by metabolic routes. Nitric oxide (NO) may also be involved as a signaling molecule and/or with an antioxidant role. NO can act as an antioxidant reducing membrane damage by interacting with lipid peroxyl radicals, or it can promote cell death (Beligni *et al.*, 2002). Incubation of barley aleurone cells with a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), accelerated GA-induced PCD, suggesting that endogenous NO was retarding PCD. This was supported by the detection of endogenous NO production using NO-sensitive fluorescent dyes (Beligni *et al.*, 2002), although there was no apparent difference in NO production between GA or cells treated with another plant growth regulator abscisic acid (ABA), which protects

endosperm cells from PCD (see later). Exogenous application of NO also retarded PCD in GA-treated cells in a similar way to BHT, suggesting an antioxidant role, and NO also delayed the downregulation of AOS metabolism gene expression.

While aleurone cells become highly vacuolated, degradation of their nuclear DNA is detectable. It proceeds rapidly and they lose over 50% of their nuclear DNA within 5 days after GA treatment. However, although TUNEL staining is positive indicating the presence of free 3′ OH ends, controversy exists over whether DNA laddering takes place. One study (Fath *et al.*, 1999) did not find DNA laddering in barley aleurone protoplasts, whereas more recently DNA laddering was detected in preparations from both wheat and maize intact aleurone cells (Domínguez *et al.*, 2004). Whether there is interspecific divergence in the mechanism for nuclear DNA degradation in aleurone cells, or whether the contrasting results are due to differing methodologies remains unclear. Where it has been detected, the timing of DNA laddering is in agreement with the TUNEL data, coinciding with extensive vacuolation. Candidate nucleases have been identified; at least three nucleases are induced by GA treatment, and their induction is delayed by ABA treatment. The timing of their activity fits in with the data on DNA degradation. Thus, strong upregulation of their activity occurs after hydrolase release is essentially complete (Fath *et al.*, 2000). It would seem, moreover, that the nuclease activity and consequent DNA degradation are a requirement for PCD in these cells. Hydrolase release, nuclease activity, and DNA degradation were all inhibited by the guanylyl-cyclase inhibitor LY83583 (Bethke *et al.*, 1999; Fath *et al.*, 1999), indicating that cGMP may also play a regulatory role in aleurone cell PCD.

The link between hydrolase release and cell death is not, however, simple because at least in the earlier stages of hydrolase release, it can be uncoupled from PCD. When aleurone cells are treated with ABA, cell death is delayed even if the cells have previously been treated with GA and induction of hydrolase release has begun (Fath *et al.*, 2000). Furthermore, in aleurone protoplasts, accumulation of proteases in the medium does not result in PCD. Thus, freshly prepared protoplasts were incubated in medium that had been recovered from GA-treated cells and that was rich in hydrolases; however, this did not accelerate PCD in the protoplasts (Fath *et al.*, 2000).

Ca^{2+} and protein phosphorylation have been implicated in the signal transduction mechanism that leads to GA-induced PCD. Okadaic acid, a protein phosphatase inhibitor, blocked GA-induced cytosolic Ca^{2+} increase (Kuo *et al.*, 1996) and microinjection of a syntide-2, synthetic substrate for calmodulin and Ca^{2+}- dependent protein kinases, inhibited the development of the large vacuole, an essential precursor of PCD in the aleurone cells (Ritchie and Gilroy, 1998).

2. The Starchy Endosperm

Unlike the aleurone, the starchy endosperm cells are dead by the time the seed reaches maturity. Researchers (Gallie and Young, 2004) suggested that the death of these cells may be necessary to allow access for the hydrolases, which break down the starch reserves, making the nutrients available to the embryo. Cell death of the starchy endosperm is thus unusual in that the corpse of the dead cell is not eliminated and persists in the developed seed. Cell death in this tissue needs to be correctly timed such that starch filling is complete before PCD is initiated. Moreover, the starch filling is not synchronous across the whole tissue; it occurs in a wave starting in the central/proximal region of the seed and moving toward the pedicel end, and PCD follows in the same pattern (Young and Gallie, 2000a). Thus, in close proximity the starchy endosperm cells are dying while the aleurone cells and the embryo remain living, suggesting a programmed coordination of signals. The plant-growth regulator ethylene plays an important role in regulating PCD in the starchy endosperm both spatially and temporally. Evidence comes from the use of inhibitors of ethylene synthesis such as AVG ([2-aminoethoxyvinyl]glycine) and of ethylene action such as 1-MCP (1-methylcyclopropane), both of which delay PCD, whereas applications of exogenous ethylene accelerate PCD in this tissue (Young *et al.*, 1997). Two peaks of ethylene production are detected in the endosperm coinciding with waves of PCD. The first is in the central/proximal region of the seed, while the second late peak occurs at the pedicel end of the seed (Young *et al.*, 1997). Evidence for involvement of ethylene in endosperm PCD also comes from starch-deficient mutants. In the maize *shrunken 2* mutant endosperm, PCD occurs prematurely (Young *et al.*, 1997) and ethylene levels are elevated coinciding with the wave of PCD. However, to achieve correct timing of PCD in different cells, and furthermore to protect the aleurone and the embryo from the ethylene signal, the cells must either be exposed differentially or sense the same ethylene levels differentially. Researchers (Gallie and Young, 2004) have isolated cDNAs both for ethylene biosynthetic genes and ethylene receptor genes. They have shown that the expression of ethylene receptors is much lower in the endosperm compared to the embryo, suggesting that the endosperm tissue is responsive to lower levels of the plant growth regulator compared to the embryo. This fits in with other experiments showing that if ethylene levels are sufficiently high, then the embryo cells will also enter PCD (Young *et al.*, 1997).

As well as ethylene, ABA may also be important in the regulation of PCD in the starchy endosperm. ABA is important during the desiccation of the seed as it reaches maturity. Evidence comes from two mutants in which the endosperm PCD occurs prematurely (Young and Gallie, 2000b). One of these mutants (*vp9*) is defective in ABA production, whereas the other

(*vp1*) is defective in ABA perception. Thus, it would seem that in the wild-type ABA signaling is protecting the endosperm cells from PCD. Ethylene production increases concomitantly with premature PCD in these mutants, suggesting a link between the two plant-growth regulators, although the exact nature of the association is as yet unclear.

Two other types of signals have also been studied in relation to endosperm PCD. AOS may play a role, but again the evidence is indirect: production of an enzyme involved in AOS removal in barley, peroredoxin (*Per1*), is only expressed in the embryo and the aleurone, but not in the endosperm (Stacy *et al.*, 1996). Also SOD production is reduced in the viviparous mutants previously described, suggesting that it may have a protective role against premature PCD (Guan and Scandalios, 1998). However, further work is required to obtain firmer evidence for a role of AOS in PCD of this tissue.

The other possible trigger for PCD is endoreduplication. During endosperm development there is a wave of endoreduplication starting at the central region, and followed by the wave of PCD. In support of a role for endoreduplication in the regulation of PCD is the observation that in lily endosperm there is no endoreduplication and the endosperm cells remain alive at maturity (Young and Gallie, 2000a). However, again the evidence is indirect and would require further data to support a causative link.

The mechanism of PCD in the starchy endosperm shares features with other types of PCD. DNA laddering is detected once the wave of cell death begins, and fragmentation of the DNA into larger fragments of 50–300 kb is detectable much earlier before cell death becomes evident (Young and Gallie, 2000a). There is also a clear link between ethylene and DNA laddering: exposure to ethylene accelerated DNA fragmentation in wild-type maize. Furthermore, in maize starch-deficient mutant *shrunken2* kernels where PCD and DNA laddering progress more rapidly than wild-type, treatment with AVG reduced the DNA breakdown. Nuclease activity is also detected in the endosperm at levels 10-fold higher than in the embryo (Young *et al.*, 1997).

3. The Endosperm of *Ricinus communis*

Although the machinery associated with protein breakdown has not been a major focus in work on cereal endosperm PCD, some very interesting results have emerged from the study of endosperm PCD in the castor bean (*Ricinus communis*). In this species the endosperm persists into the mature seed and then dies following germination as the protein and lipid reserves it contains are depleted. The lipid reserves are mobilized via the formation of glyoxysomes, but additionally the endosperm cells also contain another organelle that develops from the ER and has been called the ricinosome (Schmid *et al.*, 1999, 2001). Endosperm degeneration is accompanied by

DNA fragmentation, and the number of ricinosomes dramatically increases as DNA fragmentation becomes evident (Schmid *et al.*, 1999). A papain-like endopeptidase (Cys-EP), which shows homology to cysteine proteases and is associated to other plant PCD events, is also found in ricinosomes (Gietl *et al.*, 1997; Than *et al.*, 2004). The Cys-EP is formed as a 45-kDa proenzyme, which is later cleaved to a 35 kDa mature form. Antibodies to Cys-EP can be used to label the ricinosomes and follow the fate of the Cys-EP as PCD progresses (Schmid *et al.*, 1998). Labeling shows that the proenzyme accumulates in the intact ricinosomes in parallel with the increase in DNA fragmentation. However in the final stages of PCD, the ricinosomes rupture releasing the mature 35-kDal form. Thus, the ricinosomes seem to be playing a similar role to the large vacuole in the aleurone cell, releasing hydrolytic activity into the cytoplasm as PCD reaches its final stages. Again pH is postulated to play a part as acidification of isolated ricinosomes triggers the activation of the Cys-EP protease (Schmid *et al.*, 2001). *In vivo* this may occur as a result of cytoplasm acidification due to a change in tonoplast permeability late in PCD resulting in proton release from acidic vacuoles.

B. Cell Death in the Embryo and Suspensor

Following endosperm degeneration, the next event in the life of the seed is the development of the embryo, and PCD plays a crucial role here too. Although shaping of the embryo also relies on PCD, this has been less well-studied (Giuliani *et al.*, 2002). Two major PCD events, however, have been studied in detail, the elimination of supernumerary embryos and the degeneration of the suspensor.

In many plants, particularly gymnosperms, several embryos can develop from a single zygote, but usually only one develops to maturity (Bawa *et al.*, 1989). The elimination of the excess embryos follows a precise pattern of cell elimination starting at the basal end of the embryo and moving toward the apex. Although little is known of the signals dictating which embryo survives and which die, more is known about the mechanisms of elimination. Thus, the PCD is accompanied by DNA fragmentation and increasing vacuolization (Filonova *et al.*, 2002). The cytological features resemble those of autophagy in which the cytoplasm and organelles are engulfed and then lysed by the vacuoles.

