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Review

Programmed cell death during plant growth and development

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Abstract

This review describes programmed cell death as it signifies the terminal differentiation of cells in anthers, xylem, the suspensor and senescing leaves and petals. Also described are cell suicide programs initiated by stress (e.g., hypoxia-induced aerenchyma formation) and those that depend on communication between neighboring cells, as observed for incompatible pollen tubes, the suspensor and synergids in some species. Although certain elements of apoptosis are detectable during some plant programmed cell death processes, the participation of autolytic and perhaps autophagic mechanisms of cell killing during aerenchyma formation, tracheary element differentiation, suspensor degeneration and senescence support the conclusion that nonapoptotic programmed cell death pathways are essential to normal plant growth and development. Heterophagic elimination of dead cells, a prominent feature of animal apoptosis, is not evident in plants. Rather autolysis and autophagy appear to govern the elimination of cells during plant cell suicide.

Keywords: programmed cell death, plants, apoptosis, protease

Abbreviations: pcd, programmed cell death; GA, gibberellic acid; PARP, poly(ADP-ribose) polymerase

Introduction

In plants, as in animals, programmed cell death (pcd) results when cell suicide pathways are activated as part of normal growth and development. Cell suicide pathways may also be activated in response to certain biotic and abiotic external factors. In animals, the ultrastructural changes occurring during apoptosis, a particular type of pcd, allow pcd to be distinguished from necrosis. Necrosis results from severe injury and does not appear to rely on cell suicide programs. This review examines pcd during sex determination, anther dehiscence, pollen tube growth, fertilization, embryo suspensor growth, seed germination, aerenchyma formation, tracheary element and sclereid differentiation, sieve element differentiation, and leaf and flower petal senescence. Due to the broad range of topics covered, it was not possible to present in this space comprehensive reviews of each subject. Rather, to examine whether apoptosis is a universal pathway to pcd in plants, I have reviewed reports describing morphology of cells undergoing pcd during the processes listed above. Information concerning potential biochemical and molecular markers for plant pcd is also reviewed, with special attention to possible roles for proteases, since research in this area has established proteases as important mediators of pcd in animals.

There is continuing interest in whether apoptosis occurs in plants. Electron microscopy has revealed that during apoptosis in animals, chromatin condenses and segregates into sharply delineated masses positioned at the nuclear envelope (Ellis *et al*, 1991; Kerr and Harmon, 1991). Condensed chromatin is electron dense and is often described as pycnotic. The cytoplasm also condenses and both the nuclear and cellular outlines appear lobed. Over the next few minutes, nuclear fragmentation occurs and deeply lobed convolutions of the cell surface are evident. The protruding lobes then separate and produce membrane-bound apoptotic bodies containing intact organelles. Apoptotic bodies are phagocytosed by adjacent cells. At the biochemical level, apoptosis results in fragmentation of nuclear DNA, probably catalyzed by a calcium-dependent nuclease (Wyllie *et al*, 1980), yielding a ladder of DNA on agarose gels (Wyllie, 1980). Based on the above characteristics, it appears that apoptosis occurs in mycotoxin-treated plant protoplasts (see Gilchrist, 1997, this issue) and during plant pathogen-induced cell death (Ryerson and Heath, 1996; and see Morel and Dangl, 1997, this issue). It is concluded, however, that apoptosis is not a universal pathway to plant pcd, and that instead, many plant cell suicide programs depend on autophagic and autolytic mechanisms for cell killing. Plant cell death was recently reviewed by Greenberg (1996) and Jones and Dangl (1996) and other relevant reviews are indicated below.

Death during plant reproduction

The events that comprise plant sexual reproduction have been examined at the genetic, physiological, ultrastructural, biochemical, and molecular levels. From these studies have emerged examples of pcd as a necessary component of sex determination, gamete development, fertilization and embryogenesis. There is increasing interest in the possible utility of model systems developed for the study of plant reproduction as model systems for the study of plant pcd. In this regard, characterization of sex determination mutants and embryo-defective mutants as well as *in vitro* experiments with isolated

embryo sacs may soon begin to identify the components of plant pcd pathways.

Sex determination

Although most flowering plants bear flowers that contain both male and female sex organs (perfect flowers), unisexuality in asparagus (Bracale *et al*, 1991), maize (DeLong *et al*, 1993) and white campion (Grant *et al*, 1994), results from the programmed death of preformed sex organs (recently reviewed by Dellaporta and Calderon-Urrea, 1993). In maize, pcd in both male and female sex organs is characterized by increased vacuolation and loss of organelles (Cheng *et al*, 1983). Outwardly, cells of degenerating organs in maize and white campion (Grant *et al*, 1994) appear sunken, indicating that tonoplast rupture may be a final event in the sex organ abortion process. Vascular development in asparagus is incomplete in female organs of male flowers and in male organs of female flowers (Bracale *et al*, 1991). Whether the lack of vascular development promotes cell death in these organs via the withholding of phytohormones or other signaling molecules, or represents a secondary effect of cell death is not known. The presence of mature vascular tissue in the degraded stamen rudiment in female flowers of white campion (Grant *et al*, 1994), however, argues against vascular discontinuity as a necessary inducer of cell death.

The characterization of two recently cloned genes necessary for female sex organ abortion, Ts2 (DeLong *et al*, 1993) and Gsf1 (Li *et al*, 1997), of maize and *Tripsacum*, respectively, may eventually reveal the biochemistry responsible for the effects of exogenously applied feminizing phytohormones, such as GA (Hansen *et al*, 1976). The predicted product of these genes is similar to hydroxysteroid dehydrogenases, and may catalyze conversion of a GA-like precursor to a pcd promoter that acts either indirectly by regulating as yet unidentified genes, or directly as a cytotoxic molecule (DeLong *et al*, 1993).

