

Cell-death mechanisms in maize

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Development and differentiation in plants requires that specific cells be eliminated by cell-death mechanisms. Structural and ultrastructural observations demonstrate that cells or groups of cells in numerous maize tissues undergo cell death at predictable times. A vast collection of maize mutants exists, and many of these mutants show phenotypes that suggest aberrant cell-death mechanisms. The agent responsible for these mutations is often a characterized transposable element, making it possible to isolate the genes involved using transposon-tagging strategies. Thus, maize is developing into an excellent model system for the study of cell-death mechanisms in plants.

There are many examples of cell death in plants, the details of which are becoming the focus of intensive research¹⁻⁴. When cell death occurs as part of normal developmental processes it is considered to be a programmed cell-death (PCD) process. When normal development is perturbed, such as by a genetic lesion, cell death can occur ectopically. In addition, cell death can occur at the sites of environmental stresses, such as pathogen infection or physical wounding, or in response to low concentrations of toxins. These three distinct types of cell death – in normal development, perturbed development or because of environmental stresses – do not necessarily utilize the same cell-death mechanism; however, they do all appear to occur via a controlled disassembly of the cell. By contrast, cell death that occurs because of severe trauma (i.e. traumatic cell death or necrosis), such as after exposure to a high concentration of a toxin, heating or freezing, is not a controlled disassembly of the cell and is characterized by cellular swelling and lysis, which are not morphological characteristics of PCD (Refs 3 and 4).

PCD has been well characterized in animals and is an active process that facilitates the disassembly of the dying cell. Animal cells undergoing PCD can display cytoplasmic condensation and shrinkage; blebbing of plasma membranes and nuclear membranes; chromatin condensation and marginalization; internucleosomal DNA fragmentation; and phagocytosis of the dying cell^{5,6}. When these morphological changes are observed, the cell-death process is often termed apoptosis. However, not all cell-death events that are programmed include all of the features of apoptosis³⁻⁵.

The PCD process in animal cells can be initiated by signal transduction pathways involving Ca²⁺ fluxes and changes in protein phosphorylation; it is then executed by a proteolytic cascade that includes caspases and endonucleases^{5,6}. Caspases are a family of cysteine proteases whose function in PCD is to activate other caspases or to catalyze the cleavage of cytosolic, cytoskeletal, nuclear-scaffold or DNA repair proteins. Caspases are believed to bring about many of the morphological changes observed in cells undergoing the PCD process⁵⁻⁷. In addition, an endonuclease was recently described that is also activated by certain caspases. This 'caspase-activated DNase' appears to be normally present in cells, but is only activated during PCD, when it causes DNA fragmentation⁸. Lastly, certain caspases appear to be regulated by apoptotic protease activating factor (Apaf), a complex of three proteins found in human cell extracts⁹. Other proteins involved in the regulation of PCD have been identified and include members of the Bcl-2 (also termed Ced-9) family and the recently described Bax inhibitor-1 (BI-1). Some of the members of the Bcl-2 family can act to prevent PCD, as is the case for Bcl-2 and Bcl-X_L, but others, such as Bax and Bak, promote the PCD process^{5,6}. BI-1 localizes

to the intracellular membranes in which Bcl-2 is found and may function to enhance the activities of Bcl-2 (Ref. 10). How these proteins actually regulate PCD is unclear.