Suspensor degeneration is a more universal feature of plant embryogenesis. In the first zygotic division, the two daughter cells are differentiated into a densely cytoplasmic cell, which will divide to ultimately produce the embryo and a highly vacuolated cell, which undergoes fewer rounds of cell division to produce the suspensor. The suspensor acts as a conduit for nutrients to the developing embryo and degenerates once its function is

complete. The signals limiting growth of the suspensor are thought to come from the embryo as mutants in which normal embryo development is impaired also have enlarged suspensor cells with features usually associated with embryonic cells (Schwartz *et al.*, 1994). However, the nature of these signals remains unknown. PCD in the maize (Giuliani *et al.*, 2002) and bean (*Vicia faba*; Wredle *et al.*, 2001) suspensor is accompanied by DNA fragmentation and nuclear degeneration, which proceeds basipetally. In bean, evidence also exists of the formation of provacuoles and nuclear inclusions, which may indicate an autophagic type of PCD. However, the nuclear morphology of endosperm and suspensor cell PCD was compared in bean (Wredle *et al.*, 2001) and differences were revealed in the arrangement of the chromatin, suggesting subtle differences in the PCD program in these two cell types. In plants it is also possible to stimulate embryo development *in vitro* by the appropriate use of plant-growth regulators, and in these systems the embryonic and suspensor structures are clearly defined. This approach has been used for studying PCD in Norway spruce (*Picea abies*) embryos (Filonova *et al.*, 2000; Smertenko *et al.*, 2003). In this system as well, suspensor cell death was accompanied by DNA fragmentation initially into 50 kb fragments and later DNA laddering similar to events seen in animal apoptosis. Cytological studies showed the formation of large central vacuoles formed from smaller provacuoles (Filonova *et al.*, 2000). The nucleus also fragmented, and finally the tonoplast ruptured and there was an almost complete lysis of cytoplasm, organelles, and nucleus. Thus, the PCD shows characteristics both of apoptosis and a form of autophagy. The cytoskeleton also rearranges during PCD in this system: microtubules are disrupted in the suspensor compared to the embryonic cells, and as PCD sets in, all microtubule fragments disappear completely (Smertenko *et al.*, 2003). Conversely actin in the suspensor is organized into longitudinal and oblique cables. Disruption of these cables with actin-depolymerizing drugs such as latrunculin results in aberrant suspensor development. The authors suggest that actin may be important in this system for autophagous PCD in these cells, as has been found in animal PCD (Aplin *et al.*, 1992).

The Norway spruce model was also used to study the role of caspase-like enzymes in suspensor PCD (Bozhkov *et al.*, 2004). Using synthetic peptide substrates and appropriate inhibitors, a caspase-6-like (VEIDase) activity was detected. The activity localized to suspensor and embryonic cells fated for PCD and inhibition of the VEIDase activity resulted in blocking suspensor PCD. This strongly suggests that this caspase-like activity is required for PCD in these cells. However, how the caspase-like activity functions here during PCD is unclear, although its early expression, coincident with microtubule disassembly and vacuole enlargement, might indicate a regulatory role. Cloning of the gene or genes responsible for the caspase-like activity is clearly needed to help resolve its role.

III. Leaf, Stem, and Root Development

The major nonreproductive organs of the mature plant are the leaves, stem, and root system, and in all three of these PCD is important both in normal organ development and function and in shaping the organ in response to environmental cues. PCD is also the terminal event in the destruction of leaves in response to environmental and developmental cues known as senescence.

A. Leaf Sculpting

One of the most dramatic examples of PCD is the remarkable sculpting of some leaves such as those of *Monstera* and the lace plant, *Aponogeton madagascariensis* (Fig. 1). Most complex leaf shapes are formed through an acceleration or repression of growth during morphogenesis (Dengler and Tsukaya, 2001), however, in *Monstera* and the lace plant, sculpting occurs through PCD. In *Monstera* tiny holes are formed early in development and increase in size with leaf expansion (Melville and Wrigley, 1969). These holes finally break through the leaf margin to form the characteristic lobed pattern. In the lace plant the program is different. Here leaves form as solid shapes, and a subset of cells subsequently undergoes PCD to produce a pattern of holes (Gunawardena *et al.*, 2004). The trigger for this PCD is unknown, but the mechanism seems to parallel other better-studied systems with clear DNA degradation, chromatin condensation, and evidence of tonoplast rupture. However, DNA laddering is not detectable. Further work is clearly needed to understand how cells are targeted for PCD and how the tonoplast rupture is triggered.

Figure 1 Morphology of mature Lace plant leaf showing regular perforations arising from PCD remodeling of young expanding leaves. Bar = 150 μm. (Image kindly provided by Dr. Arunika Gunawardena.)

B. Leaf Senescence

Cell death has an additional role in plants compared to animals in that it is the terminal event of plant senescence. The process of senescence and cell death are clearly distinct at a physiological level because senescence, at least in leaves, can be a reversible process, whereas cell death is considered a terminal event (Thomas *et al*., 2003). Leaf senescence has been the focus of several reviews (Lim *et al*., 2003) and genomic-wide approaches to identify regulatory networks (Buchanan-Wollaston *et al*., 2003; Gepstein *et al*., 2003). This review will focus only on what is known about PCD at the cellular level, which is associated with leaf senescence. The signals initiating the overall process of senescence are common to other PCD events. Reduction of ethylene signaling (Grbić and Bleecker, 1995) and upregulation of cytokinin production (Gan and Amasino, 1995) delay senescence, indicating that the levels of these two PGRs are involved. Elevated cytoplasmic calcium is associated with leaf senescence in parsley (Huang *et al*., 1997), and calcium fluxes have also been implicated in two other leaf senescence systems (Chou and Kao, 1992; He and Jin, 1999). Thus, calcium signaling may play a role in leaf PCD, although further data are needed. Cytologically, PCD has been charted in rice leaves undergoing induced (by dark treatment) or natural senescence (Lee and Chen, 2002). In this system, features noted were cytoplasm depletion, organellar breakdown and expansion of the central vacuole, which at later stages contained inclusions possibly of chloroplast origin. Chromatin condensation and apoptotic bodies were not noted, and although cells became TUNEL-positive and DNA became increasingly degraded, there was no evidence of DNA laddering. However, DNA laddering was detected in senescing leaves of other species such as wheat (Caccia *et al*., 2001), olive (Cao *et al*., 2003), and five other tree species (Yen and Yang, 1998). Chromatin condensation was also reported both in tobacco and in the monocot *Ornithogalum virens* (Simeonova *et al*., 2000). There was no clear reduction in mitochondrial membrane potentials in *Pisum sativum* mesophyll cells undergoing senescence (Simeonova *et al*., 2004), indicating that if mitochondria are associated with this PCD system, it is not in the same way as in animal apoptosis. Chloroplast disassembly seems to be an early sign of senescence, but whether this is part of the PCD mechanism remains uncertain (Thomas *et al*., 2003).

C. Tracheary Element Differentiation

A much better studied system is that of tracheary element differentiation. Tracheids are part of the xylem system, which in higher plants transports water and minerals from the roots to the rest of the plant. Tracheid cells, or

tracheary elements (TEs), are dead at maturity, forming tapered hollow tubes with thickened cell walls. The formation of TEs requires targeted cell death of precursor cells derived from meristematic cells. An *in vitro* system has been developed in *Zinnia* to study this PCD in which mesophyll cells transdifferentiate into TEs (Fukuda and Komamine, 1980).

Evidence from the use of inhibitors implicates brassinosteroids, a type of steroid PGR, as a requirement for PCD progression (Yamamoto *et al.*, 1997), and brassinosteroid biosynthesis genes increase in expression during the transition to PCD (Yamamoto *et al.*, 2001) in this system. In *Arabidopsis*, mutants in brassinosteroid receptor genes also result in abnormal vascular differentiation (Cano-Delgado *et al.*, 2004), supporting the involvement of PGRs in this developmental process.

AOS seems not to be involved in tracheary element PCD because inhibitors of the AOS generating enzyme NADPH oxidase did not inhibit TE PCD and levels of H_2O_2 were not elevated in cells following TE induction (Groover *et al.*, 1997). In contrast, calcium/calmodulin signaling is involved. An increase in sequestered calcium occurs (Roberts and Haigler, 1989), and Ca^{2+} uptake is a requirement for PCD progression (Roberts and Haigler, 1990). Transiently elevated calmodulin levels are associated with PCD progression and calmodulin blockers also inhibit the process (Kobayashi and Fukuda, 1994). Calcium ionophores also induced DNA fragmentation (Groover and Jones, 1999). Furthermore, a link exists between calcium signaling and G proteins in this system. Thus, mastoparan, which is an activator of heterotrimeric G proteins, induced DNA degradation and this effect was suppressed by inhibitors of Ca influx.

Vacuolar collapse is the first visible sign of PCD in TE differentiation (Fukuda, 1996) followed by organelle degeneration. Organelle removal is a very rapid process, with nuclear degradation occurring within 10–20 min (Obara *et al.*, 2001) and complete organellar destruction within 6 hr of vacuolar collapse (Groover *et al.*, 1997). The vacuolar collapse may be triggered by changes in the organic ion permeability of the tonoplast and the collapse is preceded by loss of tonoplast integrity as the plasma membrane remains intact, but the tonoplast becomes unable to exclude fluorescein (Obara *et al.*, 2001). Also treatment of differentiating TEs with an inhibitor of organic anion transport, probenecid, resulted in an acceleration of the vacuolar collapse (Kuriyama, 1999).

In contrast to other systems, there is no evidence of nuclear condensation, fragmentation, or DNA laddering in TE PCD. DNA degradation was detectable using TUNEL staining, although this was a late event resulting from release of nucleases following vacuolar collapse (Mittler and Lam, 1995). In fact no DNA degradation is detected prior to vacuolar collapse (Obara *et al.*, 2001). Various nuclease activities have been detected and genes encoding nucleases with very similar expression patterns are expressed

during TE PCD, including an S1-type nuclease (ZEN1), which is likely to be located in the vacuole (Sugiyama *et al.*, 2000; Thelen and Northcote, 1989; Ye and Droste, 1996). ZEN1 may be of particular importance as it is specifically expressed in TEs, and both an anti-ZEN1 antibody and antisense constructs of ZEN1 are able to block DNA degradation (Ito and Fukuda, 2002). Expression of these nuclease genes is just before the start of autolysis, indicating a role in DNA breakdown following vacuole rupture.

Proteolysis appears to be required for TE PCD; in particular inhibition of both proteasome function and cysteine proteases delayed or inhibited the process (Fukuda, 2000). Two cysteine protease genes encoding papain-like cysteine proteases are expressed as TEs move into PCD (Yamamoto *et al.*, 1997; Ye and Varner, 1996) and cysteine protease activities of 20–30 kDa increase at a similar time (Beers and Freeman, 1997; Minami and Fukuda, 1995). These are probably located in the vacuole and may be involved in the autolysis of the cell following vacuolar rupture. A serine protease activity is also detected, but because it has a higher pH optimum, it is probably associated with the cytoplasm rather than the vacuole (Beers and Freeman, 1997). A protease activation cascade may also act as a possible trigger for vacuolar collapse, although the late timing of its activation makes this model less plausible (Fukuda, 2000; Groover and Jones, 1999).

Clearly TE differentiation has proven an excellent system in which to study plant PCD, and some of the intercellular and intracellular regulatory pathways such as PGR and Ca^{2+} control have been elucidated. Transcriptomic approaches to identify genes expressed early after PCD induction in this system (Milioni *et al.*, 2002) may also help to provide a more complete picture of how TE PCD is regulated, and whether such regulatory genes are shared with other PCD events during plant development.

