Apoptosis in Plants

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Abstract: Apoptosis is a feature of animal cells that explains some aspects of programmed cell death in plants. Differences between plant and animal cell development require that concepts be reexamined to signify how plant cells have evolved the need for cell elimination in the meristematic growth habit, life cycle, and alternation of generations. Central to this theme is the regulation of divisional cycles for mitosis, meiosis, apomeiosis, and their related sexual and asexual reproductive processes. Apoptosis depends on the coordinated expression of genes regulating divisional cycles and apoptotic pathways so that irreversible nuclear and cytoplasmic elimination occurs. Cellular degradation products are salvaged to sustain adaptation, viability, structural function, and ontogeny. The cell wall is usually retained and further differentiated or eliminated. A model of factors predisposing apoptosis and comprising checkpoints in cell divisional cycles is presented for comparisons among plant and animal cells.

Key words: Apoptosis, programmed cell death, DNA damage, life history, regulatory proteins, cell cycles, metabolic salvage.

Abbreviations and symbols

DAPI:	4,6-diamidino-2-phenylindole dihydrochloride
TUNEL:	TdT-mediated dUTP-biotin nick end labelling (TUNEL assay for apoptosis)
TdT:	terminal deoxynucleotidyl transferase
dUTP:	deoxyuridine triphosphate
G_0, G_1, S, G_2, M:	various phases of cell and nuclear divisional cycles including mitosis (M). G_0 resting state, G_1 expression of genes for growth and development, S DNA synthesis, and G_2 chromosome replication.

Introduction

Apoptosis (Gk *apo* away from, *ptosis* falling) is a phenotypically distinct form of controlled cell deletion or programmed cell death. It plays a major role during development and homeostasis, and in the expression of many diseases in animal cells (Kerr et al., 1972). The ancient Greek word originally referred to the loss of petals from flowers, or leaves from trees. As such, the concept is worthy of reconsideration in plant biology. Apoptosis offers a complementary but opposite function to divisional cycles that regulate cell populations. Parts of the apoptotic program have been conserved among worms, insects, vertebrates (Steller, 1995) and plants (Bell, 1994, 1996; Durzan, 1994, 1996; Wang et al., 1996; Greenberg et al., 1994; Havel and Durzan, 1996, Mittler et al., 1995; Mittler and Lam, 1995a). Differences in details between plants and animals are now evident, but many features are the same (Wang et al., 1996).

The diagnosis of apoptosis in animal cells had advanced beyond the need for characteristic DNA degradation patterns created by endonucleases. Massive DNA degradation is commonly encountered. Growth- and stress-activated pathways determine whether a cell will survive or die by apoptosis (Xia et al., 1995). The apoptotic pathway is positively and negatively regulated (Knudson et al., 1995). Regulation is linked to checkpoints that detect DNA damage and replication, and transduce signals to effectors for each phase of a divisional cycle (Lydall and Weinert, 1995; Carr, 1996).

We propose a model for plant apoptosis in the life cycle that embodies predisposing physiological states, divisional cycling, salvage of metabolic degradation products, terminal differentiation, disease resistance, and renewed growth. Apoptosis is the outcome of a set of distinct genetic processes with irreversible selective steps in terminal differentiation and divisional cycling. It contributes to the loss of petals, flowers, fruit, leaves etc. in annuals, biennials, and perennials. Distinctions are made for the deletions of genes, or other structures and functions that may or may not lead to cell death and elimination.

Preconditions for Apoptosis

Genetic and ontogenetic requirement

Genes triggering the complete elimination of the cell must be present and accessible for expression in multicellular development (Osborne and Schwartz, 1994). In plants, the accelerated cell death gene (*ACD2*) acts as a negative regulator of programmed cell death triggered by pathogens (Greenberg et al., 1994). Host-selective toxins, causing stem canker in tomato, express the characteristic apoptotic features of animal cells (Wang et al., 1996). Genetic and morphological evidence for apoptosis appears in megaspore abortion (Bell, 1996). Nogler (1984) and Maheshwari (1950) outlined charac-

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teristic ontogenetic patterns of cell death in sporogenesis and gametophytic apomixis. Endonuclease and ubiquitin-mediated apoptotic events in free nuclei of egg-equivalents, and among individual cells of the developing axial tier of early embryos, are supported ontogenetically, morphologically, cytologically, and biochemically (Havel and Durzan, 1996; Durzan, 1996). Mutations block apoptosis by controlling oxidative stress (genes *p53*, *Bcl-2*), and predispose cancer (Kinzler and Vogelstein, 1996; Hockenbery et al., 1993). Yeast mutants arresting the cell cycle when DNA is damaged provide an important connection between cell cycle progression and apoptosis (Carr, 1996).

In our model, genes for the apoptosis and their pathway products enable exits from divisional cycles before cells are eliminated (Fig. 1). Apoptotic gene expression is linked to scheduled alterations and unscheduled failures in DNA repair, specific signal pathways, and to the transcription and post-translational modification of proteins. Apoptosis integrates with many evolutionary aspects of multicellular plants and vegetative development. Elimination mechanisms enhance fitness, viability, ontogenetic programs, and reproductive multiplication.