Although PCD occurs in plants, little is known about how it is executed and regulated. Only two homologs of animal PCD-related genes have been identified in plants. The homolog of *dad1* ('defender against apoptotic death') was detected in rice and *Arabidopsis* genomes via database searching. The *Arabidopsis dad1* cDNA is capable of reducing apoptosis in a *dad1*-deficient hamster cell line, indicating that it is functionally similar to the mammalian *dad1* gene¹¹. In addition, a homolog of mammalian BI-1 has been detected in the *Arabidopsis* genome, which may also function to suppress PCD in plants¹⁰. It is possible that the PCD mechanisms in plants and animals have some unique features as a consequence of the fundamental differences in life-cycles, development, response to the environment and cellular organization. However, PCD in plants does seem to have many similar features to PCD in animals, including fragmentation of nuclear DNA, involvement of Ca²⁺, changes in protein phosphorylation, increase in nuclear heterochromatin and involvement of reactive oxygen species¹⁻⁴. The ability of plant hormones to delay or hasten a cell-death event is also an indication that a particular cell-death event is programmed^{3,4}. Here, we discuss cell-death events in maize and suggest maize mutants that may exhibit aberrant cell-death mechanisms.

From mutant morphology to gene function

Maize morphology and ultrastructure indicates that cell-death events occur throughout the sporophyte (Fig. 1). In addition, cell death occurs within both sporophytic and gametophytic cells during the generation of the female gametophyte (Fig. 1). Mutant analysis can be used to bridge the gap between morphological observation and the elucidation of biochemical processes. In transposon-tagging studies, large numbers of mutagenized plants are screened for phenotypes that are predicted to occur if genes involved in a particular biological pathway (such as PCD) are disrupted; the transposable element can then be used as a hybridization probe to identify the mutant gene within a genomic library. Transposon-tagging is well developed in maize and has been used to isolate genes involved in cell death. Gene and predicted-protein sequence comparisons can often be used successfully to predict function. However, when no similarity to known proteins is identified, there are still various strategies available to help elucidate function. In addition, a great deal can be learned regarding the pleiotropic effect of a mutant gene, and thus the biological processes that the gene influences, through analysis of the mutant compared with the wild-type plant.

Cell-death events

The hypersensitive response and disease-lesion mimics

An example of PCD in plants is the hypersensitive response (HR), which is characterized by a rapid cell death within a limited number of cells at the site of pathogen entry. The genetically controlled induction of the HR involves the interaction of a disease-resistance protein from the host with the corresponding avirulence protein from the pathogen. This interaction appears to initiate a signal transduction pathway leading to the HR. The molecular mechanisms of HR cell death involve ion fluxes, the production of reactive oxygen species and lipid peroxidations, all of which can occur during the PCD process^{1,2,4}.

The function of a resistance gene, which may encode a receptor, a kinase or a protein with both of these functions, is to control the triggering of the HR in the presence of a pathogen. It is likely that any aberration in the structure or function of a resistance gene will lead to an uncontrolled response. The presence in maize of many mutants that mimic lesions caused by plant-pathogen interactions provides support for this proposal¹². It has recently been demonstrated that certain alleles of the *Rp1* gene of maize exhibit lesion development in the absence of infection (Fig. 2c)¹³. The isolation of *Rp1* and the availability of lesion-mimic *Rp1* alleles is likely to play a key role in improving understanding of the induction and biochemistry of the HR.

The highly localized nature of the HR suggests that mechanisms must exist to keep cell death contained. There are several mutations known in maize in which this containment mechanism has been compromised, with the result that cell death ultimately engulfs the whole plant. The gene for one of these mutations, *lethal leaf spot1* (*lls1*; Fig. 2a) has recently been cloned via transposon tagging¹⁴. Its similarity to genes encoding aromatic-ring-hydroxylating enzymes suggests that one mediator of cell death in plants is a phenolic compound.

The cloning of *Les22*, a dominant lesion-mimic gene, has provided information suggesting that excessive production of oxygen free radicals leads to lesions that closely resemble HR lesions (Fig. 2b). The etiology of *Les22*, however, is mediated by a null mutation in the *urod* gene, which encodes uroporphyrinogen decarboxylase, a key enzyme of the porphyrin pathway, which produces both chlorophyll and heme in plants¹⁵. Similar to porphyria in humans, inactivation of one copy of the *urod* gene in maize results in a partial block in the pathway, leading to an accumulation of uroporphyrin. This porphyrin intermediate, which is readily