Anther dehiscence

The dehiscence program that leads to the rupture of anthers and release of pollen represents a dramatic example of the coordinated death of several different cell types in a single organ (reviewed by Goldberg *et al*, 1993) and is associated with increased expression of a cysteine protease (Koltunow *et al*, 1990). Anther rupture occurs at the stomium after degeneration of the tissue located between the stomium and the connective tissue of the pollen sacs. In addition to death of the cluster of cells directly beneath the stomium, protoplast degeneration is the fate of four other cell types in tomato anthers: the tapetum, epidermal cells adjacent to the stomium, the endothecium and the connective (Bonner and Dickinson, 1989). Some evidence for the involvement of hydrolytic enzymes in anther pcd has been presented. Cellulase protein level increases as anthers mature (del Campillo and Lewis, 1992). The TA56 thiol proteinase mRNA accumulates within the circular cluster of cells between the stomium and the connective (Koltunow *et al*, 1990). This is paralleled by an increase in ubiquitin and/or ubiquitinated protein levels in the same tissue (Li *et al*, 1995). Following degeneration of the circular cluster of cells, TA56 mRNA increases in both the stomium and connective (Koltunow *et al*, 1990). Hence proteolytic pathways involving both thiol proteases and perhaps ubiquitin conjugation appear to be active just before degeneration of specific cell types in anthers, suggesting a role for multiple proteolytic systems during anther cell death. The ubiquitin-dependent pathway of proteolysis is induced during pcd in degenerating insect muscles (Haas *et al*, 1995).

Death of incompatible pollen

Pollen released from the anther as a result of the dehiscence program will hydrate and germinate producing a pollen tube when it arrives on the epidermis of the cells of the stigma; but in some cases, death of the pollen tube may soon follow. The stigma is connected to the ovary by the transmitting tissue that passes through the style (Esau, 1965). The transmitting tissue facilitates the growth of the pollen tube through the style. One of the roles of the stigma and style is to discriminate between appropriate and inappropriate pollen grains, resulting in the prevention of the growth of interspecific and in some cases intraspecific pollen. The prevention of self-pollination, a condition known as self-incompatibility has evolved presumably to prevent the deleterious effects of inbreeding (recently reviewed by Newbigin *et al*, 1993; Nasrallah *et al*, 1994; Dodds *et al*, 1996). Both sporophytic (determined by the diploid genotype of the parent plant) and gametophytic (determined by the haploid genotype of the pollen grain) mechanisms have been described for the control of the self-incompatibility phenotype.

Self-incompatibility in members of the Solanaceae (potato and tobacco), Rosaceae (roses and many tree fruits) and Scrophulariaceae (snapdragon) is of the gametophytic type and is controlled by a highly polymorphic locus called the S-locus. In the Solanaceae, when a pollen tube is growing through a style that has the same S-alleles as the pollen tube, its growth rate slows and death soon follows. The genes known to be encoded by the S-locus in the Solanaceae are an allelic series of stylar ribonucleases known as S-RNases, and activity of S-RNases as allele-specific cytotoxins is thought to be essential for the expression of self-incompatibility (Dodds *et al*, 1996). *In vitro* assays indicate that S-RNases non-specifically degrade RNA (McClure *et al*, 1990). *In vivo*, however, during growth in *Nicotiana alata* styles, only the rRNA of incompatible and not that of compatible pollen tubes is degraded. Two models have been proposed to explain how stylar S-RNase gains specific access to RNA of incompatible pollen (Dodds *et al*, 1996; McCubbin *et al*, 1997). In the first model, S-RNases are excluded from the pollen tube but the S-locus product in pollen recognizes the corresponding S-RNase present in the extracellular matrix of the stylar transmitting tissue and allows it to enter the incompatible pollen tube. The second model proposes that S-RNase enters all pollen tubes but is inactivated or sequestered from the substrate in compatible pollen tubes. The S-locus product in incompatible pollen tubes would, in this model, prevent its corresponding S-RNase from being inactivated or sequestered. The end result

according to either model would be the non-autonomous inhibition of protein synthesis leading to the observed arrest of cell growth (Herrero and Dickinson, 1981), and ultimately to death. Although RNase activity is one of several hydrolytic markers for autolysis during tracheary element differentiation (Ye and Droste, 1996) and for leaf (Taylor *et al*, 1993) and petal (Matile and Winkenbach, 1971) senescence, stylar S-RNase appears to be the sole lytic agent necessary (Dodds *et al*, 1996, and references therein) for a process that may be initiated by the action of a single cell death promoter – the as yet unidentified pollen S-locus product.

Degeneration of haploid megaspores

Upon arrival at the ovary, pollen tubes must enter the embryo sac for successful fertilization. In the majority of angiosperms the single embryo sac develops from a surviving haploid megaspore: three of the four megaspores formed by meiosis having degenerated. In the fern *Marsilea*, megaspore death involves chromatin pycnosis (Bell, 1996). The presence of pycnotic chromatin led Bell (1996) to propose that *Marsilea* megaspore death was apoptotic. Chromatin pycnosis is a morphological marker for both apoptotic and nonapoptotic pcd (Schwartz, 1992). Pycnotic chromatin in apoptotic nuclei is typically appressed to the inner surface of the nuclear membrane in large electron dense masses with sharply delineated margins. In contrast, pycnotic chromatin in nonapoptotic nuclei may be present throughout the nucleus as smaller electron dense bodies with diffuse edges. The above distinction between the morphologies of pycnotic chromatin in nuclei of apoptotic *versus* nonapoptotic dying cells (Schwartz, 1992) indicates that reports of pycnotic nuclei not accompanied by further descriptions of chromatin as well as other aspects of nuclear morphology are probably insufficient for determining pcd type. With regard to megaspore death in angiosperms, investigations of events at the ultrastructural, biochemical and molecular levels may reveal new details of pcd in economically important seed plants.

Death of synergids

Programmed cell death also occurs within the embryo sac during fertilization. Usually the pollen tube enters the ovule through the micropyle, a narrow opening in the ovular tissue. Located at the micropylar pole of the embryo sac are the egg cell and usually two synergids, although synergids are absent from some species (Willemse and Van Went, 1984). The pollen tube penetrates one of the synergids, which then degenerates (Willemse and Van Went, 1984). It is possible that synergid degeneration after pollen tube penetration is a necrotic response to injury. However, that synergid degeneration may also occur in the absence of pollination (recently reviewed by Russell and Dumas, 1992), indicates that, at least in some species, synergids are programmed for death (Van Went and Cresti, 1988; Sumner, 1992). Once inside the synergid, pollen tube growth ceases and an opening forms in the tube wall (Willemse and Van Went, 1984), through which the pollen tube cytoplasm and two sperm cells are discharged. One sperm cell is transferred to the egg for zygote formation and the other is transferred to the central cell for endosperm formation.