Apoptosis and divisional cycles

Plants comprise at least two main groups of cells: One involved in nuclear and cell division (free-nuclear cycling, mitosis, meiosis, apomeiosis, etc.) and the other in differentiation, maturation, aging, and specialized functions. An important question is how a long-term commitment is made between the genetic control of divisional cycles, specialized functions, and terminal differentiation in cell populations. The ability to determine final ontogenetic outcomes involves DNA repair and modification of divisional cycles.

Under favorable conditions, a basic tendency of the cell is progress to the next completed division cycle (Doerner, 1994; Murray and Hunt, 1993). If conditions are unfavorable, the cell either pauses or enters a specialized resting state. If serious and irreversible DNA alterations are experienced, the nucleus or cell exits the division cycle and is eliminated. These conditions imply that a "risk pattern" exists among prior physiological states leading to premature (unscheduled) apoptosis as distinct from those scheduled ontogenetically. The risk pattern comprises signaling pathways and provides early markers for apoptosis.

DNA repair or synthesis normally corrects errors and ameliorates the risk pattern. Cells now avoid apoptosis and continue in the division cycle until the next checkpoint for DNA and structural integrity. Checkpoints are specific times in a division cycle during which progression through the cycle can be delayed in response to structural damage or to an incomplete prior event such as DNA replication. Checkpoint gene products, e.g., p53, arrest division, and ensure the fidelity of genomic replication and segregation (Cross et al., 1995). Nuclei of rescued embryos and cell suspensions of *Ephedra californica*, Norway spruce, and *Araucaria angustifolia* show reactivity to a monoclonal antibody for mouse p53 gene product (Havel and Durzan, unpublished data). In unperturbed cells, checkpoints are not essential. Apoptosis is a normal part of ontogenetic and reproductive processes (Bell, 1996).

Division cycle checkpoints comprise signal transduction systems that regulate the cell cycle (Hartwell and Kastan, 1994). For each step in a division cycle, structures (DNA, chromatids, chromosomes, nuclei, cells) are replicated, sometimes repeatedly (Brown and Dyer, 1972). Checkpoint activation leads to a variety of cycle adaptations, including structural, temporal, and functional adjustments, as well as to cell death (Fig. 1).

Predisposition of Physiological States to Apoptosis

At least two physiological states predispose the risks for scheduled or unscheduled cell elimination. One is a response to external and stressful change agents that alters or damages DNA structure (Sarafian and Bredesen, 1994; Thompson, 1995). The second arises from internal complications or ontogenetic programs that initiate divisional activity with or without apoptosis. Signaling pathways within cells are comprised of chains of intercommunicating proteins. Proteins of a pathway integrate external signals with internal targets i.e., with effector proteins. The acquisition and release of signal information is the process of signal transduction. Molecular phenogenetics is used to study relationships between the genotype and its manifestations as a phenotype e.g., elimination of organs by abscission (Durzan, 1990). Signaling pathways lead to an adjustment in fitness (Kora, 1992).

External factors

Stressful external factors have the potential to trigger the expression of apoptosis. Signaling pathways enable a cell to respond to external factors regulating cell division and differentiation. Thresholds in physiological states predisposing apoptosis are activated by natural and synthetic hormones, photoperiod, temperature, low oxygen, toxins, some pathogens, metabolic blocks, dead-end pathways, X-rays, and direct or indirect genetic damage (Durzan et al., 1973; Kinzler and Vogelstein, 1996; Mittler and Lam, 1995b; Wang et al., 1996).

For example, the short-day grass *Dicanthium* controls the proportion of both normal and aposporous embryo sacs by the length of the inductive period (Knox and Heslop-Harrison, 1963). Aposporous sacs are never found as far as known without a degenerating megaspore or sac derived from it (Bell, personal communication). The sac wall contains callose rather than cellulose. This wall eventually collapses and is digested (Heslop-Harrison, 1972). External factors may be uncertain, nondeterministic, asynchronous, uncoordinated, and local. Independent signals are not always centrally coordinated or orchestrated. Our model predicts that when external signals become internalized, a combinatorial set of physiological states emerges (e.g., Bray, 1995; Durzan, 1990) which predispose division checkpoints for apoptosis.

Zinc deficiency in pistachio under field conditions leads to embryo abortion with an accumulation of arginine nitrogen (Durzan, 1994). Both arginine and Zn are active site components of DNA polymerase. Intracellular zinc chelation triggers apoptosis in mature thymocytes (McCabe et al., 1993). In toxin-treated tomato protoplasts, the intensity of classic apoptotic DNA ladder patterns were enhanced by Ca^{2+} and inhibited by Zn^{2+} (Wang et al., 1996). DNA sequences for a family of proteins that inhibit apoptosis have open reading