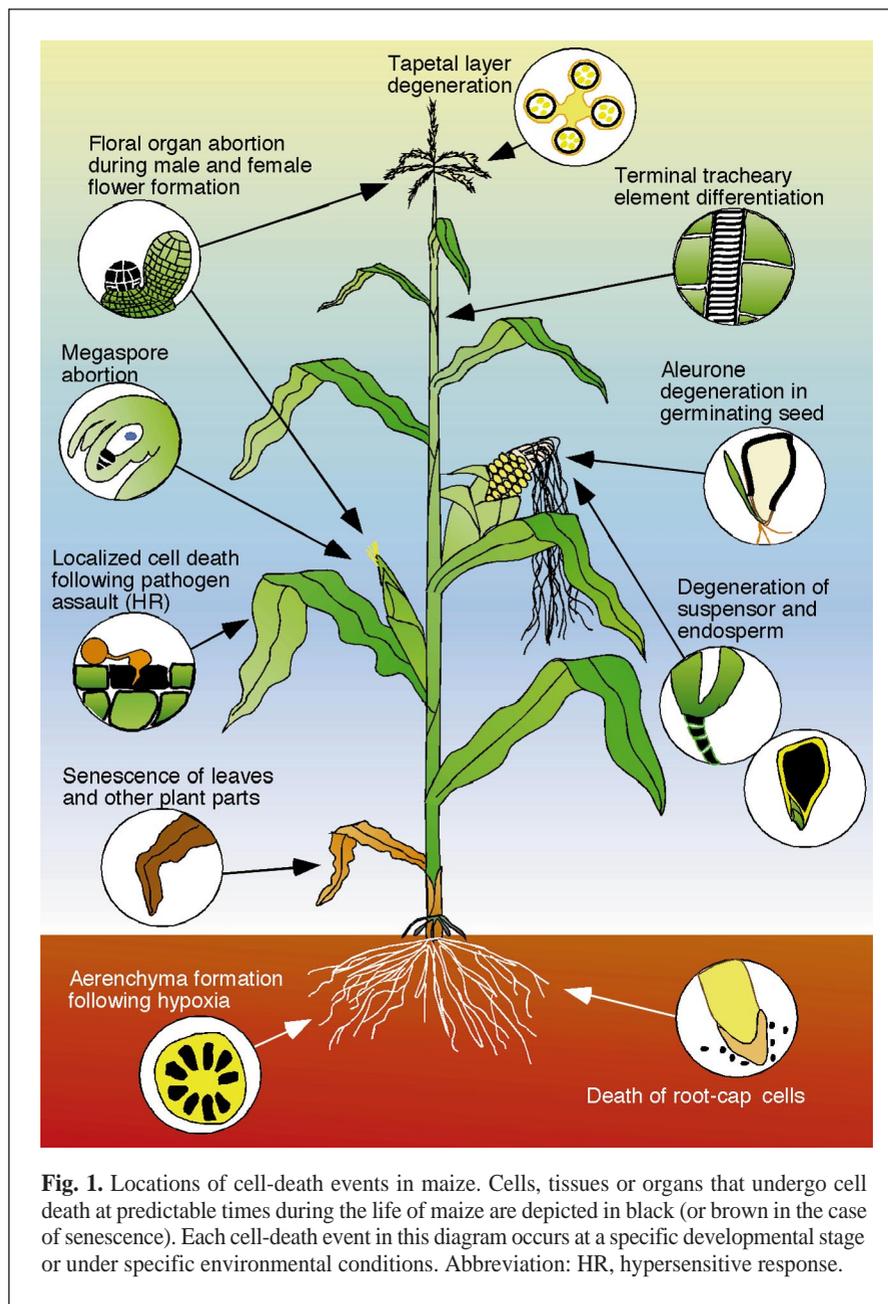


Fig. 1. Locations of cell-death events in maize. Cells, tissues or organs that undergo cell death at predictable times during the life of maize are depicted in black (or brown in the case of senescence). Each cell-death event in this diagram occurs at a specific developmental stage or under specific environmental conditions. Abbreviation: HR, hypersensitive response.