Synergid death has been extensively characterized in tobacco, pearl millet and cotton. Degeneration of the synergid membranes nearest the micropylar region is detectable when the pollen tube tip arrives at the receptive synergid in *Nicotiana tabacum* (Huang and Russell, 1994), implying that in this species initiation of the synergid death program may depend on signals from the pollen tube. Complete degeneration, however, does not occur until after penetration by the pollen tube when the synergid vacuole collapses and the pollen tube releases its contents into the synergid. Ultimately, even plasma membrane integrity is lost (Russell and Dumas, 1992). At this point cytoplasm may leak from the degenerated synergid and pass between other cells of the embryo sac. Culturing unfertilized cotton ovules in the presence of the phytohormones GA and IAA promotes synergid degeneration, with an increase in cytoplasmic density and vacuolar collapse representing early events in synergid death (Jensen *et al*, 1997). The roles of phytohormones in synergid degeneration in *planta* are not known.

Several reports have described dynamic changes in calcium distribution prior to and during synergid degeneration. In *Nicotiana tabacum*, the final stage of maturation of the embyro sac is concomitant with the accumulation of calcium in the nucellar tissue surrounding the micropylar canal (Tian and Russel, 1997). This extracellular calcium may serve as a chemotropic attractant to the pollen tube (Reger *et al*, 1992) and may ultimately enter the synergid increasing its intracellular calcium content. After examining synergid degeneration in pearl millet, which occurs in the absence of pollination, Chaubal and Reger (1994) proposed that calcium moving from the apoplast of nucellar tissue surrounding the embryo sac enters the endoplasmic reticulum and numerous small vacuoles present in the synergids. When vacuoles are saturated with calcium, calcium is diverted, perhaps via the endoplasmic reticulum, to the nucleus, which then loses its nucleolus and degenerates. These observations implicate calcium influx as an effector or synergid death, perhaps via the activation of proteases, endonucleases, or signal transduction pathways as occurs during apoptosis in certain animal systems (McConkey and Orrenius, 1995). Isolated embryo sacs (Huang *et al*, 1992) and ovules (Jensen *et al*, 1977) may represent useful systems for pharmacological studies aimed at exploring the roles of calcium, and other factors, in the death of synergids.

Degeneration of the suspensor

The developing embryo may play a role in pcd of the neighboring suspensor. After fertilization, the zygote in flowering plants usually divides to form a basal cell and a terminal cell. The embryo develops from the terminal cell, while the basal cell undergoes a few relatively rapid divisions to form the suspensor. The suspensor plays an active role in the early promotion of the growth of the embryo proper (reviewed by Schwartz *et al*, 1997). During late stages of

embryo growth, however, the suspensor degenerates. The degenerating cells of *Phaseolus* and *Tropaeolum* suspensors contain numerous and varied vesicles surrounded by 2, 4 or even 8 membranes (Nagl, 1976). These appear to be autophagic vesicles where the included cytoplasm exhibits degenerative changes. Later during autolysis, large autolytic vacuoles form and membrane whorls appear to be released into these vacuoles. Eventually the vacuole lyses and the protoplast becomes disorganized. Nuclei, along with plastids, appear most resistant to degradation, being destroyed only after the tonoplast ruptures (Gartner and Nagl, 1990). The cell morphology described for degenerating suspensors is consistent with autophagic cell death, indicating that the chromatin pycnosis observed by Nagl (1976) is probably part of a nonapoptotic pathway. In *Phaseolus* and *Tropaeolum* suspensors, the progression of autolysis exhibits polarity, beginning in cells at the basal end of the organ and proceeding toward the embryo (Nagl, 1976). Acid phosphatase (Nagl, 1977; Singh *et al*, 1980) and β-glucosidase (Singh *et al*, 1980) activities increase during suspensor autolysis. It has been proposed that the lysed material may be mobilized for use by the embryo (Nagl, 1976).

Arrested embryo growth or destruction of embryos often results in abnormally vigorous growth of suspensors (Yeung and Meinke, 1993, and references therein), including, in the embryo-defective mutant of *Arabidopsis*, *twin*, the growth of a second embryo from transformed cells within the suspensor (Vernon and Meinke, 1994). *Twin* and other embryo-defective suspensor mutants delay their autolysis program and replace it with a more embryo-like program (Yeung and Meinke, 1993). Hence the presence of a normally developing embryo proper apparently restricts growth of the suspensor and may provide the signal(s) for the induction of the cell death pathway in suspensors. Further analysis of embryo-defective mutants may provide clues leading to the initiators of pcd in the suspensor.

Death during vegetative growth

From seed germination through maturation and senescence, several examples of pcd, can be found during vegetative growth. One dramatic example is provided by the foliar tissue sculpting that leads to the production of fenestrated leaves in *Monstera* species. The fenestrated morphology results from the death of just a few cells in the developing leaf primordia. These areas then expand along with the growing leaf resulting in a perforated lamina. Although some morphological and physiological aspects of this process have been characterized (Melville and Wrigley, 1968), nothing is known about the molecular biology of this interesting phenomenon. In contrast, more extensive information is available on the biology of cell death programs engaged during germination, vascular differentiation, and stress- and age-dependent death of mature tissues and organs, and these processes are discussed below.

Aleurone death

In some species, seed germination is accompanied by cell death in the aleurone layer (Reid and Meier, 1972; Kuo *et al*, 1996). The aleurone functions during germination by secreting hydrolases for mobilization of stored seed reserves and this aspect of aleurone physiology has been extensively studied (reviewed by Jones and Jacobsen, 1991). Both cysteine and aspartic acid proteinases localize to vacuoles in barley aleurone (Okita and Rogers, 1996, and references therein). Less is known, however, about the aleurone pcd pathway. Cell death in aleurone and endosperm cells during germination of seeds appears to involve autophagic vacuoles (Vigil, 1970; Reid and Meier, 1972), and, in wheat aleurone, is preceded by an increase in cytosolic calcium levels (Kuo *et al*, 1996). It was also recently discovered that death of wheat aleurone cells is prevented by treatment with okadaic acid and hence appears to depend on protein phosphatase activity (Kuo *et al*, 1996). Further probing of the wheat aleurone system with pharmacological agents may identify other components of the aleurone cell death pathway.

Pith autolysis

Under certain conditions the autolysis and disappearance of cortex or pith parenchyma cells leads to the formation of lysigenous aerenchyma, i.e. stems or roots containing large air-filled cavities (Armstrong and Armstrong, 1994). Stem pith autolysis is a widespread phenomenon (Carr *et al*, 1995), that in some species is positively correlated with rapid stem elongation and increasing sink strength of reproductive structures (Carr and Jaffe, 1995). Temperature- (Lu *et al*, 1991), drought- (Aloni and Pressman, 1981), hypoxia- (McPherson, 1939 , as reviewed by Kawase, 1979) and N or P deficiency-induced (He *et al*, 1994) root and stem pith autolysis have also been described.