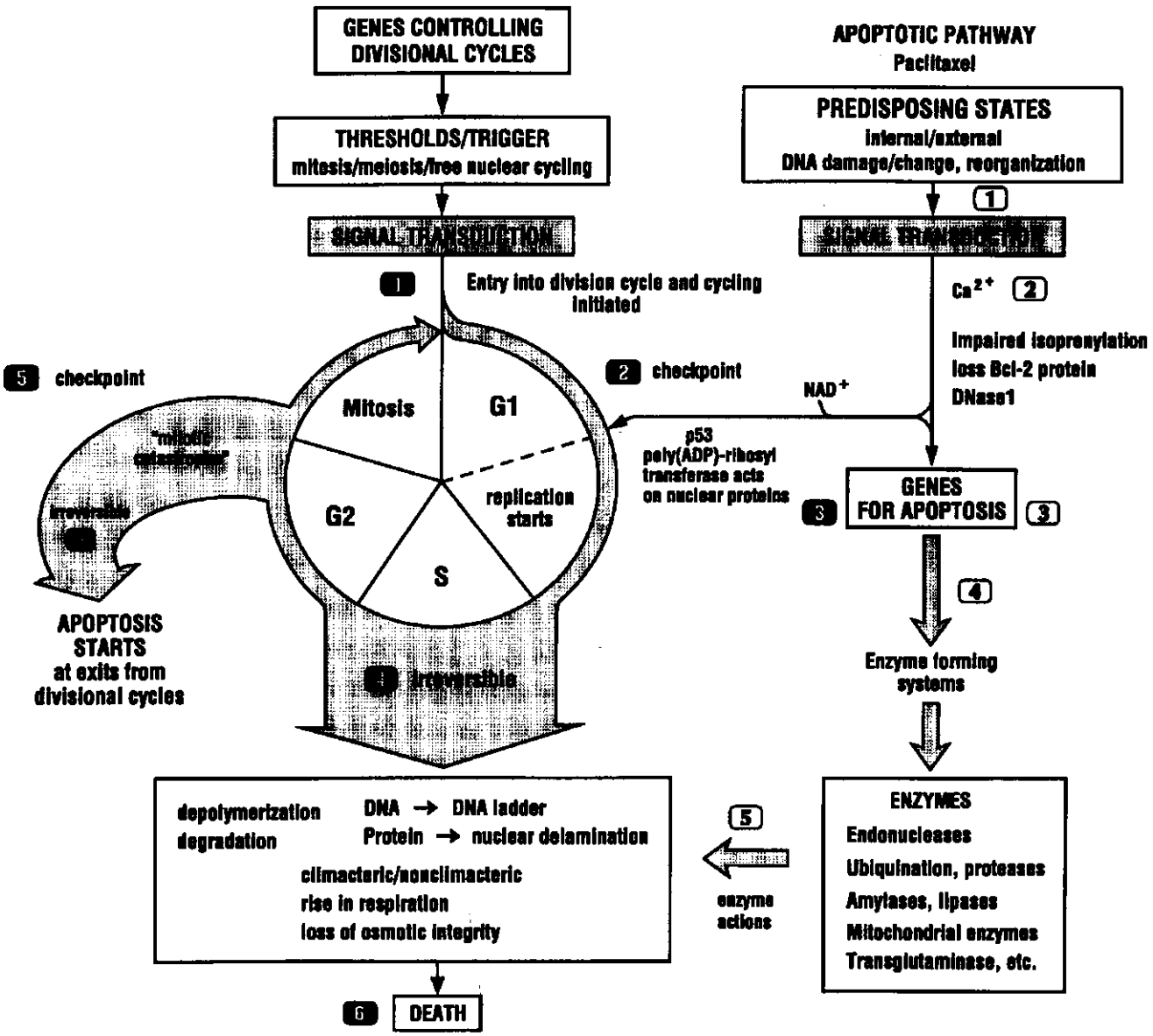

Fig. 1 Model for apoptosis in plant ontogeny based on internal and external factors predisposing scheduled or unscheduled entry into and exit from divisional cycles. The model shows a mitotic cycle, but meiotic, apomeiotic, and free nuclear cycles may be substituted. Key to shaded numbered boxes: **1.** Entry into a division cycle is determined ontogenetically and initiated by signal transduction of external or internal factors (e.g. plant hormones) that represent potentially preapoptotic states. **2.** A division cycle checkpoint for DNA damage occurs at a G_1/S transition (broken line). If an error is found, *p53* genes are expressed to initiate DNA repair and replication. Failing this, specific signalling pathways monitoring DNA damage (e.g., Carr, 1966) predispose apoptosis. **3.** In animals, a separate signalling pathway involving proteases and other enzymes activated by Ca^{2+} cleaves poly(ADP-ribose) polymerase to trigger and establish a set point for apoptosis. A ribosyl transferase adds multiple ADP-ribose units from NAD^+ to the carboxyl groups of nuclear proteins. The apoptotic pathway can be controlled, extended and suppressed (Liston et al., 1996). Enzyme-forming and activating systems for endonucleases give the morphological indications of apoptosis. **4.** Exit from the cycle is irreversible and is characterized by multiple biochemical, cytochemical, morphological, and physiological criteria. **5.** If the cycle passes the first checkpoint, others occur at S phase and at the G_2 transition to mitosis. Repair at the chromosomal and structural levels enables division cycles to proceed. Other checkpoints occur for free nuclear cycling and the meiotic cycle. **6.** Death occurs and cells are lysed with or without wall elimination by enzymes of the apoptotic pathway. Apoptotic degradation products are salvaged for growth and development of other cells. Turnover and salvage sustains the totipotency of the remaining cells (Durzan and Steward, 1983).
Key for the white numbered boxes in the apoptotic pathway: **1.** An inducing factor predisposes apoptosis. For example, paclitaxel (Taxol™) binds to microtubules and blocks mitosis. **2.** Signals for apoptosis may arise from: the loss of cell compartmentation (reorganization), DNase entry into the nucleus, Ca^{2+} increases, impaired isoprenylation of proteins at nuclear membrane or endoplasmic reticulum, and Bcl-2 protein losses. These signals impact the control of divisional cycles. **3.** Genes for apoptosis are expressed for **4.** specific enzymes leading to activities **5.** that complete cell death.