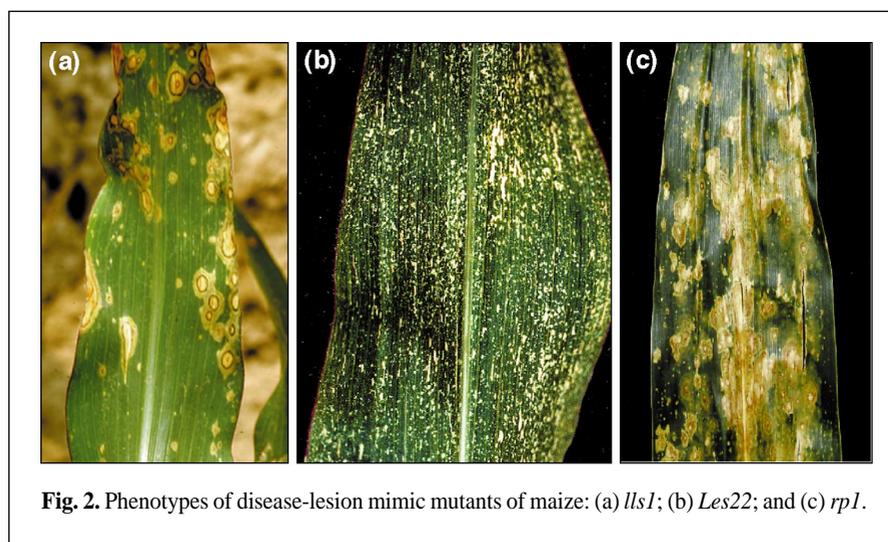


Fig. 2. Phenotypes of disease-lesion mimic mutants of maize: (a) *lls1*; (b) *Les22*; and (c) *rp1*.