In roots of maize (He *et al*, 1996) and sunflower (Kawase and Whitmoyer, 1980) and stems of numerous herbaceous plants (Kawase, 1981, and references therein) hypoxia induces aerenchyma formation that is thought to facilitate the diffusion of oxygen from normoxic portions of the plant to hypoxic tissues. In maize (He *et al*, 1996) and sunflower (Kawase, 1981), the requirement for hypoxia can be replaced by treatment with ethylene. He *et al* (1996) found it possible to modify transduction of the ethylene signal using second messenger antagonists. These authors concluded that an increase in intracellular calcium is necessary for cell death. He *et al* (1996) also found that contrary to its effect on aleurone death (see above) okadaic acid promoted cell death.

Cellulase activity appears to be a valid biochemical marker for lysigenous cavity formation (Kawase, 1981; Huberman *et al*, 1993; He *et al*, 1996), and it may act with other cell-degrading enzymes in a coordinated cell death program (He *et al*, 1996). Although the localization and timing of cellulase gene expression and activity relative to dying cells need to be more rigorously determined, the apparent coupling of cellulase activity and autolysis of maize root cells (He *et al*, 1996) is consistent with the observation that application of cellulase to detached stems of numerous plant species induces aerenchyma formation (Kawase, 1981). Polygalacturonase (Huberman *et al*, 1993) and α-amylase (Davis, 1985) activities are also increased during lysigenous cavity formation.

Lu *et al* (1991) and Niki *et al* (1995) have characterized the temperature-sensitive formation of lysigenous cavities in the roots of pea. The pithlike central xylem parenchyma cells of pea roots differentiate normally under the permissive temperature (less than 15°C) to form late maturing metaxylem tracheary elements, but often autolyse to form cavities at nonpermissive temperatures. Cell degeneration in developing pea root vascular cavities is characterized by the following sequence of events: loss of tonoplast, appearance of electron-dense inclusions in the nucleus, swelling and loss of organellar integrity, thinning of the cell wall and plasma membrane breakdown (Niki *et al*, 1995). Protoplast breakdown also precedes the disappearance of the cell wall during drought-induced tomato stem pith autolysis (Aloni and Pressman, 1981). Niki *et al* (1995) suggested that pea vascular cavity formation could be the result of (a) misdirected lysis due to hydrolytic enzymes produced in adjacent autolysing tracheary elements, or (b) the initiation of a premature tracheary element death program. The latter possibility raises the interesting question of whether the tracheary element death program exists as a parallel pathway that can be uncoupled from the normally preceding events of cell wall thickening and lignification (Fukuda, 1997, this issue).

Differentiation of tracheary elements and fiber-sclereids

Over the last 15 years, experimental systems optimized for tracheary element differentiation have received increasing attention as model systems for studying plant pcd. The ontogeny of tracheary elements and fiber-sclereids terminates with the production of a thickened cell wall that is usually lignified. The thickened secondary cell walls of both tracheary elements and fiber-sclereids provide support and flexibility and often lack a protoplast at maturity. In the xylem, tracheary elements are joined end-to-end in long continuous tubes and serve as the water-conducting tissue of the vascular system. The xylem is typically spacially associated with the phloem, the principle food-conducting tissue of vascular plants. A method for studying sclereid development in *Arabidopsis* was recently presented (Lev-Yadun, 1994) and may represent an opportunity for the development of an additional experimental system for the molecular genetics of plant pcd. The death of water-storage cells in the xeromorphic leaves of certain species of *Sanseveria* and *Oncidium* is another example of terminal differentiation resulting in persistent modified cell walls that lack protoplasts (Koller and Rost, 1988). Details of the biochemistry and molecular biology of fiber-sclereid and water-storage cell pcd are not known, although it has been suggested that fiber-sclereids and tracheary elements share genetic pathways leading to their differentiation (Esau, 1965; Savidge, 1983). Experimental systems, most notably the *Zinnia* mesophyll cell culture system (Kohlenbach and Schmidt, 1975; Fukuda and Komamine, 1980), have been developed for *in vitro* induction of tracheary element differentiation from parenchyma cells. The ease of identification of tracheary elements, with their distinctive cell wall thickenings, and the semisynchronous autolysis of cultured differentiating *Zinnia* tracheary elements has made this an especially accessible system for pharmacological studies and for the cloning of tracheary element and pcd cDNAs.

While some indirect evidence for DNA fragmentation during tracheary element pcd has been presented (Mittler *et al*, 1995; Wang *et al*, 1996), the ontogeny of intracellular disorganization does not mirror that of mammalian cell apoptosis (Groover *et al*, 1997). Rather, death of tracheary elements more closely resembles that occurring in cells of senescent leaves, where extensive cellular disorganization is the last event, apparently following tonoplast lysis (Thomson and Platt-Aloia, 1987; Fukuda, 1996; Jones and Dangl, 1996). Whether the release of hydrolytic enzymes following tonoplast rupture is the primary cause or a secondary effect of pcd has not been established (Thompson and Platt-Aloia, 1987; Jones and Dangl, 1996).

Although no genes or proteins controlling the initiation of the autolytic phase of tracheary element differentiation have been identified, hydrolytic enzyme activity increases dramatically late in the differentiation process. Markers for autolysis include an endonuclease (Thelen and Northcote, 1989), an RNAse (Ye and Droste, 1996) and proteases (Minami and Fukuda, 1995; Ye and Varner, 1996; Beers and Freeman, 1997). Activities of both cysteine and serine proteases increase late in the differentiation process. The enzymes have been partially characterized using model substrates *in vitro*, but their *in vivo* substrates have not been identified. Localization of these proteases has not been determined; however, the sequence encoding the prepropeptide for at least one cysteine protease is consistent with vacuolar residence (Ye and Varner, 1996). Cysteine and serine protease inhibitors are known to inhibit apoptosis in several model systems (Kumar and Lavin, 1996). Cysteine protease inhibitors also block tracheary element differentiation if applied before wall thickenings are detected (Fukuda, 1996). The ubiquitin-dependent pathway of proteolysis may also be required for vascular development, as the overexpression in transgenic tobacco of a mutant form of ubiquitin unable to form polyubiquitin chains resulted in aberrant vascular development (Bachmair *et al*, 1990). While these results point to roles for cysteine proteases and perhaps ubiquitin-protein conjugation as mediators of tracheary element differentiation, they do not specifically address the role of proteases in the regulation of plant pcd.