D. PCD in Aerenchyma Formation

Another plant tissue in which hollow tubes are formed via PCD is aerenchyma. This is a specialized root tissue common in wetland species, which can be induced in other plants including some major crops by water stress (Evans, 2004) (Fig. 2). It can be formed either by differential growth and cell separation or in the case of lysogenic aerenchyma by the selective death of cells in the mid cortex, spreading radially to form channels of air spaces, separated by living cells. The function of this architecture is to allow better gas exchange in waterlogged root systems (Armstrong, 1979). Induction of aerenchyma formation has been studied in more detail as the timing of events is easier to follow; however, the mechanisms are probably very similar in constitutive aerenchyma development. Low oxygen is the initial trigger for aerenchyma formation. This signal is transduced via ethylene: both

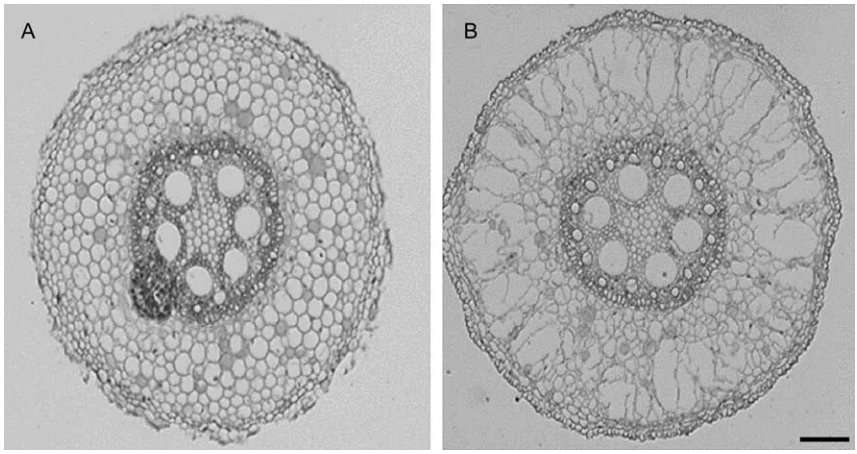

Figure 2 Development of aerenchyma. Hematoxylin- and eosin-stained maize root sections. (B) 2.5-day-old root treated with low oxygen levels (3%) to induce aerenchyma formation compared to (A) in 21% oxygen in which aerenchyma does not develop. Scale bar = 100 μm. Reproduced with permission from Gunawardena *et al.*, 2001.

endogenously produced or exogenously elevated ethylene induces the formation of lysogenous aerenchyma (Drew *et al.*, 1981). Presumably, the ethylene is released and then detected via receptors in the cells fated to die. A plausible system for distinguishing between cell fates would relate to the receptors as was seen for endosperm cells; however, this has not been shown directly for aerenchyma formation (Evans, 2004). Calcium signaling is again required: Ca^{2+} chelators and inhibitors of Ca^{2+} release from internal stores block aerenchyma formation (He *et al.*, 1996). It has also been reported that cytosolic Ca^{2+} levels rise (Drew *et al.*, 2000), and that this is linked to a signal transduction pathway involving inositol 1,4-5-triphosphate, and protein phosphorylation, although details are as yet unpublished. It is also possible that AOS signaling may be active in aerenchyma PCD based on data showing an increase in AOS in cells beginning to degenerate (Bouranis *et al.*, 2003).

In induced aerenchyma formation in maize, the first cytological signs of cell death are plasma membrane (PM) invagination and the formation of small vesicles just beneath it (Gunawardena *et al.*, 2001a). At a very similar time but probably afterwards, chromatin condensation is detected, followed by membrane-enclosed organelles closely resembling animal apoptotic bodies (Gunawardena *et al.*, 2001a). TUNEL staining is also detected in maize, although evidence for DNA laddering was less convincing (Gunawardena *et al.*, 2001a). Changes in the cell wall are detected early in the PCD using specific antibodies, although they are not evident by

electron microscopy until much later (Gunawardena, 2001b). In rice where aerenchyma is constitutive, a similar pattern is also observed with descriptions also of vacuolation (Webb and Jackson, 1986) and cytoplasm acidification probably resulting from loss of tonoplast integrity (Kawai *et al.*, 1998). However in rice there was no TUNEL staining suggesting either a species-specific difference in the PCD mechanism or perhaps reflecting a more rapid PCD progression in this system (Evans, 2004). What happens to the cell contents is an interesting question. Evidence from cryo-SEM suggests that there may be rapid ionic reabsorption of cell contents by neighboring cells (van der Weele *et al.*, 1996).

Sadly, despite the wealth of knowledge about the cytological events in aerenchyma formation, and a relatively clear-cut case for PGR triggering of PCD, there is very little biochemical data on aerenchyma PCD. Unlike the tracheary element work, no information is available to date on proteases, either as part of potential cascades or involved in the mopping up after vacuolar collapse, or on potential nuclease genes. This may be due to the inaccessibility of the aerenchyma tissue and the lack of a cell culture model (Evans, 2004).

E. Cell Death in the Root Cap

A more exposed root tissue that undergoes PCD is the root cap. From early embryogenesis, root cap cells stain positively for TUNEL, indicating DNA degradation (Giuliani *et al.*, 2002), whereas other areas of the root remain TUNEL-negative. Root cap cells are derived continuously from the apical meristem and are sloughed off at the surface of the root in a manner reminiscent of skin cells. Staining of mature onion roots reveals that only the outermost 2–3 cells of the root cap are TUNEL positive (Wang *et al.*, 1996). In these cells nuclear fragmentation was also detected and the formation of apoptotic-like bodies noted. Similar results were obtained with *Arabidopsis* roots (Møller and McPherson, 1998; Zhu and Rost, 2000). One study (Zhu and Rost, 2000) also showed chromatin condensation, vacuolation, and organelle destruction. Notably, the frequency of plasmodesmata fell in the outer root cap cells before DNA fragmentation was detected, suggesting an exclusion of the dying cells from the living root cap. Two *Arabidopsis* mutants give important insight into the determination of root cap cell fate. Mutants in *TORNADO1* and *TORNADO2* genes are defective in the early divisions of the epidermal/lateral root cap cells (Cnops *et al.*, 2000). In these mutants, although root cap cells develop ectopically in the epidermal positions, they still die, suggesting that root cap cell death is a highly programmed event under genetic control. Signaling may involve AOS, as an *Arabidopsis* gene encoding an H_2O_2-generating diamine oxidase

(ATAO1) is expressed in lateral root cap cells (Møller and McPherson, 1998), which are undergoing vacuolation and are destined for PCD. Little is known about the machinery that executes PCD in these cells. However, two proteases from barley, aleurain and barley vacuolar aspartic proteinase (phytepsin), are expressed in root cap cells (Runeberg-Roos and Saarma, 1998), suggesting a proteolytic mechanism, perhaps following the autophagic model seen in tracheary elements and aleurone cells.

IV. Flowering and Reproduction

Plant reproduction requires the development of complex structures that interact with each other and that have an inherently limited life span. Thus, PCD is involved here both in shaping the sexual and nonsexual organs of the flower and in their removal once they are no longer needed. In addition, PCD also plays a crucial role in self-incompatibility where genetically similar pollen is rejected by the female reproductive structures.

A. Sexual Organ Abortion

While most flowering plants produce bisexual flowers containing both male and female organs, approximately 10% produce unisexual flowers either on the same or on different individuals (Irish and Nelson, 1989). In these flowers sex determination is achieved by arrest or abolition of the sexual organ primordia and the role of PCD in this process has been reviewed (Wu and Cheung, 2000). Two types of plant growth regulator have been implicated in regulation of sexual organ abortion in maize. GA treatment partially abolished the phenotype of *anther ear 1* and *dwarf* mutants, which produce male sexual organs (stamens) on the female flowers (Irish and Nelson, 1989), and which encode biosynthetic GA genes (Bensen *et al.*, 1995; Winkler and Helentjaris, 1995). In another mutant *ts2*, the male tassels show varying degrees of feminization and the TS2 gene encodes a protein with homology to steroid dehydrogenases (DeLong *et al.*, 1993). This enzyme may act on brassinosteroids, another class of PGRs mentioned previously (Wu and Cheung, 2000).

B. PCD in the Male Sexual Organs

Pollen develops within locules of the anther, which are lined with a tissue known as the tapetum. This plays a nutritive role during pollen development, coating the pollen grains with proteins and lipids and forming the

intricately sculpted outer layer of the pollen wall known as the exine. However, the tapetum degenerates once its function is completed. This degeneration is accompanied by cell shrinkage, vacuolation, and thinning of the cell walls (Bedinger, 1992). Chromatin condensation and ER inclusions have been reported in *Lobivia rauschii* and *Tillandsia albida* (Papini *et al*., 1999), as well as DNA fragmentation in barley anthers (Wang *et al*., 1999). In *Brassica oleracea, B. napus, Digitalis purpurea* and a cultivated form of *Fuchsia*, nuclear blebbing has also been reported in some cells (A. Stead, unpublished data). However, little seems to be known about the signaling or mechanism of tapetal PCD despite its importance in plant fertility. One study (Lesniewska *et al*., 2004) reported a depolarization of mitochondrial membranes in tapetal cells in advanced PCD which may indicate an involvement of the mitochondria in this process. A rice cysteine protease gene has been isolated, which is expressed in the tapetum and developing pollen grains, late during pollen development (Lee *et al*., 2004). Mutants in this gene are impaired in pollen development. It will be interesting to understand the function of this gene more fully; perhaps it may have relevance to the PCD mechanism operating in tapetal cells.

Two distinct types of mutation, nuclear or cytoplasmic, result in an early onset of tapetal PCD. The best known of these is cytoplasmic male sterility, which has been described in over 150 species (Schnable and Wise, 1998). In these plants, mutations in the mitochondrial genome result in male sterility through aberrations in anther development, or more frequently, tapetal degeneration and pollen abortion soon after meiosis. Note that although the mitochondrial defect is present in all the cells of the plant, the major defect is in anther function. Because the mutation is known to be in the mitochondrial genome, it has long been assumed that the death of the anther cells is due to a failure of the mitochondria to sustain the large energy needs of these tissues (Levings, 1993). Thus, PCD in these cells may well be triggered by mitochondrial dysfunction. Evidence for the mechanism at play has come from studies of the sunflower CMS system, PET1-CMS (Balk and Leaver, 2001). Here cell condensation, DNA laddering, and chromatin condensation are associated with the early death of the tapetum (Balk and Leaver, 2001). Mitochondria persist in the early PCD stages; however, release of cytochrome c into the cytosol is detected before the changes in cell morphology. This is followed by a loss of outer mitochondrial membrane integrity and a fall in the respiratory control ratio. Thus, in this system there appears to be a central role for the mitochondrion in PCD reminiscent of its role in mammalian PCD (Krishnamurthy *et al*., 2000).

Numerous nuclear mutants exhibiting male sterility have also been described. In some cases these too owe their male sterility to a premature degeneration of the tapetum. Tapetal PCD was studied in one male sterile rice mutant (Ku *et al*., 2003) and was associated with cytoplasmic shrinkage,

membrane blebbing, vacuolation, changes in mitochondrial morphology, and DNA fragmentation. The DNA fragmentation detected by TUNEL was seen premeiotically, which is earlier than in CMS. However, there is as yet no precise data on the mechanism of PCD in this system.