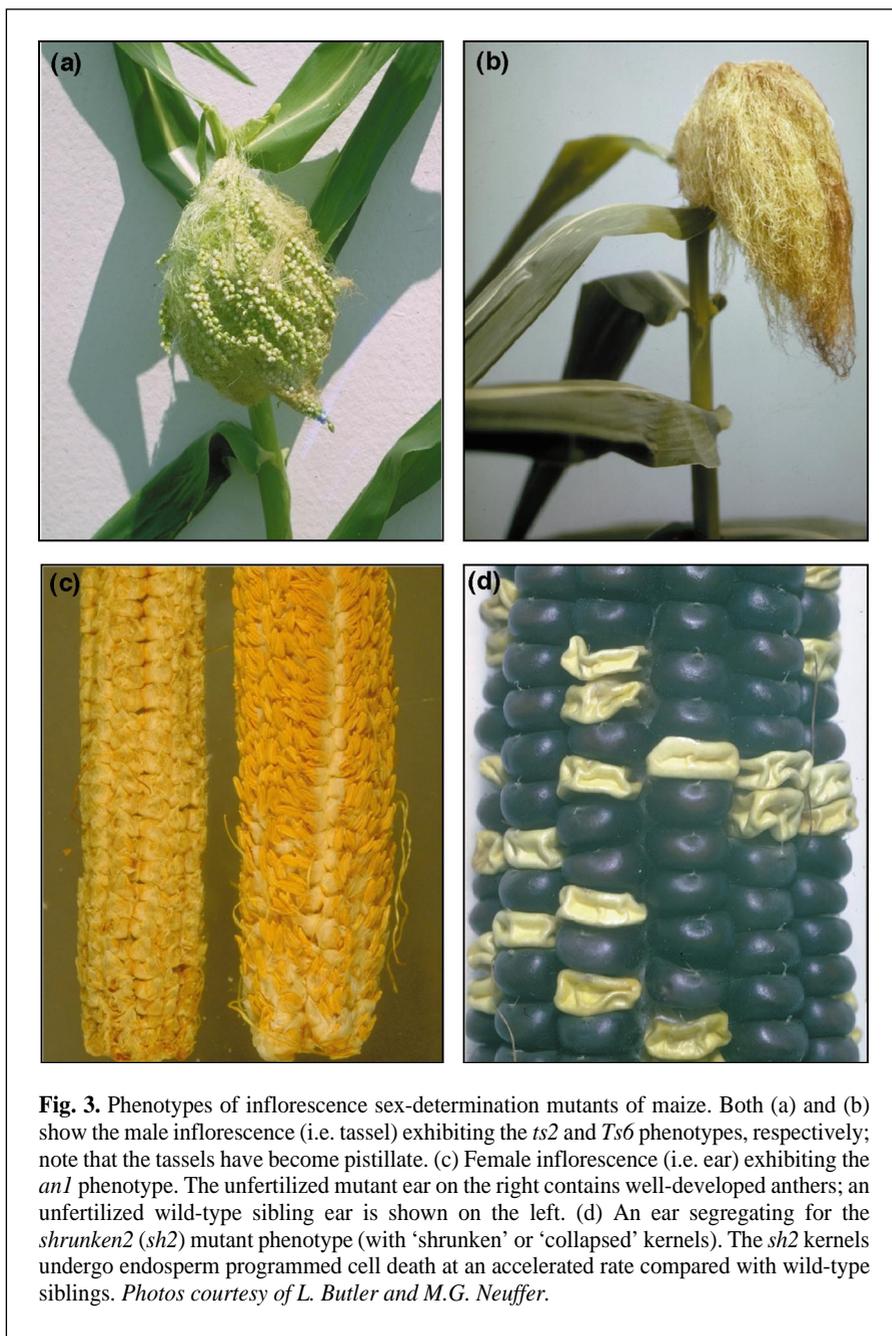


Fig. 3. Phenotypes of inflorescence sex-determination mutants of maize. Both (a) and (b) show the male inflorescence (i.e. tassel) exhibiting the *ts2* and *Ts6* phenotypes, respectively; note that the tassels have become pistillate. (c) Female inflorescence (i.e. ear) exhibiting the *an1* phenotype. The unfertilized mutant ear on the right contains well-developed anthers; an unfertilized wild-type sibling ear is shown on the left. (d) An ear segregating for the *shrunken2* (*sh2*) mutant phenotype (with 'shrunken' or 'collapsed' kernels). The *sh2* kernels undergo endosperm programmed cell death at an accelerated rate compared with wild-type siblings. Photos courtesy of L. Butler and M.G. Neuffer.

photo-excited, leads to an excessive formation of singlet oxygen and other cell-damaging reactive oxygen species in the presence of light. The *Les22* mutation illustrates that some of the disease-lesion mimic mutants are likely to result from perturbation of normal metabolic pathways. Whether the reactive oxygen species in *Les22* mutants initiate a PCD pathway or accumulate to levels that cause traumatic cell death remains to be determined. In either case, the lesion formation in *Les22* mutants is an example of ectopic cell death.

Xylem formation

The tracheary elements of vascular plants conduct water from the roots to aerial structures, and are composed of dead, hollow cells with thick secondary walls. These cells are derived from cambial initials in immature tissues or from the dedifferentiation of parenchyma cells in mature tissue. In both cases, the cell death that

occurs probably involves PCD mechanisms. Arabinogalactan proteins (AGPs) have been shown to be present on the secondary-wall thickenings of cells destined to undergo PCD, producing xylem. These AGPs might mark cells that are committed to PCD (Ref. 16). Although morphological studies have been carried out¹⁷, very little is known regarding the cellular, subcellular and biochemical events that occur during PCD of maize xylem elements.