The retention, *in situ*, of protoplasts by some tracheary elements and fiber-sclereids (Esau, 1965) indicates that pcd can be uncoupled from the differentiation process. It is not known, however, whether death is dependent on signals generated during tracheary element differentiation or results from the activity of an independent parallel pathway. Initiation of a premature tracheary element death program (i.e. autolysis in the absence of cell wall modification) has been proposed as one explanation for autolysis of cells occurring in the vascular cavity of pea roots (Niki *et al*, 1995). Conclusive experimental evidence demonstrating that the tracheary element cell death program can be activated independent of cell wall thickening and lignification is lacking, however. It is not known whether the tracheary element cell death program,

or components thereof, are active during other pcd events in plants.

Selective autolysis of sieve elements

Sieve elements are the principle conduit for the movement of assimilates through the phloem and they exhibit a unique selective autolysis during their differentiation (see Sjolund, 1997, for a recent review). Although mature sieve elements retain their protoplast, selective autolysis results in the degradation of the nucleus, the tonoplast, ribosomes, dicytosomes and microtubules. The endoplasmic reticulum persists as do plastids and mitochondria in many species, although the latter two organelle types are usually modified (Esau, 1965). During sieve element differentiation in *Ephedra*, pycnotic degeneration of the nucleus is detectable just prior to the decline of populations of dictyosomes and ribosomes and collapse of the tonoplast (Cresson and Evert, 1994). Chromatin pycnosis was also noted in differentiating sieve elements of *Mimosa* (Esau, 1972) and in wheat (Eleftheriou, 1986). Although electron micrographs prepared from *Ephedra* and *Mimosa* documented nuclear pycnosis similar to that occurring in nonapoptotic pcd (Schwartz, 1992), chromatin pycnosis and nuclear morphology in wheat sieve elements were identical to that reported for apoptotic nuclei (Kerr and Harmon, 1991), including lobing and fragmentation. The envelope of the degrading nucleus in sieve elements is also closely associated with endoplasmic reticulum cisternae (Esau, 1972). It was suggested that the endoplasmic reticulum may serve as a source of acid phosphatase (Oparka *et al*, 1981) involved in autolysis.

Ubiquitin-dependent proteolysis may be important to sieve element differentiation. A cDNA encoding polyubiquitin protein has been cloned from pine phloem (Alosi-Carter *et al*, 1995), and immunoblot analysis has demonstrated the presence of ubiquitin protein in phloem exudate (Schobert *et al*, 1995). *In vivo*, however, ubiquitin may be inactivated by trypsin-like activity catalyzing the removal of the *C*-terminal gly-gly (Wilkinson, 1988). This modification is not detectable by SDS-PAGE or immunoblot analysis and hence the presence of immunologically reactive ubiquitin in sieve elements does not establish pathway activity. The detection of other proteolytic systems active during sieve element differentiation has not been reported. A *Streptanthus tortuosus* tissue culture system developed for the characterization of proteins present in differentiated sieve elements (Wang *et al*, 1995) has led to the identification of β-amylase as a sieve element marker and may prove useful for identifying additional biochemical and molecular markers for this unique selective autolysis.

Abscission

Sexton and Roberts (1982) noted that abscission is sometimes erroneously referred to as a process requiring cell senescence or degeneration. Abscission is a phenomenon affecting leaves, flowers, fruits, branches and bark that results both in the separation of these organs from the parent body without injury to living tissue and in the protection of the newly exposed tissue, via suberization and lignification (Esau, 1965). This shedding of organs is a normal process usually occurring after the mature organ has degenerated (see leaf and flower senescence below) or following fruit ripening. Alternatively, premature abscission may be induced following stress (Williams and Whitham, 1986; Morgan *et al*, 1990). Separation of organs from the parent plant follows dissolution of cell walls in a few layers of specialized cells, known as the abscission zone, usually at the base of the abscising organ (Sexton and Roberts, 1982). Three types of breakdown are listed by Addicott and Lynch (1955): (1) the removal of the middle lamella, (2) removal of the middle lamella and part of the primary cell wall, and (3) separation of entire cells. The enzymes responsible for cell separation include cellulases (Sexton *et al*, 1980; del Campillo *et al*, 1990; Brummell *et al*, 1994) and polygalacturonases (Taylor *et al*, 1990; Kalaitzis *et al*, 1995). In dicotyledonous plants, abscission is accelerated by ethylene and inhibited by auxin (Abeles, 1968) and brassinolide (Iwahori *et al*, 1990). A precise understanding of the role of these and other signaling molecules (Thompson and Osborne, 1994) regulating abscission would have important implications for crop production.

Despite the occurrence of pith autolysis during *Phaseolus* leaf abscission (Webster, 1970), other anatomical and ultrastructural changes occurring in the layer of cells where organ separation occurs are not consistent with pcd directly participating in abscission. On the contrary, abscission zone cells are apparently metabolically active at the time of organ shedding. Abscission zone cells contain more organelles (Goren *et al*, 1984) and exhibit greater secretory activity (Ramina *et al*, 1989) than neighboring cells. Moreover, in the case of *Phaseolus* leaves (Webster, 1970) and citrus fruit (Goren *et al*, 1984), events leading to abscission include an increase in cell division and cell enlargement in the abscission zone. In *Phaseolus*, it is the thin walled, newly dividing cells through which separation takes place, and nuclei in these cells are intact and often still dividing when separation occurs and cell contents are lost (Webster, 1970). Separation in peach leaf abscission zone occurs when digestion of the outer thicker wall, rather than thin walls produced by secondary cell division, leads to cell breakage and the release of cytoplasm (Ramina *et al*, 1989). Intracellular separation occurs after the dissolution of cell wall components in citrus leaf abscission zones, while plasmalemma and tonoplasts of separation layer cells remained intact throughout abscission (Goren *et al*, 1984).

The above observations considered with the fact that the cellulosic and polygalcturonate components of the cell wall are responsible for support and adhesion, respectively, suggest that the action of cell wall lytic enzymes leading to dissolution and/or mechanical cell wall disruption in the separation layer is necessary and sufficient for organ shedding to occur. Degeneration of the cytoplasm in the separation layer does not appear to be necessary and, in fact, may be disadvantageous, as continued synthesis and secretion of lytic enzymes by metabolically active cells are likely to favor organ separation. The competence of stem pith to initiate autolysis following cellulase or ethylene treatment (described above) indicates that pith autolysis observed in the abscission zones of *Phaseolus* may have been a secondary effect induced by external factors, such

as cellulase, originating in the separation layer. Pith autolysis, therefore, may not be necessary for organ shedding in *Phaseolus* (Webster, 1970). That dissolution of cell wall material appears to be sufficient to ensure organ shedding does not exclude the possibility that examination of additional experimental systems may reveal conclusive examples of pcd in the separation layer of abscising organs.