frames, repeat sequences encoding the inhibitory protein, and a carboxy-terminal zinc-finger (Liston et al., 1996).

Internal factors

Genes for metabolic regulation determine a spectrum of physiological states that may predispose apoptosis. The consequences of metabolic regulation, divisional cycling, DNA repair and transcription need to be sorted out. DNA is rearranged, reorganized, recombined, degraded or modified (e.g. deleted, amplified, imprinted, etc.) by nuclease-mediated events (Barlow, 1993; Britt, 1995; Krimer and Van't Hoff, 1983). Nucleases also reposition transposable elements and telomeres on chromosomes (Kipling, 1995). The new genomic DNA pattern is now organized differently from the embedded DNA pattern of the prior divisional cycle. Endonucleases are transitionally activated by many processes, and patterns of endonuclease activity, as the sole criterion for apoptosis, are inadequate. Indirect changes could occur from protein modification and degradation, endoglucanases, free radicals, accumulation of toxic products, etc. Damage leads to nonenzymatic and enzymatic browning reactions, advanced glycosylation end products, the methylation of compounds by S-adenosylmethionine, and to ethylene production. This means that combinations of external and internal factors could predispose apoptosis.

Gaps created in DNA produce lengthy, single-stranded regions. This activates mono- and poly(ADP)-ribosyltransferases that posttranslationally modify the carboxyl groups of nuclear proteins by adding single or multiple ADP-ribose units (Fig. 1). The source of these units is NAD^+. ADP-ribosylation depletes the nucleotide pools including ATP, and predisposes cell death (Gaal and Pearson, 1986). A protease, responsible for the cleavage of poly(ADP-ribose) polymerase, is a *pivotal trigger* for apoptosis (Nicholson et al., 1995). A heterodimeric protein promoting cell death "wrestles" with another protein (Bcl-2) through conserved motifs. This establishes a *set point* for cell death. The Bcl-2 protein occurs in mitochondria, endoplasmic reticulum and nuclear membranes. *Bcl-2* mutations block apoptosis (Hockenbery et al., 1993; Liston et al., 1996).

Senescence, while distinct from apoptosis, provides markers predispositioning apoptosis. For example, in peas, ovary senescence occurs in the absence of putrescine biosynthetic enzymes and with increased levels of putrescine oxidase. By contrast, fruit development is characterized by an increase in arginine decarboxylase and a constant level of putrescine oxidase (Perez-Amador and Carbonell, 1995). One factor autoregulating leaf senescence is cytokinin production controlled by a gene that encodes a rate-limiting isopentenyl transferase (Gan and Amasino, 1995). Ethylene is a multipurpose signalling molecule that controls senescence and defense against pathogens. A multigene families encodes ethylene sensors that are differentially expressed during plant growth and development (Theologis, 1995). Fruit ripening occurs with significant ethylene-induced changes in cell walls and fruit texture (Fisher and Bennett, 1991). Controlled atmospheres, low temperatures, and gentle handling of fruit slow ripening and senescence. Other phytohormones and signal substances, e.g., abscisic acid, auxin, salicylic acid, etc. affect the fate of cell populations. DNA replication, and terminal differentiation.

By contrast, apoptosis is regulated by specific signal detection and effector pathways for the monitoring of divisional checkpoints (Carr, 1996), and by gene-regulated pathways for cell elimination (Fig. 1). Controls are imposed by redox states, the transduction of several signalling systems by Ca^{2+} activated endonucleases, proteases, phosphorylation, phosphatidylinositol-3′ subgroup of kinases, and posttranslational modifications as in terminal differentiation.

Longevity and cell death

Some coniferous species live many thousands of years, e.g. *Sequoia, Fitzroya, Pinus aristata* var. *longaeva* (dated at 5000 years old). Fruit tree meristems have shorter life spans and become senile (e.g. Schaffalitzky de Muckadell, 1959). The continuation of cell division for thousands of years depend on the ability of meristems to repair and recover from damage to the genome, i.e. on the ability to adjust fitness over the long term and in harsh physical environments. As for longevity *in vitro*, the irreversible loss of organogenetic totipotency in the absence of pathogens has been proposed as a typical trait of plant cancers (Gaspar, 1995).

Cells are effectively eliminated by processes such as abscission and differentiation (Klekowski, 1988). Lifespans of vascular plants are not always limited by endogenous programs for senescence (Finch, 1990). The loss of viability of seeds is closely correlated to the accumulation of chromosome aberrations (Dourado and Roberts, 1984).