A null mutation in a gene that is essential for xylem production would probably result in an embryo or seedling lethal, and such mutants are inherently difficult to isolate. However, it is possible that leaky mutant alleles of genes involved with xylem PCD will only reduce or modify xylem production, and these would be more readily identifiable. For example, plants that are homozygous for the recessive mutant allele of *wilted 1* (*wi1*) exhibit retarded differentiation of the metaxylem elements of the stem vascular bundles; this results in a viable plant that has characteristic wilted leaves¹⁸. Two other wilted phenotype mutants (*wi2* and *wi3*) that are non-allelic to *wi1* have also been described¹⁸.

Tapetal cells

Microsporogenesis in maize takes place within the anther locule of the male inflorescence. Prior to its death, the interior cell layer of the anther locule (the tapetum) plays an active role in the production of the microgametophyte. However, at the time of microspore mitosis the tapetal cells die, forming a proteinaceous and lipoidal residue that coats the mature pollen surface¹⁹. This cell death is certainly developmentally programmed, but hallmarks of PCD have not been described.

Mutants that alter tapetal cell structure and viability in maize have been isolated¹⁸. The tapetal cells of the *male sterile 26* (*ms26*) mutant prematurely vacuolate and die. It has been suggested that the defect in *ms26* mutants might cause a toxic product

to accumulate in tapetal cells and that these cells are sensitive to this toxin because of their high metabolic activity. If this is true, it will be interesting to determine whether the tapetal cells respond to the toxin by initiating a PCD pathway or if the toxin causes traumatic cell death. However, it is also possible that *ms26* is a gene that is directly involved in PCD and when mutated results in premature tapetal cell death. The *ms26* mutant was isolated from active *Spm* transposable element lines²⁰, which should facilitate its isolation by transposon tagging.

Tapetal cell death also occurs in plants that contain the Texas male sterile cytoplasm (T-cytoplasm). The mitochondrial DNA of T-cytoplasm plants contains a unique gene, *Turf-13*, whose product (URF13) is thought to be involved in causing tapetal cell death and subsequent pollen sterility. The nuclear gene *rf2*, which encodes an aldehyde dehydrogenase (ALDH), can restore male fertility to T-cytoplasm plants. It has been suggested that ALDH

might detoxify the effects established in mitochondria that contain URF13 (Ref. 21). It is presently not clear whether tapetal cell-death events associated with T-cytoplasm are an example of ectopic cell death or traumatic cell death caused by the toxic effects of URF13.

Female gametophytes

The development of the female gametophyte (embryo sac) of maize can be divided into two stages: megasporogenesis and megagametogenesis. During megasporogenesis, the diploid megaspore mother cell undergoes meiosis to produce four haploid megaspores, three of which die. The factors that determine which three megaspores will die are not well understood; however, in maize, megaspore cell death is known to be developmentally programmed and is correlated with an increase in hydrolytic activity and severance of plasmodesmatal connections²². Mutations that influence megaspore cell death have not been described in maize.

Floral organ abortion

Maize is a monoecious plant that produces unisexual male (tassel) and female (ear) inflorescences. Each inflorescence begins development with both male (staminate) and female (gynoecium) primordial cells; however, gynoecium cells abort in the tassel, and staminate cells abort in the ear. In both cases, these cells do develop for a time – this is most evident in the ear, where staminate development proceeds to the extent that anthers with discrete lobes can be seen. Ultimately the cells die, exhibiting vacuolization and loss of cytoplasmic integrity²³.