Conflicting results have emerged concerning the involvement of pcd in the abscission of root-cap cells. Abscission of single root-cap cells occurs beneath the soil surface where mucilage-coated cells are lost and replaced at the root-cap meristem. *Phaseolus* root-cap cells contain high levels of hydrolytic enzymes (Thomas *et al*, 1977), as do senescing plant organs and autolysing tracheary elements. Histological evidence that fragmented nuclei are present in root-cap cells has also been presented (Wang *et al*, 1996). Despite these observations supporting pcd of root-cap cells, cultured detached maize root-cap cells remain alive without dividing for up to three months (McCully, 1989, and references therein). It has been suggested that partially separated root-cap cells are involved in transducing signals controlling hydrotropic and thigmotropic responses, and that live separated cells are engaged in regulating bacterial growth in the rhizosphere (McCully, 1989). Perhaps a division of labor exists among root-cap cells requiring death of only a subset of cells, or if separation occurs prematurely or inappropriately.

Senescence

The physiological and biochemical events that comprise age-dependent senescence represent a controlled remobilization of intracellular components for export to plant storage tissues and/or reproductive structures. In leaves, extensive cellular disorganization and cell death occur only after the majority of starch and protein have been remobilized (Wittenbach *et al*, 1982; Thomson and Platt-Aloia, 1987), suggesting that it may be possible to uncouple cell death from the remobilization phase of senescence. The ultrastructure, biochemistry and molecular biology related to remobilization have been extensively characterized. In contrast, relatively little is known about the control of cell death during senescence. Senescence of ripening fruits (see Hadfield and Bennett, 1997, this issue), leaves (see reviews by Thomas and Stoddart, 1980; Kelly and Davies, 1988; Smart, 1994) and flower parts (petal senescence was reviewed by Borochov and Woodson, 1989) is developmentally regulated. Senesence can also be induced by environmental stress or experimentally in both detached and attached organs. As with abscission and pith autolysis, ethylene can accelerate the senescence process in many species. Cytokinins have an effect opposite to that of ethylene and in many species are capable of delaying (Gan and Amasino, 1995) or even reversing senescence (Smart, 1994). Naturally occurring leaf senescence is an orderly, and sometimes slow, process. For example, the near complete loss of chlorophyll from senescing soybean leaves, which begins approximately 4 weeks after flowering, is accomplished over a 6 week period (Wittenbach *et al*, 1980). During soybean leaf senescence, approximately 80% of starch and 90% of soluble protein are lost from the leaves. Leaf breakdown products are rapidly translocated to sink tissues such as fruits, stems, or roots. Senescence of flower petals and detached leaves can proceed more rapidly, terminating in just a few days. Senescence can be inhibited by anaerobic conditions and cycloheximide (Martin and Thimann, 1972; Valpuesta *et al*, 1995); hence, it is an active degradative process which requires *de novo* protein synthesis.

Ultrastructural analyses indicate that chloroplasts and other organelles remain intact throughout senescence (Thomson and Platt-Aloia, 1987). Intact chloroplasts can be isolated from protoplasts prepared from dark-induced senescent wheat leaves from which up to 80% of the chlorophyll and protein have been lost (Wittenbach *et al*, 1982). Wittenbach *et al* (1982) suggested that autophagy of chloroplasts within the intact vacuole may be occurring during soybean leaf senescence. Respiratory activities of the mitochondria and catabolic activities of peroxisomes increase or remain steady during senescence (Smart, 1994) and mitochrondria remain intact. The endoplasmic reticulum appears at least partially labile during senescence, while the nucleus appears to remain intact up to an advanced stage of chlorophyll loss (Thomson and Platt-Aloia, 1987). It is possible to isolate intact vacuoles from senescing leaves (Wittenbach *et al*, 1982). Moreover, that leaves remain turgid until advanced senescence provides indirect evidence for the persistence of an intact tonoplast (Smart, 1994). Thus it appears that the organized disassembly program active in leaves during senescence maintains cellular compartmentation throughout most of senescence, with death occurring semi-synchronously only at the very end of the process (Thomson and Platt-Aloia, 1987). In contrast, petal senescence is characterized by early wilting (Matile and Winkenbach, 1971; Smith *et al*, 1992) and/or a water-soaked appearance, indicating the loss of tonoplast integrity. Ultrastructural changes in senescing carnation petals confirm this apparent cellular disorganization including early rupture of the tonoplast and plasmalemma and dissolution of cell wall components leading to complete loss of cellular contents (Smith *et al*, 1992). Autophagic activity of vacuoles has also been noted in senescing petals (Matile and Winkenbach, 1971).

Hydrolytic enzyme activity increases during senescence, and much attention has been focused on the role of proteases during senescence. Numerous biochemical characterizations of increased proteolytic activity during senescence have been presented (Matile and Winkenbach, 1971; Martin and Thimann, 1972; Drivdahl and Thimann, 1977; Wittenbach *et al*, 1980, 1982; Miller and Huffaker, 1982; and reviewed by Feller, 1986), including evidence for activity of both thiol (Miller and Huffaker, 1982; Drivdhal and Thimann, 1977) and serine proteases (Miller and Huffaker, 1982). Further support for an important role for proteases in leaf senescence has come from the identification of senescence-related genes. Several investigators characterizing cDNA libraries from senescing leaves (Hensel *et al*, 1993; Lohman *et al*, 1994; Smart *et al*, 1995; Drake *et al*, 1996) and flower petals (Jones *et al*,

1995; Valpuesta *et al*, 1995) have identified sequences with homology to cysteine proteases.

In leaves, the vast majority of protease activity detectable with model substrates appears to localize to the large central vacuole (Boller and Kende, 1979; Wittenbach *et al*, 1982; Canut *et al*, 1987); and hence, senescence-related proteases are likely to be vacuolar. Because this acidic compartment exhibits the ability to regulate the degradation of included protein (Canut *et al*, 1986; also reviewed by Staswick, 1994; and by Herman, 1994), it is possible that some mechanism, not yet described, exists for transport of senescence-targeted proteins to the vacuole and their subsequent degradation in that organelle. That plastids contains $>60\%$ of intracellular leaf protein suggests involvement of plastidic proteinases in remobilization of N during senescence (reviewed by Dalling and Nettleton, 1986). Chloroplastic ATP-dependent (Malek *et al*, 1984; Liu and Jagendorf, 1986) and non-ATP-dependent proteases (Bushnell *et al*, 1993) have been detected. However, one candidate for the ATP-dependent activity (the *E. coli* homolog ClpP system) is expressed constitutively and therefore does not appear to be a senescence-related protease (Shanklin *et al*, 1995).