Replicative senescence refers to the control by telomeres of the number of divisions programmed by the ontogenetic algorithm (Kipling, 1995). As animal cells age, telomeres shorten with each cell division. Telomere length is now a biomarker for the potential number of cellular divisions remaining before death. If this observation holds for some plant cells, then replicative senescence, when combined with markers for apoptosis, will provide a new approach to the loss of totipotency and expression of apoptosis.

Division Cycle Checkpoints

Several checkpoints appear in response to a scheduled or unscheduled changes in genomic DNA (Fig. 1). Plant cells may cease divisional cycling and remain at rest for a variety of reasons (Brown and Dyer, 1972). In yeast, single and double strand breaks in DNA caused by an endonuclease are sufficient to arrest the cell cycle (Bennett et al., 1993). Damaged DNA delays cycle progression at the G_1-S checkpoint, S phase, DNA damage checkpoints, and at the G_2-M transition (Carr, 1996). DNA repair or changes in DNA sequence may initiate an entry into the next cycle or to a terminal devision cycle (Colombel et al., 1992).

The activation of checkpoint pathways involves proteins as detectors of DNA damage, and as blockers of cycle progression. Pathways detect changes in DNA structure and signal this information to effector mechanisms for repair or elimination. In animals, the p34 protein kinase encoded by a *cdc2* gene is a key component for the G_1/S transition. DNA damage actuates a transcription factor for the expression of a *p53* gene whose product is a nuclear phosphoprotein. The *p53* gene encodes a transcriptional activation of genes for cellular arrest at G_1 and its expression gives a high proportion of apoptotic

cells (Hartwell and Kastan, 1994). Loss of p53 function contributes to tumor formation, immunodeficiency, autoimmune disease, and neurodegenerative disorders (Montes de Oca Luna et al., 1995; Vito et al., 1996). In *Arabidopsis* a cdc2-gene product establishes the competence for meristem cell proliferation without overt processing errors (Martinez et al., 1992). At the G_2/M transition, cells are in the 4 C predivisional state, and ready for nuclear and cell replication. A serine-threonine kinase is essential for entry into mitosis. Premature activation of this $p34^{cdc2}$ kinase induces a process similar to apoptosis. This controversial activation is called mitotic catastrophe (Martin et al., 1995). Heterochromatin-like domains at telomeres make the double-stranded terminus inaccessible to nucleases and recombination enzymes. Natural chromosome ends cannot activate cell cycle checkpoints.

Divisions in plants are based on variations in mitosis, meiosis, and free nuclear cycling. In female sporogenesis of some plants, three of the four daughter-cell meiotic products will die and may provide stimuli for the division of the remaining spore (Johri, 1984; Nogler, 1984).

Fidelity in completing division cycles is achieved by the coordinated activity of cyclin-dependent kinases, checkpoint controls, and repair pathways (Hartwell and Kastan, 1994; Carr, 1996). Nonapoptotic plant cells passing the checkpoints develop spatially by polarization and strict control over the plane of division. Interphase microtubules are aligned perpendicular to the cell's growth axis. The preprophase band, girdles the cell at G_2. At prophase, it determines the plane of division. *Arabidopsis* mutants are unable to form cortical microtubular arrays. Mutants show irregular cell expansion, and cannot align division planes, but differentiation patterns are already in correct relative positions (Traas et al., 1995). Cells arrested in G_0/G_1 contain only one of four types of cortical microtubule arrays (Dawson and Lloyd, 1985).

DNA Replication and Repair

Genes are expressed for monitoring cell cycle progression, DNA damage and repair, or for apoptosis. An intact nuclear membrane and a "licensing factor" ensures the eukaryotic chromosomal DNA is replicated exactly once in each cell cycle (Chong et al., 1995; Madine et al., 1995). Initiation of DNA replication in nuclei from quiescent cells requires permeabilization of the nuclear membrane (Lano and Munshi, 1994). The "licensing factor" is removed from chromatin during replication (Chong et al., 1995). DNA polymerases correct errors at checkpoints (Kunkel, 1992). A proof-reading 3′ exonuclease removes any mispaired bases and the chain extension can continue. Proof-reading is not perfect and is error-prone. The yeast *Rad53* gene encodes a protein kinase for cell cycle arrest, and for control of transcriptional responses to DNA damage (Sanchez et al., 1996).

Deficient p53 cells undergo many rounds of DNA replication (e.g., endomitosis), even in the presence of spindle-inhibiting agents. The p53 protein is also a component of a spindle checkpoint that controls ploidy (Cross et al., 1995). Spindle errors produce aneuploidy (Singh, 1993). Replication errors produce chromosome aberrations (Hartwell and Kastan, 1994). In maize, broken chromosomes are healed only in sporophytic cells and not in the endosperm (McClintock, 1942). If a chromosome breaks and is not healed, the cell is predisposed for elimination (Kipling, 1995). UV-induced damage has both toxic and mutagenic effects. Dark repair pathways in plants do not directly reverse DNA damage. Instead, the damaged DNA is replaced with new, undamaged nucleotides (Britt, 1995). Base-excision repair restores nicked DNA to its original sequence through the combined actions of exonucleases, repair polymerase, and DNA ligase. Nucleotide-excision repair uses an endonuclease complex to initiate removal of the damage by initiating nicks, which are then excised by a helicase. This supports the view that endonuclease action is insufficient by itself as a marker for apoptosis.