Other anther tissues also undergo PCD: pollen is released from the anther locule by death of the stomium cells, and death of cells within the endothecium provides a mechanism for the ejection of the pollen at dehiscence. In the *Arabidopsis* mutant *delayed dehiscence1*, there is a delay in stomium degeneration resulting in male sterility (Sanders *et al.*, 2000). The gene encodes an enzyme in the jasmonic acid biosynthetic pathway, and application of jasmonic acid restored wild-type dehiscence. Thus, this plant growth regulator may be important directly or indirectly in the correct timing of death in these cells. In tobacco, ethylene may also be involved, as blocking ethylene perception delayed stomium degeneration (Rieu *et al.*, 2003). Although the biochemical mechanisms of PCD in these cells are relatively under studied, a report of a cysteine protease expressed in the endothecium of brinjal (Xu and Chye, 1999) and a thiolendopeptidase expressed in tobacco stomium (Koltunow *et al.*, 1990) may lead to further characterization of the processes involved.

C. PCD in the Female Gametophyte

Numerous cells and tissues undergo PCD throughout female gametophyte development and function. The first step in the production of the female gametophyte is meiosis of the megaspore mother cell to yield four meiotic products. However, in most plant species, three of these degenerate, and the eight cells of the mature female gametophyte contained within the embryo sac derive from two rounds of mitosis of just one meiotic product. The three degenerating cells show some classical signs of PCD including cell shrinkage, cytoplasmic disorganization, and chromatin condensation (Bell, 1996). The next PCD event concerns four of the eight embryo sac cells. These are known as the synergids and the antipodal cells. The synergids are positioned close to the micropyle, the pore through which the pollen tube enters the ovule to effect fertilization, whereas the antipodal cells are at the opposite end of the embryo sac. During or shortly before fertilization, one of the two synergids degenerates, and in some species the antipodal cells also degenerate after fertilization. Because the synergids in some species degenerate independently from pollination, it seems likely that the signals are intrinsic to their development (Drews and Yadegari, 2002). In *Nicotiana*, the degeneration of the synergids is characterized by a change in nuclear morphology, collapse of the vacuole, and disappearance of organelles (Huang and Russell, 1994). There is also a loss of membrane integrity (Huang and Russell, 1992) and

the generation of "pinched-off" cytoplasmic bodies (Huang *et al*., 1993). Likewise in wheat (*Triticum aestivum*) synergid PCD is accompanied by nuclear shrinkage and deformation, and chromatin condensation (An and You, 2004). Thus, the symptoms of PCD evident in synergid degeneration may fit with the autophagous model of PCD. However, an *Arabidopsis* female gametophyte mutant (*gfa2*) has been described in which one of the two synergids that normally degenerates following pollination, fails to die (Christensen *et al*., 2002). *GFA2* encodes a member of the DnaJ protein family, which acts as a chaperone in the mitochondrial matrix. The authors suggest that GFA2 may be required for correct folding of mitochondrial proteins, which in turn are required for PCD in the synergid. Thus an involvement of mitochondrial function in synergid PCD at least in *Arabidopsis* seems to have been established. A comparison of antipodal and synergid PCD in wheat (An and You, 2004) indicates that the PCD mechanisms may differ in these closely positioned cells. In particular, there are clear differences in chromatin and nuclear morphology, with only slight changes in nuclear volume in antipodal cells and only partial chromatin condensation. Also, in the *gfa2* mutant previously described, antipodal PCD is not affected. This may reflect the different function of the cells and of course may vary in different species, but would be an interesting area for further research.

D. PCD During Self-Incompatibility Interactions

One reproductive tissue that has received a lot of attention is the pollen tube and its interactions during pollination and fertilization. Over 50% of higher plants protect against self-fertilization by self-incompatibility (SI) mechanisms that are thought to have evolved independently in different plant families several times. SI involves highly specific interactions between the pollen and the pistil. In incompatible reactions fertilization is prevented and in some systems is associated with PCD of the pollen tube. Research (Thomas and Frankin-Tong, 2004) has shown that SI in *Papaver* is associated with DNA fragmentation and caspase-3-like activity. An inhibitor of caspase-3, the peptide Ac-DEVD-CHO, inhibited DNA fragmentation and pollen tube growth. Poly(ADP) ribose polymerase (PARP) is involved in nuclear DNA repair (Smith, 2001) and is a classic substrate for caspase-3 enzymes. This study (Thomas and Franklin-Tong, 2004) showed that the caspase-3-like activity elicited by SI in the *Papaver* pollen tubes was able to cleave PARP, and that detection of this activity preceded DNA fragmentation. SI also stimulated the release of cytochrome c into the cytosol. *Papaver* SI results in increases in cytosolic $[Ca^{2+}]$ (Franklin-Tong *et al*., 1993), and elevating cytosolic $[Ca^{2+}]$ in the pollen tubes using Mastoparan resulted in the release

of cytochrome c, providing a link between SI, Ca signaling, and cytochrome c release. Mastoparan treatment also stimulated caspase-3-like activity linking the DNA degradation and cytochrome c release to increases in cytosolic [Ca^{2+}]. Thus, at least in this SI system there is compelling evidence for a PCD mechanism with several of the hallmarks of animal PCD.

E. Petal Senescence and Cell Death

Petal senescence, unlike leaf senescence, inevitably ends with PCD; however again, whether the two processes should be treated as one remains hotly debated (Thomas *et al*., 2003; van Doorn and Woltering, 2004). Global genomic approaches are being used in addition to studies of individual processes to identify genes involved in petal senescence (e.g., Breeze *et al*., 2004), and the expression of several of the known homologues to animal PCD-related genes—namely Beclin, Bax inhibitor I, and dad-1, (Hückelhoven, 2004; Orzàez and Granell, 1997; Wagstaff *et al*., 2003; C. Wagstaff, unpublished results)—has been revealed, although the role of these genes in regulating petal PCD remain unresolved.

Evidence from electron microscopy across *Alstroemeria* senescent petals (Wagstaff *et al*., 2003) clearly shows that PCD is already occurring at relatively early stages of petal senescence. In many flowers including *Arabidopsis*, tobacco, and many others, senescence and thus PCD is triggered by ethylene (Stead and van Doorn, 1994) sometimes associated with pollination (O'Neill, 1997). However, in another group, including lilies such as *Alstroemeria*, the role of ethylene is less clear-cut. Given the very tightly regulated life span of many flowers (van Doorn and Stead, 1997), endogenous cellular factors may determine onset of PCD, but this has not been clearly demonstrated. Once the trigger for PCD has been perceived by the cell, the intracellular mechanisms operating in petal PCD closely resemble those of the other systems described. Calcium signaling and GTP-binding proteins have been implicated in some species (Porat *et al*., 1994). ROS accumulation has also been reported both in ethylene-induced (Bartoli *et al*., 1996) and in ethylene-independent (Panavas and Rubinstein, 1998) petal senescence, together with a drop in antioxidants (Bartoli *et al*., 1997). There is often evidence of tonoplast invagination (Matile and Winkenbach, 1971; Phillips and Kende, 1980) or the formation of vesicles (Smith *et al*., 1992), and in the final stages only a thin layer of cytoplasm remains (Stead and van Doorn, 1994; Wagstaff *et al*., 2003) and organelles disappear (Stead and van Doorn, 1994). Changes in membrane composition, fluidity, and peroxidation occur in several species (Rubinstein, 2000). In some cases, such as carnation (*Dianthus caryophyllus*; Fobel *et al*., 1987), daylily (*Hemerocallis hybrid*; Panavas and Rubinstein, 1998), and rose (*Rosa hybrid*; Fukuchi-Mizutani *et al*., 2000),

lipid peroxidation (e.g., through the action of lipoxygenases [LOX]) may be one of the key factors effecting loss of membrane integrity. However, in other species like *Alstroemeria* (Leverentz *et al.*, 2002), loss of membrane semipermeability was chronologically separated from LOX activity that had declined by over 80% by the onset of electrolyte leakage. Thus, in this species loss of membrane function was not related to LOX activity or accumulation of lipid hydroperoxides per se representing a novel pattern of flower senescence.

DNA laddering has been found in some petals such as *Alstroemeria* (Wagstaff *et al.*, 2003) and pea (Orzàez and Granell, 1997), and both nucleases (Breeze *et al.*, 2004; Langston *et al.*, 2005; Panavas *et al.*, 1999) and proteases (Stephenson and Rubinstein, 1998; Wagstaff *et al.*, 2002) are upregulated during petal senescence in several species. Significantly in *Sandersonia aurantiaca,* a member of a class of cysteine proteases carrying the KDEL C-terminal motif is expressed during senescence (Eason *et al.*, 2002), which shows homology to a protease from *Ricinus communis* implicated in ricinosome–mediated endosperm PCD (Gietl *et al.*, 1997) (see earlier discussion). This suggests a mechanism for petal cell PCD, at least in this species, which may parallel the vacuole-driven autophagous model previously described in the *Ricinus* endosperm.

V. Conclusions and Future Directions

The examples of developmental PCD in the plants reviewed here illustrate a diversity of data sets but also some fundamental differences between the different cell fates and PCD mechanisms. It is perhaps useful to compare the data available for each of the systems reviewed (Table I) to look for commonalities. It can be asked how useful is the animal model of apoptosis or autophagy in explaining plant PCD mechanisms? In common with most animal PCD systems, a signal extrinsic to the cell, often hormonal, is usually involved. However there is no one PGR that induces PCD, although ethylene, GA, and brassinosteroids appear in more than one system. There are few clear-cut examples of completely autonomous PCD, although perhaps the root cap comes close. In this case the evidence from mutant studies (Cnops *et al.*, 2000) supports a direct genetic control of PCD for these cells, and more data from mutants in other systems where extrinsic signals have not been clearly defined, such as PCD during ethylene-independent petal senescence, may reveal the degree of PCD autonomy. Another interesting issue is how cells adjacent to cells undergoing PCD protect themselves from the "death signals." Data from ethylene signaling in the endosperm (Gallie and Young, 2004) indicate that at least in some systems this may be achieved by a differential in sensitivity. However, in the TE system another mechanism seems to be operating. Here there is evidence for an inhibitor of

Table I Comparison of PCD Characteristics from Different Plant Tissues

	Possible Signaling		Possible Mechanism			
Plant Tissue	PGR Control	Ca^{2+}/Phosphatase Signaling	Apoptosis?	Autophagy?	Fate of Corpse	Some Key References
Aleurone cells	GA (+); ABA (−)	Ca^{2+} increases inhibited by protein phosphatase and protein kinase inhibitors NO(−)? AOS (+)	No DNA laddering, or PM blebbing	Possible proteolytic cascade, lytic vacuoles	Disappears	Fath *et al.*, 1999; Kuo *et al.*, 1996; Lam *et al.*, 2000
Starchy endosperm	Ethylene (+); ABA (−)	AOS(+)?	DNA laddering nucleases		Remains	Stacy *et al.*, 1996; Young and Gallie, 2000a; Young *et al.*, 1997
Ricinus endosperm	Germination			Lytic ricinosomes, from ER, proteolytic cascade	Disappears	Schmid *et al.*, 1999, 2001
Suspensor	Signals from embryo		DNA laddering Caspase-6 activity	Vacuolarization, autophagic vacuole	Disappears	Bozhkov *et al.*, 2004; Giuliani *et al.*, 2002; Wredle *et al.*, 2001
Supernumary embryos	Signals from Female gametophyte nutrient depletion			Vacuolarisation, vacuole rupture TUNEL positive	Disappears	Filonova *et al.*, 2002
Leaf sculpting	Unknown		DNA fragmentation but no laddering	Vacuole rupture,	Disappears	Gunawardena *et al.*, 2004