Recent work provides a strong link between the cytosolic/nuclear-localized ubiquitin pathway of proteolysis and leaf senescence. Genschik *et al* (1994) found that senescing tobacco accumulated high levels of mRNA encoding two different ubiquitin carrier proteins (E2s), homologous to *Ubc4At4a* and *Ubc4At4b*, and a polyubiquitin gene. Using a GUS reporter construct, Garbarino and Belknap (1994) followed the expression of the ubiquitin-ribosomal protein fusion gene, *ubi3*, during potato leaf maturation. Activity was highest in meristematic tissues, declined during leaf expansion and rose again to near meristematic levels in senescent leaves. As rRNA content decreases during senescence (Makrides and Goldthwaite, 1981), increased *ubi3* expression during senescence may reflect a need for a higher level of ubiquitin for protein degradation rather than a need for higher levels of ribosomal proteins for senescence-related gene expression. The investigations described above indicate that multiple proteolytic systems are induced late in the senescence process; however, such experiments do not specifically address the role of proteolysis as a regulator of the commitment to senescence and/or cell death.

As senescence of organs is followed by abscission, which serves to remove from the parent plant cells that are no longer needed, the purpose served by a cell death program in senescing tissues is not readily apparent. The endogenously controlled intracellular deterioration that occurs during organ senescence may be sufficient to cause cell death (Nooden, 1988). Perhaps, therefore, the entire senescence process should be considered a unique cell death program. It remains possible, however, that

Table 1 Summary of programmed cell death during plant growth and development

	Biochemical and molecular markers	Vacuolar rupture precedes complete cellular disorganization	Autophagic vesicles reported	Chromatin pycnosis reported	Cell fate[2]
Sex organ abortion[1]	*Ts2/Gsf1* (hydroxysteroid dehydrogenases)	?	no	no	2
Anther dehiscence	Cellulase, ubiquitin, TA56 (cysteine protease)	?	no	no	2
Death of incompatible pollen	S-RNases	?	no	no	3
Degeneration of haploid megaspores	?	?	no	yes	2
Synergid death	nuclear calcium increase	yes	no	no	2
Suspensor death	acid phosphatase, β-glucosidase	yes	yes	yes	1,2
Aleurone/endosperm death	intracellular calcium increase, protein phospatase (aleurone death prevented by phosphatase inhibitor)	?	yes	no	1,3
Pith autolysis	cellulase, α-amylase polygalacturonase, intracellular calcium increase, protein phosporylation (death prevented by kinase inhibitor, death promoted by phosphatase inhibitor)	yes	no	no	1
Tracheary element autolysis	nucleases, proteases, ubiquitin	yes	no	no	3
Sieve element selective autolysis	β-amylase, ubiquitin	yes, but autolysis is selective	no	yes	3 (with protoplast)
Senescence	nucleases, proteases, carbohydrases, ubiquitin, ubiquitin conjugating enzymes	yes	yes	no	4

[1]See text for references. [2]Cell fate as indicated in figure 1

death is due to a separate pcd pathway active in concert with remobilization of cellular components. If so, the identification of molecular markers for plant pcd would help to reveal (a) the point during senescence at which pcd is initiated, (b) whether the senescence pcd pathway shares elements with other plant pcd pathways, and (c) whether cell death can be uncoupled from the preceding events of senescence.

Conclusion

This review has described cell death that is initiated by stress, such as hypoxia-induced aerenchyma formation, and cell death that appears to depend on communication between neighboring cells, such as (a) incompatible pollen tube death, (b) suspensor degeneration, and (c) synergid death in some species (Table 1). Also described was pcd as it signifies the terminal differentiation of cells in anthers, xylem, and senescing leaves. Some of the processes considered in this review are arguably not examples of cell suicide. The death of incompatible pollen tubes is perhaps better described as cell murder where the victim is somehow prevented from protecting itself from lethal external factors (Martin, 1993). The unique selective autolysis occurring during sieve element maturation is neither suicide nor murder, but produces an enucleate protoplast.

The genes responsible for initiation of plant cell suicide programs are unknown. Sequences with identity to the highly conserved inhibitors of animal pcd, e.g. the *Caenorhabditis elegans ced-9* (Hengartner *et al*, 1992) and human *bcl-2/Bax* (reviewed by Rubin *et al*, 1994) have not been retrieved from the expressed sequence tags (ESTs) from plant sources. However, *Arabidopsis* and rice do express a protein similar to DAD-1, a recently identified animal pcd supressor, suggesting that plants and animals do share certain components of pcd pathways (Sugimoto *et al*, 1995). Cloning of genes sharing identity with members of the cell death-promoting caspase protease family (Alnemri *et al*, 1996) has not been reported for plants. Some members of this family (e.g., caspase 3, Tewari *et al*, 1995) cleave poly(ADP-ribose) polymerase (PARP), a nuclear enzyme found in most eukaryotes, including plants (Lepiniec *et al*, 1995). Hence assays for PARP-cleaving activity and sensitivity of plant pcd to peptidic caspase inhibitors may reveal the presence in plants of homologs to animal cell death proteases. In addition, since the ubiquitin pathway is responsible for the degradation of some short-lived regulatory proteins (Hochstrasser, 1995), strategies