Not all errors may be corrected in divisions, as in meiotic mutants (Baker et al., 1976; Hurst, 1993). Geminiviruses replicate through DNA intermediates, and their activities involve cell cycle regulation (Greenberg et al., 1994; Nagar, 1995). This contrasts with most other known plant viruses, which replicate through RNA intermediates. Geminiviruses provide an opportunity to study DNA synthesis, repair, and division cycle regulation in relation to apoptosis.

Markers for Apoptosis

Gahan (1984) has distinguished between reversible and irreversible types of damage in plant cells of different ages. The morphological features of apoptosis, however, comprise an active, inherently different set of genetically programmed events subject to a variety of stimuli (Kerr et al., 1972). Several pathways and a repertoire of proteins induce, control, extend, and suppress apoptosis. In plants, cell elimination can be a slower process than in animals (e.g., Wang et al., 1996). Energy is needed for the loss of structrual organization causing in some cases respiratory rises (Newmeyer et al., 1994).

Variations in plant cell elimination are evident. First, a complete cell elimination is ontogenetically prescribed. A gradual suicide may occur with complete protoplast and wall elimination. This contributes to age-related biological changes. Second, the cell wall persists as in xylogenesis. Third, selective organelle deletion and selection may occur as in the plant zygote. Protoplasts without a nucleus can persist for 1- to 2-years as in sieve element formation in wine grapes (e.g. Luxová, 1974). Fourth, the plant's nuclear genome may be fragmented by amitosis or by formation of a micronucleus that may or may not become apoptotic. Variations in apoptosis are diagnosed by multiple markers showing how the cell exits from a division cycle and is deleted from cell populations.

Morphological markers

Morphology changes in 2 arbitrary stages (Kerr et al., 1972). First, nuclear and cytoplasmic condensation breaks the cell up into many membrane bound well-preserved fragments. Second, these fragments (apoptotic bodies) are shed, degraded, and taken up by other cells. A multinucleated giant cell may be created (Knudson et al., 1995). In plants, apoptosis is also marked by the localized collapse of nuclear domains (pycnosis), loss of the nuclear membrane, nucleolar release, and fragmentation of nuclei and cytoplasm into distinct bodies (Bell, 1996; Havel and Durzan, 1996; Wang et al., 1996). For example, when cells of the early embryo are

enucleated by the ontogenetic program for suspensor formation, nucleoli are released into the cytoplasm (Havel and Durzan, 1996).

The nuclear genome is fragmented sometimes into unequal parts by amitosis in plant cells. Resultant micronuclei may or may not remain viable (Sing, 1993; Kipling, 1995; Nuti Ronchi, 1995). Genomic separation may start at the centromere or telomere. Telomeric DNA is inaccessible to proteins such as exonucleases, ligases, and recombination enzymes. This distinguishes a natural chromosome end from a double-strand break. The breakdown of telomeres changes DNA replication and gives "position effects" that may abolish gene expression in daughter cells.

The selective degradation of a specific nucleus in binucleate egg-equivalents of conifers is illustrated where the ventral canal nucleus alone becomes apoptotic (Durzan et al., 1994; Havel and Durzan, 1996). Just after fertilization in conifers, the paternal chloroplast genome is retained in the neocytoplasm around the zygotic nucleus (Camefort, 1969). Maternal chloroplasts are deleted (Neale and Sederoff, 1989). In angiosperms, the maternal chloroplasts are usually retained.

At the level of an individual nucleus, apoptosis is diagnosed by a terminal deoxynucleotidyl transferase (TdT). This enzyme labels the 3′ OH ends of DNA, generated by DNA fragmentation or nicking, with biotin-conjugated dUTP. Labelling is visualized with a secondary detection system and a colorimetric substrate (Gavrieli et al., 1992; Gorczyca et al., 1993; Mittler and Lam, 1995a). The TdT-mediated dUTP-biotin nick-end labelling (TUNEL) assay, when combined with DAPI, identifies nuclei that are or are not destined for elimination (Havel and Durzan, 1996). This detection method reaffirms earlier observations where the ventral canal nucleus is apoptotic as determined by permeability to Evan's blue, and the strongly acetocarmine reactive egg-equivalent nucleus, is not (Gupta and Durzan, 1987).

Moreover, along the axial tier of Norway spruce early embryos, transitional cells leading to suspensor development show endonuclease activity at localized nuclear genomic domains. Nuclei become TUNEL positive before pycnosis is evident and before nucleoli are released into the cytoplasm (Havel and Durzan, 1996). In female spores of many angiosperms, the collapse of the pycnotic nucleus is followed by shrinkage of protoplasm and cell wall collapse. Apoptosis is expressed after a conspicuously unequal division that precedes cell death e.g., megasporogenesis in heterosporous ferns and seed plants (cf., Maheshwari, 1950).