Leaf senescence	Ethylene (+) and cytokinin (−)	Ca^{2+} increases	DNA laddering in some species	Vacuolation	Disappear	Caccia *et al.*, 2001; Gan and Amasino, 1995; Grbić and Bleecker, 1995; Huang *et al.*, 1997; Lee and Chen, 2002
Tracheary elements	Brassinosteroids	Ca^{2+} increases, calmodulin, G proteins		Vacuolar collapse, rapid organelle degradation, nucleases No DNA laddering	Cytoplasm disappears cell wall structural	Roberts and Haigler, 1989, 1990
Aerenchyma	Ethylene	Ca^{2+} increases, protein phosphorylation	Apoptotic bodies, DNA laddering (?)	Vacuolation, loss of tonoplast integrity	Cytoplasm disappears cell wall structural	Drew *et al.*, 2000; Gunawardena *et al.*, 2001a,b
Root cap	Genetic control	Possible AOS	Apoptotic bodies, chromatin condensation	Vacuolation, organelle destruction	Sloughed off	Møller and McPherson, 1998; Wang *et al.*, 1996; Zhu and Rost, 2000
Sex organ abortion	GA and brassinosteroids		Chromatin condensation			Wu and Cheung, 2000
Tapetum			DNA laddering, nuclear blebbing; mitochondrial membrane depolarization	Vacuolation	Disappear, cell contents taken up by microspores	Lesniewska *et al.*, 2004; Papini *et al.*, 1999; Wang *et al.*, 1999
CMS	Mitochondrial disfunction	Cytochrome c release	DNA laddering, chromatin condensation		Disappear	Balk and Leaver, 2001

(*Continued*)

Table I *Continued*

Plant Tissue	Possible Signaling		Possible Mechanism		Fate of Corpse	Some Key References
	PGR Control	Ca^{2+}/Phosphatase Signaling	Apoptosis?	Autophagy?		
Endothecium	Jasmonic acid, ethylene				Disappear	Rieu *et al.*, 2003; Sanders *et al.*, 2000
Megaspores	Embryo sac expansion			Cell shrinkage, chromatin condensation	Disappear	Bell, 1996
Synergids	Pollination in some species		Chromatin condensation; requirement for mitochondrial chaperone	Vacuolar collapse	Burst releasing contents	An and You, 2004; Christensen *et al.*, 2002; Huang and Russell, 1992, 1994
Petal senescence	Ethylene in some species	Ca^{2+}/phosphatase signalling, ROS increases	DNA laddering	Tonoplast invagination formation of vesicles	Mesophyll cells disappear	Bartoli *et al.*, 1996; Orzàez and Granell, 1997; Panavas and Rubinstein, 1998; Phillips and Kende, 1980; Porat *et al.*, 1994; Wagstaff *et al.*, 2003
Pollen tube	SI interactions	Cytochrome c release, increased Ca^{2+}	DNA fragmentation Caspase-3 activity		Burst	Thomas and Franklin-Tong, 2004

proteasome-mediated protein degradation, which is released into the apoplastic space (Endo *et al.*, 2001) and which protects living cells from the hydrolases released during TE PCD. A more thorough comparison of gene expression and biochemistry between cells undergoing PCD and their living neighbors may be helpful to understand more fully the complexity of extrinsic cellular signaling leading to PCD.

Intracellular calcium signaling seems almost universal where it has been assayed and may well be a common feature in all plant PCD. Involvement of protein kinase and phosphatase control also seems an area worthy of further study. Release of cytochrome c has only been reported in two PCD systems, CMS and SI, both of which might be considered special cases of PCD. Whether cytochrome c release is more widespread is not clear at present, but it would be very useful to resolve the extent to which it occurs in any of the other systems, as this may shed light on the involvement of the mitochondria in the plant PCD systems. DNA laddering appears in many of the PCD systems but is not universal, and may not ultimately be very useful in understanding the PCD mechanism. Laddering clearly suggests activation of nucleases, which cut the DNA in a specific manner but does not explain much about how the activation occurs. The cytological evidence is perhaps more useful. Vacuolation accompanies PCD in more than half of the examples reviewed, and in general rupture of the vacuole coincides with release of hydrolases into the cytoplasm. This suggests a possible model that would account for at least some of the systems reviewed (Fig. 3). In this model an external signal activates increases in cytoplasmic calcium, which in turn stimulates the coalescence of small vacuoles derived from either the ER or the Golgi to form a large vacuole. This vacuole accumulates hydrolytic enzymes. Its collapse, resulting either from ROS accumulation or activation of a proteolytic cascade, releases hydrolases into the cytoplasm. The hydrolase release results in organellar breakdown and macromolecule degradation ending in cell death. This model has clear parallels to animal autophagy, although the vacuole rather than the lysosome is the primary organelle involved. However, this model is clearly not universal, and some PCD systems such as CMS, SI, and PCD during leaf senescence may follow a very different pattern, perhaps more similar to animal apoptosis.

An important distinction in the role of cell death in organ development is also the fate of the dead cell. Thus, in some cases such as aerenchyma formation, the corpse of the cell is removed leaving a space; however, in other cases the dead cell has a structural (e.g., TE formation) or nutritive (e.g., the starchy endosperm) role. However, from the evidence reviewed here, the fate of the cell and the mechanism of its destruction do not seem to be closely linked; thus, both TE formation and endosperm destruction in *Ricinus* follow quite closely the model outlined, although the fate of the cell

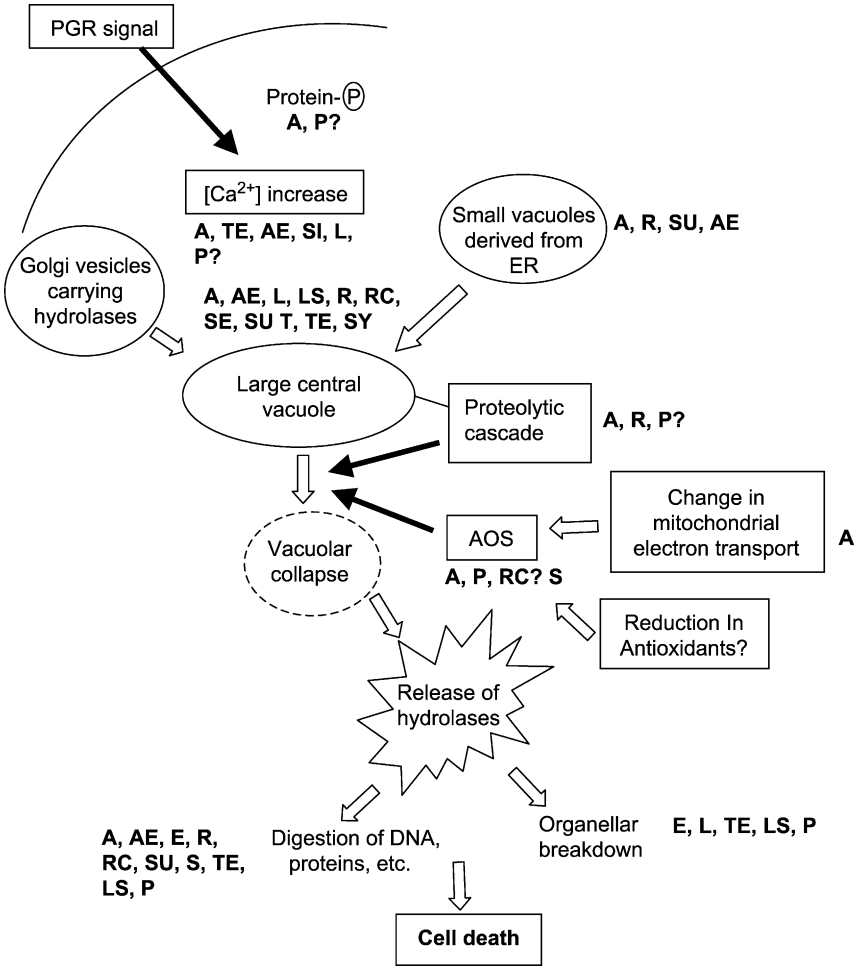

Figure 3 Model showing major signals (black arrows) and cytological/biochemical events (open arrows) during autophagic-type PCD in plants. Cell types/tissue in which these features have been reported are indicated as: A, aleurone cells; AE, aerenchyma; L, leaf sculpting; LS, leaf senescence; P, petal senescence; R, *Ricinus* endosperm; RC, root cap; S, starchy endoasperm; SE, supernumerary embryos; SI, pollen tube during SI interaction; SU, suspensor; SY, synergids; T, tapetum; TE, tracheary elements.

is very different. There may thus be parallel pathways that diverge at the point of activation of cell wall-degrading enzymes.

This review has focused largely on the mechanisms of PCD seen in different stages of plant development. This mechanistic approach seemed important in an attempt to understand the diversity of developmental PCD

in plants. The future challenge lies in filling in biochemical and molecular gaps, which will explain not only how plant cells die during development but also what is regulating their demise.

Acknowledgments

I would like to thank Dr. Tony Stead and Dr. Carol Wagstaff for helpful comments on the manuscript and Dr. David Evans and Dr. Arunika Gunawardena for supplying the images in this review.

References

An, L. H., and You, R. L. (2004). Studies on nuclear degeneration during programmed cell death of synergid and antipodal cells in *Triticum aestivum*. *Sex. Plant Reprod.* **17,** 195–201.

Aplin, A., Jasionowski, T., Tuttle, D. L., Lenk, S. E., and Dunn, W. A. (1992). Cytoskeletal elements are required for the formation and maturation of autophagic vacuoles. *J. Cell. Physiol.* **152,** 458–466.

The *Arabidopsis* Genome, Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408,** 796–815.

Armstrong, W. (1979). Aeration in higher plants. *Adv. Bot. Res.* **7,** 225–332.

Balk, J., and Leaver, C. J. (2001). The PET1-CMS mitochondrial mutation in sunflower is associated with premature programmed cell death and cytochrome *c* release. *Plant Cell* **13,** 1803–1818.

Bartoli, C. G., Simontacchi, M., Montaldi, E., and Puntarulo, S. (1996). Oxidative stress, antioxidant capacity and ethylene production during ageing of cut carnation (*Dianthus caryophyllus*) petals. *J. Exp. Bot.* **47,** 595–601.

Bartoli, C. G., Simontacchi, M., Montaldi, E., and Puntarulo, S. (1997). Oxidants and antioxidants during ageing of chrysanthemum petals. *Plant Sci.* **129,** 157–165.

Bawa, K. S., Hedge, S. G., Ganeshaiah, K. N., and Uma Shaanker, R. (1989). Embryo and seed abortion in plants. *Nature* **342,** 625.

Bedinger, P. (1992). The Remarkable Biology of Pollen. *Plant Cell* **4,** 879–887.

Beers, E. P., and Freeman, T. B. (1997). Proteinase activity during tracheary element differentiation in *Zinnia* mesophyll cultures. *Plant Physiol.* **113,** 873–880.

Beligni, M. V., Fath, A., Bethke, P. C., Lamattina, L., and Jones, R. L. (2002). Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol.* **129,** 1642–1650.