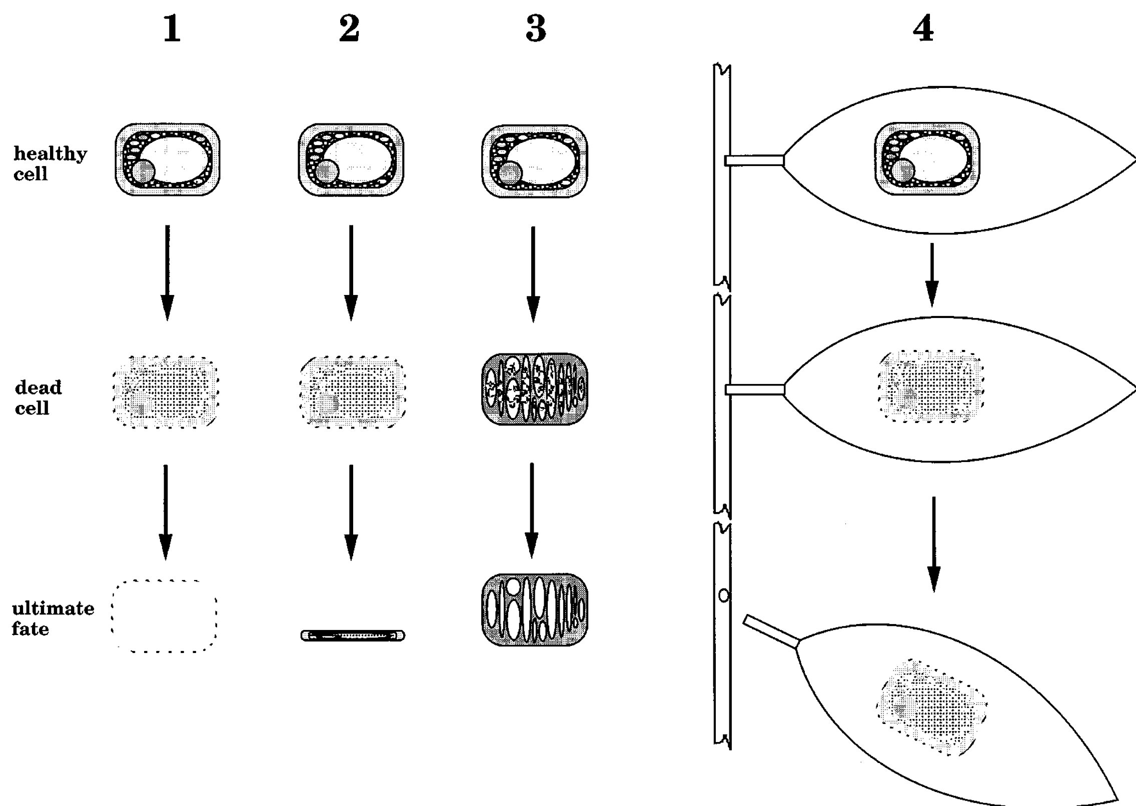

Figure 1 Cell fate following plant programmed cell death. Fate 1, both protoplasm and cell wall disappear completely after death. Precise details of complete cell removal are not yet known. Fate 2, cell death is followed by crushing or tearing of cell remnants by expanding neighboring cells. Fate 3, cell wall persists following protoplast death. In some cases, the wall is modified by thickening and lignification, as in the mature tracheary element shown here. Fate 4, cell death follows the ordered remobilization of cellular components during senescence, and organs consisting of dead cells are then shed from the parent plant (cell and leaf are not drawn to scale)

aimed at identifying nuclear proteins whose ubiquitin-dependent turnover promotes pcd may also prove fruitful. Finally, molecular genetic approaches using, for example, embryo-defective suspensor mutants (Vernon and Meinke, 1994) and sex-determination mutants (DeLong *et al*, 1993) may also identify genes controlling plant pcd.

In animals, heterophagic elimination of dead cells (phagocytosis by adjacent cells) is a prominant characteristic of apoptotic cell death (Clarke, 1990). Heterophagy is less important following nonapoptotic cell death, which relies more on autophagic and autolytic mechanisms. While the potential for phagocytosis of apoptotic bodies by plant cells could be explored using protoplast experimental systems, such as that described by Wang *et al* (1996), heterophagy of wall-bounded plant cells has not been reported. Four fates for plant cells during pcd are presented in Figure 1: (1) complete disappearance of cells, including cell walls (e.g., during aerenchyma formation and the production of fenestrated leaves (Melville and Wrigley, 1968)), (2) crushing, tearing or overgrowth of death cells by their expanding neighbors, as in the case of megaspore development, (3) persistence of cell walls with or without degraded components of dead protoplast (e.g., mature tracheary elements), and (4) post-senescence shedding of entire organs or tissues consisting of dead cells. That autolysis and perhaps autophagy rather than heterophagy appear to be the predominant mechanisms employed during both the suicide and the elimination of plant cells may in part be due to the presence of the plant cell wall. Autolysis does not rule out a role for microphagy of proteins and other soluble components released by dead cells (Herman, 1994), but the presence of the cell wall clearly presents a barrier to macrophagy by adjacent cells. It seems reasonable to assume, therefore, that, by necessity, plant cells employ controlled autolytic methods leading to the 4 cell fates shown in Figure 1.

Although apoptosis does not appear to be a universal mechanism for pcd in plants, certain elements of apoptosis have been detected during some plant pcd processes. Fragmentation of nuclear DNA during apoptosis may be catalyzed by a calcium-dependent nuclease (Wyllie *et al*, 1980). Calcium influx appears to trigger nuclear degeneration in degenerating synergids (Chaubal and Reger, 1994). Evidence for DNA fragmentation during pathogen-induced pcd (Ryerson and Heath, 1996), tracheary element differentiation (Mittler *et al*, 1995) and root-cap cell shedding (Wang *et al*, 1996) has been presented. To date, however, host-selective phytotoxin-induced pcd exhibits the most apoptotic-like characteristics, including calcium-enhanced DNA laddering, lobing or fragmentating nuclei and lobing cells separating into apoptotic bodies (Wang *et al*, 1996). These events were characterized at the biochemical and light microscope levels. It would be interesting to view electron micrographs of the phytotoxin-induced process in comparison with apoptotic animal cells. Nuclear pycnosis, visualized at the electron microscope level, has been reported to be part of three additional plant cell death programs occurring in intact plants: haploid megaspore degeneration (Bell, 1996), suspensor degeneration (Nagl, 1976) and sieve element enucleation (Eleftheriou, 1986; Cresson and Evert, 1994). Of these three, however, only the pycnotic nuclei of wheat sieve elements described by Eleftheriou (1986) contained dense masses of sharply delineated chromatin visible in lobing and fragmenting nuclei, i.e. pycnosis truly characteristic of apoptosis. While further examination of the biochemistry of the degeneration of haploid megaspores, synergids and suspensors and of the selective autolysis of sieve elements may reveal additional apoptotic-like qualitites in these processes, the observed autolytic and autophagic mechanisms active during the killing and elimination of dead cells during pith autolysis, tracheary element differentiation and senescence support the conclusion that nonapoptotic pcd pathways are essential components of normal plant growth and development.

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