Changes in the cell and nucleus are coupled with cytoskeletal reorganization. Tubulin isotypes are developmentally regulated by multiple-site posttranslational modifications e.g., acetylation, glutamylation and C-terminal detyrosination/tyrosination. In animals, these occur either during or immediately following a terminal mitosis (Lee et al., 1990). Microtubules are involved in segregation of chromosomes, cytoskeletal reorganization and nuclear integrity.

Time-lapse photomicroscopy of individual live cells shows that the first markers for cell death, induced by exposure to high levels of L- or D-glutamine, are the loss of cyclosis, transvacuolar strand breakage, and disruption of the osmotic integrity of the nucleus (Durzan and Bourgon, 1976). With high L-glutamine, the normal rhythmic cycles of changes in nuclear volume are followed within 4 minutes by a rapid expansion and contraction. Next, nuclear contents precipitate and become granular. D-glutamine feeding stops cyclosis and within an hour the nucleolar vacuoles expand. Addition of L-glutamine reverses this process.

Differentiation of phloem and xylem (cf., Esau and Cheadle, 1965) involves endonuclease activity ending in cell death (Mittler and Lam, 1995b; Northcote, 1995; Thelen and Northcote, 1989). Sequential nuclear atrophy is marked by endonucleases (Fig. 2). The nucleus decreases in volume, chromatin collapses, fragments, and condenses, and nuclear-matrix proteins are solubilized. The cell wall is retained and terminally differentiated. At low oxygen centres of vascularizing sphaeroblasts, the pattern of vascularization mimics the cross section of stems (Durzan, 1984; Durzan et al., 1973). Dead cells carry out the transport of water and food supply that maintains correlations among living parts. In *Theobroma cacao*, xylem parenchyma, xylem vessel cell walls, and gels occluding vessels accumulate elemental sulfur for disease resistance (Cooper et al., 1996).

Fig. 2 The TUNEL reaction for apoptosis reveals how nuclei and protoplasts are eliminated sequentially in a population of terminally differentiating cells during xylogenesis in *Cupressus sempervirens*. **1.** A non-apoptotic nucleus in a cell (nonreactive assay color). **2.** Start of endonuclease activity is shown by the dark area (start of red assay coloration in the nucleus). **3.** Increased endonuclease activity at the dark left inner edge of a nucleus, showing the start of pycnosis. **4.** Strong endonuclease activity throughout the collapsed nucleus (black) and thickening of cell walls during xylogenesis. Xylem-like cells are ca. 100 μm long. (Unpublished data: L. Havel, T. Scarano, and D. J. Durzan).

Biochemical markers

The regulation of apoptosis is mostly known from work with neoplastic tissue (Korsmeyer, 1995). Physiological regulation by high calcium predisposes intracellular acidification, activa-

tion of proteases, phospholipases, phosphatases, acidic endonucleases, and DNA fragmentation (Schwartzman and Cidlowski, 1993). An ATM protein functions as a detector or signaling pathway for the G_1/S checkpoint. p53 is a biochemical marker for this checkpoint (Carr, 1996). Phosphorylation of p56 is an assay for DNA damage at checkpoint G_2 (Walworth and Bernards, 1996). Cell death linked to these markers can be measured by the release of mononucleosomes and oligonucleosomes. Commercial kits are available for quantifying the number of BrdU-labelled DNA fragments that damaged cells release into the cytoplasm (e.g., Anon, 1994).

Apoptosis has been characterized by the formation on agarose gels of a ladder of small fragments of double-stranded DNA (oligonucleosomal size 180–200bp lengths) (e.g. Walker et al., 1993; Wang et al., 1996). Numerous single-strand cuts are detected after electrophoresis under denaturing conditions (Peitsch et al., 1993). Single-strand nicks are very frequent in the internucleosomal regions and in the core particle-associated DNA. Peitsch et al. (1993) propose that DNA fragmentation is not due to a double-strand cutting enzyme as postulated earlier. Rather it results from single-strand breaks. Larger DNA fragments and now single-strand events are being recognized in apoptosis (Bortner et al., 1995). In yeast, phosphorylation of *Rad* gene products comprise a biochemical assay for damage at cell cycle checkpoints. Rad proteins are involved in the processing of single-strang breaks into regions of single-stranded DNA (Sanchez et al., 1996; Walworth and Bernards, 1996).

In maize, apoptotic cells do not necessarily produce the characteristic diagnostic ladders during sex determination. A nonspecific degradation of DNA is observed as a smear (Calderon et al., 1995). The massive action of endonucleases is representative of terminal events. Damaged or released fragments are not repaired. By contrast, classic ladder patterns for DNA degradation by the apoptotic pathway have been found in tomato (Wang et al., 1996).

The timed destruction of damaged or misfolded proteins, by proteasomes regulates metabolism and division cycles. Cell regulatory proteins are marked for destruction by tagging with the protein ubiquitin (Delic et al., 1993). Ubiquination orders the timely destruction of cyclins, kinase inhibitors, and proteins that move the cell cycle forward (Barinaga, 1995; Seufert et al., 1955). In the ontogenetic program for enucleating embryonal suspensors of Norway spruce (Havel and Durzan, 1996), proteasome-like assemblages degrade nuclear and nucleolar proteins. Degradation products form a complex mucilage that is released into the culture medium (Durzan, 1996). Ubiquitin-specific protease are now recognized as cell type and substrate specific regulators of cell fate in multicellular organisms (Huang et al., 1995). The control over ubiquination is not unique to apoptosis. Ubiquination represents a ontogenetically programmed removal of regulatory protein function.