Bell, P. R. (1996). Megaspore abortion: A consequence of selective apoptosis? *Intl. J. Plant Sci.* **157,** 1–7.

Bensen, R. J., Johal, G. S., Crane, V. D., Tossberg, J. T., Schnable, P. S., Meeley, R. B., and Briggs, S. P. (1995). Cloning and characterization of the maize *An1* gene. *Plant Cell* **7,** 75–84.

Bethke, P. C., Hillmer, S., and Jones, R. L. (1996). Isolation of intact protein storage vacuoles from barley aleurone—Identification of aspartic and cysteine proteases. *Plant Physiol.* **110,** 521–529.

Bethke, P. C., Lonsdale, J. E., Fath, A., and Jones, R. L. (1999). Hormonally regulated programmed cell death in barley aleurone cells. *Plant Cell* **11,** 1033–1045.

Bouranis, D. L., Chorianopoulou, S. N., Siyiannis, V. F., Protonotarios, V. E., and Hawkesford, M. J. (2003). Aerenchyma formation in roots of maize during sulphate starvation. *Planta* **217,** 382–391.

Bozhkov, P. V., Filonova, L. H., Suarez, M. F., Helmersson, A., Smertenko, A. P., Zhivotovsky, B., and von Arnold, S. (2004). VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. *Cell Death Diff.* **11,** 175–182.

Breeze, E., Wagstaff, C., Harrison, E., Rogers, H. J., Stead, A. D., Bramke, I., Thomas, B., and Buchanan-Wollaston, V. (2004). Gene expression patterns to define stages of post-harvest senescence in *Alstroemeria* petals. *Plant Biotech. J.* **2,** 155–168.

Buchanan-Wollaston, V., Earl, S., Harrison, E., Mathas, E., Navabpour, S., Page, T., and Pink, D. (2003). The molecular analysis of leaf senescence—a genomics approach. *Plant Biotech. J.* **1,** 3–22.

Caccia, R., Delledonne, M., Levine, A., De Pace, C., and Mazzucato, A. (2001). Apoptosis-like DNA fragmentation in leaves and floral organs precedes their developmental senescence. *Plant Biosys.* **135,** 183–189.

Cano-Delgado, A., Yin, Y. H., Yu, C., Vafeados, D., Mora-Garcia, S., Cheng, J. C., Nam, K. H., Li, J., and Chory, J. (2004). BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in *Arabidopsis*. *Development* **131,** 5341–5351.

Cao, J., Jiang, F., and Sodmergen Cui, K. M. (2003). Time-course of programmed cell death during leaf senescence in *Eucommia ulmoides*. *J. Plant Res.* **116,** 7–12.

Chou, C. M., and Kao, C. H. (1992). Methyl jasmonate, calcium, and leaf senescence in rice. *Plant Physiol.* **99,** 1693–1694.

Christensen, C. A., Gorsich, S. W., Brown, R. H., Jones, L. G., Brown, J., Shaw, J. M., and Drews, G. N. (2002). Mitochondrial GFA2 is required for synergid cell death in *Arabidopsis*. *Plant Cell* **14,** 2215–2232.

Cnops, G., Wang, X., Linstead, P., Van Montagu, M., Van Lijsebettens, M., and Dolan, L. (2000). TORNADO1 and TORNADO2 are required for the specification of radial and circumferential pattern in the *Arabidopsis* root. *Development* **127,** 3385–3394.

Cohen, J. J. (1993). Apoptosis. *Immunol. Today* **14,** 126–130.

DeLong, A., Calderon-Urrea, A., and Dellaporta, S. L. (1993). Sex determination gene TASSELSEED2 of maize encodes a short-chain alcohol dehydrogenase required for stage-specific floral organ abortion. *Cell* **74,** 757–768.

Dengler, N., and Tsukaya, H. (2001). Leaf morphogenesis in dicotyledons: Current issues. *Intl. J. Plant Sci.* **162,** 459–464.

Domínguez, F., Moreno, J., and Cejudo, F. J. (2004). A gibberellin-induced nuclease is localized in the nucleus of wheat aleurone cells undergoing programmed cell death. *J. Biol. Chem.* **279,** 11530–11536.

Drew, M. C., He, C. J., and Morgan, P. W. (2000). Programmed cell death and aerenchyma formation in roots. *Trends Plant Sci.* **5,** 123–127.

Drew, M. C., Jackson, M. B., Giffard, S. C., and Campbell, R. (1981). Inhibition by silver ions of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to exogenous ethylene or to oxygen deficiency. *Planta* **153,** 217–224.

Drews, G. N., and Yadegari, R. (2002). Development and function of the angiosperm female gametophyte. *Ann. Rev. Genet.* **36,** 99–124.

Eason, J. R., Ryan, D. J., Pinkney, T. T., and O'Donoghue, E. M. (2002). Programmed cell death during flower senescence: Isolation and characterization of cysteine proteinases from *Sandersonia aurantiaca*. *Funct. Plant Biol.* **29,** 1055–1064.

Ellis, R. E., Yuan, J. Y., and Horvitz, H. R. (1991). Mechanisms and functions of cell-death. *Ann. Rev. Cell Biol.* **7,** 663–698.

Endo, S., Demura, T., and Fukuda, H. (2001). Inhibition of proteasome activity by the TED4 protein in extracellular space: A novel mechanism for protection of living cells from injury caused by dying cells. *Plant Cell Physiol.* **42,** 9–19.

Evans, D. E. (2004). Aerenchyma formation. *New Phytolog.* **161,** 35–49.

Fath, A., Bethke, P., Beligni, V., and Jones, R. (2002). Active oxygen and cell death in cereal aleurone cells. *J. Exp. Bot.* **53,** 1273–1282.

Fath, A., Bethke, P., Lonsdale, J., Meza-Romero, R., and Jones, R. (2000). Programmed cell death in cereal aleurone. *Plant Mol. Biol.* **44,** 255–266.

Fath, A., Bethke, P. C., and Barley Jones, R. L. (1999). Aleurone cell death is not apoptotic: Characterization of nuclease activities and DNA degradation. *Plant J.* **20,** 305–315.

Fath, A., Bethke, P. C., Belligni, M. V., Spiegel, Y. N., and Jones, R. L. (2001). Signalling in the cereal aleurone: Hormones, reactive oxygen and cell death. *New Phytol.* **151,** 99–107.

Ferri, K. F., and Kroemer, G. (2001). Organelle-specific initiation of cell death pathways. *Nat. Cell Biol.* **3,** E255–E263.

Filonova, L. H., Bozhkov, P. V., Brukhin, V. B., Daniel, G., Zhivotovsky, B., and von Arnold, S. (2000). Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. *J. Cell Sci.* **113,** 4399–4411.

Filonova, L. H., von Arnold, S. Daniel,G.,, and Bozhkov, P. V. (2002). Programmed cell death eliminates all but one embryo in a polyembryonic plant seed. *Cell Death Diff.* **9,** 1057–1062.

Fincher, G. B. (1989). Molecular and cellular biology associated with endosperm mobilization in germinating cereal-grains. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **40,** 305–346.

Fobel, M., Lynch, D. V., and Thompson, J. E. (1987). Membrane deterioration in senescing carnation flowers. *Plant Physiol.* **85,** 204–211.

Franklin-Tong, V. E., Ride, J. P., Read, N. D., Trewavas, A. J., and Franklin, F. C. H. (1993). The self-incompatibility response in papaver-rhoeas is mediated by cytosolic-free calcium. *Plant J.* **4,** 163–177.

Fukuchi-Mizutani, M., Ishiguro, K., Nakayama, T., Utsunomiya, Y., Tanaka, Y., Kusumi, T., and Ueda, T. (2000). Molecular and functional characterization of a rose lipoxygenase cDNA related to flower senescence. *Plant Sci.* **160,** 129–137.

Fukuda, H. (1996). Xylogenesis: Initiation, progression and cell death. *Ann. Rev.Plant Physiol. Plant Mol. Biol.* **47,** 299–325.

Fukuda, H. (2000). Programmed cell death of tracheary elements as a paradigm in plants. *Plant Mol. Biol.* **44,** 245–253.

Fukuda, H., and Komamine, A. (1980). Establishment of an experimental system for the tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol.* **65,** 57–60.

Gallie, D. R., and Young, T. E. (2004). The ethylene biosynthetic and perception machinery is differentially expressed during endosperm and embryo development in maize. *Mol. Gen. Genom.* **271,** 267–281.

Gan, S. S., and Amasino, R. M. (1995). Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270,** 1986–1988.

Gepstein, S., Sabehi, G., Carp, M. J., Hajouj, T., Nesher, M. F., Yariv, I., Dor, C., and Bassani, M. (2003). Large-scale identification of leaf senescence-associated genes. *Plant J.* **36,** 629–642.

Gietl, C., Wimmer, B., Adamec, J., and Kalousek, F. (1997). A cysteine endopeptidase isolated from castor bean endosperm microbodies processes the glyoxysomal malate dehydrogenase precursor protein. *Plant Physiol.* **113,** 863–871.

Giuliani, C., Consonni, G., Gavazzi, G., Colombo, M., and Dolfini, S. (2002). Programmed cell death during embryogenesis in maize. *Ann. Bot.* **90,** 287–292.

Goff, S. A., Ricke, D., Lan, T. H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P., Varma, H., Hadley, D., Hutchison, D., Martin, C., Katagiri, F., Lange, B. M., Moughamer, T., Xia, Y., Budworth, P., Zhong, J., Miguel, T., Paszkowski, U., Zhang, S., Colbert, M., Sun, W. L., Chen, L., Cooper, B., Park, S., Wood, T. C., Mao, L., Quail, P., Wing, R., Dean, R., Yu, Y., Zharkikh, A., Shen, R., Sahasrabudhe, S., Thomas, A., Cannings, R., Gutin, A., Pruss, D., Reid, J., Tavtigian, S., Mitchell, J., Eldredge, G., Scholl, T., Miller, R. M., Bhatnagar, S., Adey, N., Rubano, T., Tusneem, N., Robinson, R., Feldhaus, J., Macalma, T., Oliphant, A., and Briggs, S. (2002). A draft sequence of the rice genome (Oryza sativa L. ssp japonica). *Science* **296,** 92–100.

Grbić, V., and Bleecker, A. B. (1995). Ethylene regulates the timing of leaf senescence in *Arabidopsis*. *Plant J.* **8,** 595–602.

Greenberg, J. T., and Yao, N. (2004). The role and regulation of programmed cell death in plant-pathogen interactions. *Cell. Microbiol.* **6,** 201–211.

Groover, A., and Jones, A. (1999). Tracheary element differentiation uses a novel mechanism coordinating programmed cell death and secondary cell wall synthesis. *Plant Physiol.* **119,** 375–384.

Groover, A., DeWitt, N., Heidel, A., and Jones, A. (1997). Programmed cell death of plant tracheary elements differentiating *in vitro*. *Protoplasma* **196,** 197–211.

Guan, L., and Scandalios, J. G. (1998). Two structurally similar maize cytosolic superoxide dismutase genes, *Sod4* and *Sod4A*, respond differentially to abscisic acid and high osmoticum. *Plant Physiol.* **117,** 217–224.