Salvage and Recycling of Degradation Products

The metabolic turnover and recycling of macromolecules in plants provide an important renewed source of primary and secondary products, growth regulators, energy, and nutrition (Durzan and Steward, 1983; Fig. **1**). Salvage invigorates multicellular development and adjusts the plant's overall fitness. Salvage is not restricted to products of apoptosis nor to other degradative processes e.g., oligosaccharins released by wall degradation (Darvill et al., 1992). Metabolic turnover products remove constraints to mitosis, meiosis, viability, and may represent a form of cannibalistic plant survival (Mogie, 1992; Bell, 1994; Godfray, 1995, Haig, 1992).

The occurrence of metabolic DNA has been advocated by Berlyn (Renfroe and Berlyn, 1983; G. Berlyn, personal communication). Plants producing considerable caffeine and other purine or pyrimidine products raise the notion that DNA turnover could provide substrates for these secondary products (Durzan and Steward, 1983). The salvage of pyrimidine bases and the turnover of histone and nonhistone chromosomal proteins was demonstrated in conifer seedlings (Durzan et al., 1973; Pitel and Durzan, 1975,1980). Methylation of turnover products e.g., β-alanine, glycine, and proline offers osmoprotection under drought and salinity (Hanson and Rathinasabapathi, 1993).

Necrosis

Cell deletion is distinguished from necrosis, which is unprogrammed and involves the decay of injured groups of dying cells (Kerr and Harmon, 1991). Animal cell necrosis is accompanied by phagocytosis and inflammation (Kett et al., 1972). Mitochondria swell and lyse with little or no requirement for energy. Neither protein and nucleic acid synthesis, nor new gene transcription occurs. DNA is randomly digested. Necrosis is characterized by the early disappearance of membrane ion-pumping activities, due to membrane damage or cellular energy depletion (cf. Mittler et al., 1995).

In plants, injury by frost, heat etc., causes necrosis and in some cases abscission of the injured part. Haberlandt (1922) postulated that degenerating cells produced "necrohormones" removed limiting factors and stimulated viability in other living cells. Stebbins (1941) viewed necrohormonal stimuli as the least attractive of several hypotheses explaining apomixis. His main reason was that diplospory or apospory (apomeiosis) may start before somatic cells of the ovule start to degenerate. Environmental factors and recessive gene(s) were considered secondary to control by a constellation of complementary factors. Among these complementary factors, the stimuli from nearby degenerating cells continues to dominate recent ideas explaining apomixis (Bell, 1994; Mogie, 1992).

We postulate that stimuli arising from degradation products of apoptosis in plants may be distinct from those arising from necrosis because of the connection of apoptosis to a coordinated set of gene expressions. Apoptotic degradation products are not the result of a genetically unprogrammed disaster, but of terminal events offering continuity, survival, and protection in the plant's life cycle. Other examples of programmed cell death exist e.g., senescence of cotyledons, leaves, but these processes, while they may ultimately predispose apoptosis, have not yet been linked to control of divisional cycling in the life cycle. Apoptosis offers protection for cell populations against disease through hypersensitive reactions in neighboring cells, and in perennials, accounts for the falling off of petals, leaves and fruits in the context of seasonal changes, dormancy, and limiting internal and external factors, as implied by the original Greek meaning of apoptosis.

Conclusions

For plants and animals many genes for the control of divisional cycling and apoptotic pathways appear comparable. Morphological, physiological, and biochemical markers are similar. Variations in endonuclease DNA degradation patterns exist with or without elimination of the cell wall. Apoptosis is a distinct process from the deletion of genes. Multiple criteria combining genetics, biochemical signaling pathways, cytochemistry, physiology, morphology, and developmental biology are needed to distinguish variations in plant apoptosis from other types of programmed cell death. Variations in apoptosis indicate that a cascade of multiple paths control the extent of cell elimination.

The diversity of factors initiating scheduled or unscheduled apoptosis may be external or internal. Irreversible steps are determined at division cycle checkpoints for integration with apoptosis. Apoptotic proteases, posttranslational events, lytic and salvage enzymes comprise a separate but coordinated path of of informational signals. Some enzymes are common to all forms of cell death, programmed or not. Apoptosis may be scheduled by an ontogenetic programs for terminal differentiation, but strict hierarchies may not always exist. Apoptosis produces necrohormones and nutrients that are recycled to invigorate other cells. Disease resistance has been linked to products of apoptosis.

The older literature can now be reinterpreted to show how apoptosis integrates with normal development of multicellular plants. Apoptosis adjusts fitness by being complementary to mitosis, and other divisional cycles. It contributes structure and function to the life history of the organism. Adaptability, viability, survival, disease resistance, reproduction (mictic and apomictic), heterochrony, alternate bearing, alternation of generations, and the regeneration of new or missing parts are all impacted by apoptosis.

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