Gunawardena, A., Pearce, D. M. E., Jackson, M. B., Hawes, C. R., and Evans, D. E. (2001b). Rapid changes in cell wall pectic polysaccharides are closely associated with early stages of aerenchyma formation, a spatially localized form of programmed cell death in roots of maize (*Zea mays* L.) promoted by ethylene. *Plant Cell Environ.* **24,** 1369–1375.

Gunawardena, A. H. L. A. N., Greenwood, J. S., and Dengler, N. G. (2004). Programmed cell death remodels lace plant leaf shape during development. *Plant Cell* **16,** 60–73.

Gunawardena, A. H. L. A. N., Pearce, D. M., Jackson, M. B., Hawes, C. R., and Evans, D. E. (2001a). Characterisation of programmed cell death during aerenchyma formation induced by ethylene or hypoxia in roots of maize (*Zea mays* L.). *Planta* **212,** 205–214.

He, C. J., Morgan, P. W., and Drew, M. C. (1996). Transduction of an ethylene signal is required for cell death and lysis in the root cortex of maize during aerenchyma formation induced by hypoxia. *Plant Physiol.* **112,** 463–472.

He, P., and Jin, J. Y. (1999). Relationships among hormone changes, transmembrane Ca2+ flux and lipid peroxidation during leaf senescence in spring maize. *Acta Bot. Sinica* **41,** 1221–1225.

Huang, B. Q., and Russell, S. D. (1992). Synergic degeneration in Nicotiana—a quantitative, fluorochromatic and chlorotetracycline study. *Sex. plant Reprod.* **5,** 151–155.

Huang, B. Q., and Russell, S. D. (1994). Fertilization in *nicotiana tabacum*—cytoskeletal modifications in the embryo sac during synergid degeneration—a hypothesis for short-distance transport of sperm cells prior to gamete fusion. *Planta* **194,** 200–214.

Huang, B. Q., Strout, G. W., and Russell, S. D. (1993). Fertilization in *Nicotiana tabacum*—ultrastructural organization of propane-jet-frozen embryo sacs *in-vivo*. *Planta* **191,** 256–264.

Huang, F. Y., Philosoph Hadas, S., Meir, S., Callaham, D. A., Sabato, R., Zelcer, A., and Hepler, P. K. (1997). Increases in cytosolic Ca2+ in parsley mesophyll cells correlate with leaf senescence. *Plant Physiol.* **115,** 51–60.

Hückelhoven, R. (2004). BAX Inhibitor-1, an ancient cell death suppressor in animals and plants with prokaryotic relatives. *Apoptosis* **9,** 299–307.

Huttly, A. K., and Phillips, A. L. (1995). Gibberellin-regulated plant genes. *Physiol. Plant.* **95,** 310–317.

Irish, E., and Nelson, T. (1989). Sex determination in monoecious and dioecious plants. *Plant Cell* **1,** 737–744.

Ito, J., and Fukuda, H. (2002). ZEN1 is a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements. *Plant Cell* **14,** 3201–3211.

Jones, A. M., and Dangl, J. L. (1996). Logjam at the Styx: Programmed cell death in plants. *Trends Plant Sci.* **1,** 114–119.

Kawai, M., Pan, L., Reed, J. C., and Uchimiya, H. (1999). Evolutionally conserved plant homologue of the Bax Inhibitor-1 (BI-1) gene capable of suppressing Bax-induced cell death in yeast. *Febs Letters* **464,** 143–147.

Kawai, M., Samarajeewa, P. K., Barrero, R. A., Nishiguchi, M., and Uchimiya, H. (1998). Cellular dissection of the degradation pattern of cortical cell death during aerenchyma formation of rice roots. *Planta* **204,** 277–287.

Kelleher, D. J., and Gilmore, R. (1997). DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase. *Proc. Natl. Acad. Sci. USA* **94,** 4994–4999.

Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001). Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Reports* **2,** 330–335.

Kobayashi, H., and Fukuda, H. (1994). Involvement of calmodulin and calmodulin-binding proteins in the differentiation of tracheary elements in *Zinnia* cells. *Planta* **194,** 388–394.

Koltunow, A. M., Truettner, J., Cox, K. H., Wallroth, M., and Goldberg, R. B. (1990). Different temporal and spatial gene-expression patterns occur during anther development. *Plant Cell* **2,** 1201–1224.

Krishnamurthy, K. V. (2000). The programme of cell death in plants and animals—A comparison. *Curr. Sci.* **79,** 1169–1181.

Ku, S., and Hyejin Yoon Hak Soo Suh Chung, Y.-Y. (2003). Male-sterility of thermosensitive genic male-sterile rice is associated with premature programmed cell death of the tapetum. *Planta* **217,** 559–565.

Kuo, A. L., Cappelluti, S., Cervantes, M., Rodriguez, M., and Bush, D. S. (1996). Okadaic acid, a protein phosphatase inhibitor, blocks calcium changes, gene expression, and cell death induced by gibberellin in wheat aleurone cells. *Plant Cell* **8,** 259–269.

Kuriyama, H. (1999). Loss of tonoplast integrity programmed in tracheary element differentiation. *Plant Physiol.* **121,** 763–774.

Lam, E. (2004). Controlled cell death, plant survival and development. *Nat. Rev. Mol. Cell. Biol.* **5,** 305–315.

Lam, E., and del Pozo, O. (2000). Caspase-like protease involvement in the control of plant cell death. *Plant Mol. Biol.* **44,** 417–428.

Langston, B. J., Bai, S., and Jones, M. L. (2005). Increases in DNA fragmentation and induction of a senescence-specific nuclease are delayed during corolla senescence in ethylene-insensitive (etr1-1) transgenic petunias. *J. Exp. Bot.* **56,** 15–23.

Laporte, M., Harrison-Lowe, N., Yerram, M., and Olsen, L. (2004). Autophagy in plants: Identification of putative homologs in *Arabidopsis* and rice, expression of autophagy genes in response to nutrient stress, and molecular characterization of AtATG6. Abstract # 17002, Minisymposium on Protein turnover, American Society of Plant Biologists, http://abstracts.aspb.org/pb2004/public/Mo7/9062.html.

Lee, R. H., and Chen, S. C. G. (2002). Programmed cell death during rice leaf senescence is nonapoptotic. *New Phytol.* **155,** 25–32.

Lee, S., Jung, K.-H., An, G., and Chung, Y.-Y. (2004). Isolation and characterization of a rice cysteine protease gene, *OsCP1*, using T-DNA gene-trap system. *Plant Mol. Biol.* **54,** 755–765.

Lesniewska, J., Simeonova, E., and Charzynska, M. (2004). Subcellular heterogeneity of mitochondrial membrane potential in anther tapetum. *Sex. Plant Reprod.* **16,** 283–288.

Leverentz, M. K., Wagstaff, C., Rogers, H. J., Stead, A. D., Chanasut, U., Silkowski, H., Thomas, B., Weichert, H., Feussner, I., and Griffiths, G. (2002). Characterization of a novel lipoxygenase-independent senescence mechanism in *Alstroemeria peruviana* floral tissue. *Plant Physiol.* **130,** 273–283.

Levings, C. S., III (1993). Thoughts on cytoplasmic male sterility in *cms-T* maize. *Plant Cell* **5,** 1285–1290.

Lim, P. O., Woo, H. R., and Nam, H. G. (2003). Molecular genetics of leaf senescence in *Arabidopsis*. *Trends Plant Sci.* **8,** 272–278.

Matile, P., and Winkenbach, F. (1971). Function of lysosomes and lysosomal enzymes in the senescing corolla of the morning glory. *J. Exp. Botany* **22,** 759–771.

Melville, R., and Wrigley, F. A. (1969). Fenestration in the leaves of *Monstera* and its bearing on the morphogenesis and colour patterns of leaves. *Bot. J. Linn. Soc.* **62,** 1–16.

Milioni, D., Sado, P. E., Stacey, N. J., Roberts, K., and McCann, M. C. (2002). Early gene expression associated with the commitment and differentiation of a plant tracheary element is revealed by cDNA-amplified fragment length polymorphism analysis. *Plant Cell* **14,** 2813–2824.

Minami, A., and Fukuda, H. (1995). Transient and specific expression of a cysteine endopeptidase during autolysis in differentiating tracheary elements from *Zinnia* mesophyll cells. *Plant Cell Physiol.* **36,** 1599–1606.

Mittler, R., and Lam, E. (1995). *In situ* detection of nDNA fragmentation during the differentiation of tracheary elements in higher plants. *Plant Physiol.* **108,** 489–493.

Møller, S. G., and McPherson, M. J. (1998). Developmental expression and biochemical analysis of the *Arabidopsis atao1* gene encoding an H_2O_2-generating diamine oxidase. *Plant J.* **13,** 781–791.

O'Neill, S. D. (1997). Pollination regulation of flower development. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 547–574.

Obara, K., Kuriyama, H., and Fukuda, H. (2001). Direct evidence of active and rapid nuclear degradation triggered by vacuole rupture during programmed cell death in zinnia. *Plant Physiol.* **125,** 615–626.

Ogier-Denis, E., Pattingre, S., El Benna, J., and Codogno, P. (2000). Erk1/2-dependent phosphorylation of Ga-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. *J. Biol. Chem.* **275,** 39090–39095.

Orzàez, D., and Granell, A. (1997). The plant homologue of the defender against apoptotic death gene is down-regulated during senescence of flower petals. *FEBS Letters* **404,** 275–278.

Panavas, T., and Rubinstein, B. (1998). Oxidative events during programmed cell death of daylily (Hemerocallis hybrid) petals. *Plant Sci.* **133,** 125–138.

Panavas, T., Pikula, A., Reid, P. D., Rubinstein, B., and Walker, E. L. (1999). Identification of senescence-associated genes from daylily petals. *Plant Mol. Biol.* **40,** 237–248.

Papini, A., Mosti, S., and Brighigna, L. (1999). Programmed-cell death events during tapetum development of angiosperms. *Protoplasma* **207,** 213–221.

Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., Mac Donald, H. R., Mannherz, H. G., and Tschopp, J. (1993). Characterization of the endogenous deoxyribonuclease involved in nuclear-DNA degradation during apoptosis (programmed cell-death). *EMBO J.* **12,** 371–377.

Pennell, R. I., and Lamb, C. (1997). Programmed cell death in plants. *Plant Cell* **9,** 1157–1168.

Petiot, A., Ogier-Denis, E., Blommaart, E. F., Meijer, A. J., and Codogno, P. (2000). Distinct classes of phosphatidylinositol 3Ä-kinases are involved in signalling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.* **275,** 992–998.

Phillips, H. L., Jr., and Kende, H. (1980). Structural changes in flowers of *Ipomea tricolor* during flower opening and closing. *Protoplasma* **102,** 199–215.

Porat, R., Borochov, A., and Halevy, A. H. (1994). Pollination-induced senescence in phalaenopsis petals-relationship of ethylene sensitivity to activity of GTP-binding proteins and protein-phosphorylation. *Physiol. Plant.* **90,** 679–684.

Rieu, I., Wolters-Arts, M., Derksen, J., and Mariani, C. (2003). Ethylene regulates the timing of anther dehiscence in tobacco. *Planta* **217,** 131–137.

Ritchie, S., and Gilroy, S. (1998). Calcium-dependent protein phosphorylation may mediate the gibberellic acid response in barley aleurone. *Plant Physiol.* **116,** 765–776.

Roberts, A. W., and Haigler, C. H. (1989). Rise in chlortetracycline accompanies tracheary element differentiation in suspension cultures of *Zinnia*. *Protoplasma* **152,** 37–45.

Roberts, A. W., and Haigler, C. H. (1990). Tracheary-element differentiation in suspension-cultured cells of *Zinnia* requires uptake of extracellular calcium ion: Experiments with calcium-channel blockers and calmodulin inhibitors. *Planta* **180,** 502–509.

Rubinstein, B. (2000). Regulation of cell death in flower petals. *Plant Mol. Biol.* **44,** 303–318.

Runeberg-Roos, P., and Saarma, M. (1998). Phytepsin, a barley vacuolar aspartic proteinase, is highly expressed during autolysis of developing tracheary elements and sieve cells. *Plant J.* **15,** 139–145.

Salvesen, G. S., and Dixit, V. M. (1999). Caspase activation: The induced-proximity model. *Proc. Natl Acad. Sci. USA* **96,** 10964–10967.

Sanders, P. M., Lee, P. Y., Biesgen, C., Boone, J. D., Beals, T. P., Weiler, E. W., and Goldberg, R. B. (2000). The *Arabidopsis DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. *Plant Cell* **12,** 1041–1061.

Sarkkinen, P., Kalkkinen, N., Tilgmann, C., Siuro, J., Kervinen, J., and Mikola, L. (1992). Aspartic proteinase from barley grains is related to mammalian lysosomal cathepsin-D. *Planta* **186,** 317–323.

Schmid, M., Simpson, D., and Gietl, C. (1999). Programmed cell death in castor bean endosperm is associated with the accumulation and release of a cysteine endopeptidase from ricinosomes. *PNAS* **96,** 14159–14164.

Schmid, M., Simpson, D., Kalousek, F., and Gietl, C. (1998). A cysteine endopeptidase with a C-terminal KDEL motif isolated from castor bean endosperm is a marker enzyme for the ricinosome, a putative lytic compartment. *Planta* **206,** 466–475.

Schmid, M., Simpson, D. J., Sarioglu, H., Lottspeich, F., and Gietl, C. (2001). The ricinosomes of senescing plant tissue bud from the endoplasmic reticulum. *PNAS* **98,** 5353–5358.

Schnable, P. S., and Wise, R. P. (1998). The molecular basis of cytoplasmic male sterility and fertility restoration. *Trends Plant Sci.* **3,** 175–180.

Schwartz, B. W., Yeung, E. C., and Meinke, D. W. (1994). Disruption of morphogenesis and transformation of the suspensor in abnormal suspensor mutants of *Arabidopsis*. *Development* **120,** 3235–3245.

Simeonova, E., Garstka, M., Koziol-Lipinska, J., and Mostowska, A. (2004). Monitoring the mitochondrial transmembrane potential with the JC-1 fluorochrome in programmed cell death during mesophyll leaf senescence. *Protoplasma* **223,** 143–153.

Simeonova, E., Sikora, A., Charzynska, M., and Mostowska, A. (2000). Aspects of programmed cell death during leaf senescence of mono- and dicotyledonous plants. *Protoplasma* **214,** 93–101.

Smertenko, A. P., Bozhkov, P. V., Filonova, L. H., von Arnold, S., and Hussey, P. J. (2003). Re-organisation of the cytoskeleton during developmental programmed cell death in *Picea abies* embryos. *Plant J.* **33,** 813–824.

Smith, M. T., Saks, Y., and van Staden, J. (1992). Ultrastructural changes in the petals of senescing flowers of *Dianthus caryophyllus*. *Ann. Bot.* **69,** 277–285.

Smith, S. (2001). The world according to PARP. *Trends Biochem. Sci.* **26,** 174–179.

Stacy, R. A. P., Munthe, E., Steinum, T., Sharma, B., and Aalen, R. B. (1996). A peroxiredoxin antioxidant is encoded by dormancy related gene, *Per1*, expressed during late development in the aleurone and embryo of barley grains. *Plant Mol. Biol.* **31,** 1205–1216.

Stead, A. D., and van Doorn, W. G. (1994). Strategies of flower senescence—a review. *In* "Molecular and Cellular Aspects of Plant Reproduction" (R. J. Scott and A. D. Stead, Eds.), pp. 215–328. Cambridge University Press, Cambridge, UK.

Stephenson, P., and Rubinstein, B. (1998). Characterization of proteolytic activity during senescence in daylilies. *Physiol. Plant.* **104,** 463–473.

Stromhaug, P. E., and Klionsky, D. J. (2001). Approaching the molecular mechanism of autophagy. *Traffic* **2,** 524–531.

Sugiyama, M., Ito, J., Aoyagi, S., and Fukuda, H. (2000). Endonucleases. *Plant Mol. Biol.* **44,** 387–397.

Swanson, S. J., and Jones, R. L. (1996). Gibberellic acid induces vacuolar acidification in barley aleurone. *Plant Cell* **8,** 2211–2221.

Than, M. E., Helm, M., Simpson, D. J., Lottspeich, F., Huber, R., and Giet, C. (2004). The 2.0 A° crystal structure and substrate specificity of the KDEL-tailed cysteine endopeptidase functioning in programmed cell death of *Ricinus communis* endosperm. *J. Mol. Biol.* **336,** 1103–1116.

Thelen, M. P., and Northcote, D. H. (1989). Identification and purification of a nuclease from *Zinnia elegans* L.: A potential molecular marker for xylogenesis. *Planta* **179,** 181–195.

Thomas, H., Ougham, H. J., Wagstaff, C., and Stead, A. D. (2003). Defining senescence and death. *J. Exp. Bot.* **54,** 1127–1132.

Thomas, S. G., and Franklin-Tong, V. E. (2004). Self-incompatibility triggers programmed cell death in *Papaver* pollen. *Nature* **429,** 305–309.

van der Weele, C. M., Canny, M. J., and McCully, M. E. (1996). Water in aerenchyma spaces in roots. A fast diffusion path for solutes. *Plant Soil* **184,** 131–141.

van Doorn, W. G., and Stead, A. D. (1997). Abscission of flowers and floral parts. *J. Exp. Bot.* **48,** 821–837.

van Doorn, W. G., and Woltering, E. J. (2004). Senescence and programmed cell death: Substance or semantics? *J. Exp. Bot.* **55,** 2147–2153.

Wagstaff, C., Leverentz, M. K., Griffiths, G., Thomas, B., Chanasut, U., Stead, A. D., and Rogers, H. J. (2002). Protein degradation during senescence of *Alstroemeria* petals. *J. Exp. Bot.* **53,** 233–240.

Wagstaff, C., Malcolm, P., Rafiq, A., Leverentz, M., Griffiths, G., Thomas, B., Stead, A., and Rogers, H. (2003). Programmed Cell Death (PCD) processes begin extremely early in *Alstroemeria* petal senescence. *New Phytol.* **160,** 49–59.

Wang, M., Hoekstra, S., Van Bergen, S., Lamers, G. E., Oppedijk, B. J., van der Heijden, M. W., de Priester, W., and Schilperoort, R. A. (1999). Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. *Plant Mol. Biol.* **39,** 489–501.

Wang, M., Oppedijk, B. J., Lu, X., Van Duijn, B., and Schilperoort, R. A. (1996). Apoptosis in barley aleurone during germination and its inhibition by abscisic acid. *Plant Mol. Biol.* **32,** 1125–1134.

Watanabe, N., and Lam, E. (2005). Two *Arabidopsis* metacaspases AtMCP1b and AtMCP2b are arginine/lysine-specific cysteine proteases and activate apoptosis-like cell death in yeast. *J. Biol. Chem.* **280,** 14691–14699.

Webb, J., and Jackson, M. B. (1986). A transmission and cryo-scanning electron microscopy study of the formation of aerenchyma (cortical gas filled space) in adventitious roots of rice. *J. Exp. Bot.* **37,** 832–841.

Winkler, R. G., and Helentjaris, T. (1995). The maize dwarf3 gene encodes a cytochrome p450-mediated early step in gibberellin biosynthesis. *Plant Cell* **7,** 1307–1317.

Wredle, U., Walles, B., and Hakman, I. (2001). DNA fragmentation and nuclear degradation during programmed cell death in the suspensor and endosperm of *Vicia faba*. *Intl. J. Plant Sci.* **162,** 1053–1063.

Wu, H.-M., and Cheung, A. Y. (2000). Programmed cell death in plant reproduction. *Plant Mol. Biol.* **44,** 267–281.

Xu, F. X., and Chye, M. L. (1999). Expression of cysteine proteinase during developmental events associated with programmed cell death in brinjal. *Plant J.* **17,** 321–327.

Yamamoto, R., Demura, T., and Fukuda, H. (1997). Brassinosteroids induce entry into the final stage of tracheary element differentiation in cultured *Zinnia* cells. *Plant Cell Physiol.* **38,** 980–983.

Yamamoto, R., Fujioka, S., Demura, T., Takatsuto, S., Yoshida, S., and Fukuda, H. (2001). Brassinosteroid levels increase drastically prior to morphogenesis of tracheary elements. *Plant Physiol.* **125,** 556–563.

Ye, Z.-H., and Droste, D. L. (1996). Isolation and characterization of cDNAs encoding xylogenesis-associated and wound-induced ribonucleases in *Zinnia elegans*. *Plant Mol. Biol.* **30,** 697–709.

Ye, Z.-H., and Varner, J. E. (1996). Induction of cysteine and serine proteases during xylogenesis in *Zinnia elegans*. *Plant Mol. Biol.* **30,** 1233–1246.

Yen, C. H., and Yang, C. H. (1998). Evidence for programmed cell death during leaf senescence in plants. *Plant Cell Physiol.* **39,** 922–927.

Yeung, E. C., and Meinke, D. W. (1993). Embryogenesis in angiosperms—development of the suspensor. *Plant Cell* **5,** 1371–1381.

Young, T. E., and Gallie, D. R. (2000a). Programmed cell death during endosperm development. *Plant Mol. Biol.* **44,** 283–301.

Young, T. E., and Gallie, D. R. (2000b). Regulation of programmed cell death in maize endosperm by abscisic acid. *Plant Mol. Biol.* **42,** 397–414.

Young, T. E., Gallie, D. R., and DeMason, D. A. (1997). Ethylene-mediated programmed cell death during maize endosperm development of wild-type and *shrunken2* genotypes. *Plant Physiol.* **115,** 737–751.

Yuan, J., and Horvitz, H. R. (1990). The *Caenorhabditis elegans* genes *ced-3* and *ced-4* act cell autonomously to cause programmed cell death. *Dev. Biol.* **138,** 33–41.

Yuan, X.-M., Li, W., Dalen, H., Lotem, J., Kama, R., Sachs, L., and Brunk, U. T. (2002). Lysosomal destabilization in p53-induced apoptosis. *Proc. Natl. Acad. Sci. USA* **99,** 6286–6291.

Zhu, T., and Rost, T. L. (2000). Directional cell-to-cell communication in the *Arabidopsis* root apical meristem III. Plasmodesmata turnover and apoptosis in meristem and root cap cells during four weeks after germination. *Protoplasma* **213,** 99–107.