In maize, several sex-organ mutants have been described in which the inflorescence either has the opposite sex or is bisexual. Some of these mutants probably involve the genes that regulate the cell-death events in the inflorescence. The 'dwarf' mutants of maize (*d1*, *d2*, *d3* and *d5*) and the *anther ear 1* (*an1*; Fig. 3c) mutant have andromonoecious ears (i.e. fully developed anthers within the ear). Each of these mutants responds to gibberellic acid (GA) treatment, which produces apparently normal plants¹⁸. The *an1* gene has been cloned following transposon tagging and shown to encode isoprenoid cyclase (kaurene synthase A), which catalyzes the first committed step in GA biosynthesis²⁴. The *d3* gene has also been isolated and shown to encode a cytochrome p450 enzyme involved in GA biosynthesis²⁵. Biochemical evidence suggests that *d1*, *d2* and *d5* might also encode proteins necessary for the biosynthesis of GA. Thus, GA apparently plays a role in floral sex determination or floral-organ abortion in maize.

The *tasselseed* (*ts*) 4, 5 and 6 genes of maize promote varying degrees of bisexuality in the tassel, and both *ts1* and *ts2* condition a transition to entirely pistillate (feminized) tassels (Fig. 3a and 3b)¹⁸. The *ts2* gene encodes a protein with sequence similarity to short-chain alcohol dehydrogenases²⁶. The mRNA has been localized to gynoecium primordia within the tassel, suggesting that the *ts2* gene product is an enzyme involved in gynoecium abortion, which presumably occurs by a PCD mechanism²⁶. Morphological and developmental studies of *ts4* and *Ts6* indicate that these mutations prevent or delay the development of specific spikelet meristems within the tassel and ear of maize²⁷. These genes might also act to suppress pistil development in the tassel²⁷.



Fig. 4. Premature senescence mutant of maize. (a) Plant exhibiting the *pre1* mutant phenotype; note leaves are senescing prior to anthesis (pollen shedding). (b) *pre1* leaf; the arrow indicates the location of a somatic revertant sector exhibiting the wild-type phenotype.

The immature male and female inflorescences are composed of two spikelets, each containing two florets. In the female inflorescence, but not the male, one floret in each spikelet degenerates through what is probably a PCD mechanism²³. The degeneration of one floret in each spikelet results in the characteristic paired rows of eggs and subsequently kernels on the ear. Mutations in this process, if viable, should disrupt this pattern and be readily observed. In some lines of maize, floret abortion does not occur and thus the ear lacks paired rows because of crowding of the kernels²⁸.

The suspensor

The first division of the zygote in maize gives rise to two cells: an apical cell, which develops into the embryo; and a basal cell, which develops into the suspensor. The suspensor mechanically supports the embryo in the embryo sac, transfers nutrients from maternal tissue to the developing embryo, and may provide signals for embryonic development²⁹. Once these functions have been accomplished, the suspensor degenerates. From studies of plants with large suspenders (e.g. *Phaseolus coccineus* and *Tropaeolum majus*), which has facilitated their examination, it appears that the cells comprising the suspensor undergo PCD (Refs 2 and 4).

Although no specific characterization of suspensor degeneration in maize has been performed, its development and degeneration during embryogenesis is well documented^{28,29}. Two classes of mutants, *dek* and *emb*, could potentially be sources of suspensor PCD mutants. The *dek* mutants display an altered endosperm and embryo phenotype, whereas in *emb* mutants the endosperm appears normal, but the development of the embryo is severely altered. In an analysis of 51 *emb* mutants, several were found to be arrested at stages prior to suspensor degradation. Although some of these displayed a swollen or enlarged suspensor, this often correlated with the necrosis or failed development of the embryo. The suspensor died in *emb* mutants arrested at later stages²⁹, indicating that these are not likely to be suspensor PCD mutants. Maize

mutants with altered suspensor PCD would not necessarily be restricted to *emb* and *dek* mutants, but the abaxial location of the suspensor will make identifying such mutants difficult.

Aleurone and endosperm tissue

Nutrients stored in the kernel endosperm are exported and used by the developing embryo during germination. Endosperm cells normally die during the development of the kernel, following pollination. In maize, endosperm cell death is accompanied by internucleosomal DNA fragmentation, indicating that it is probably a PCD event. This process is accelerated in the *shrunk2* mutant, which is defective in starch biosynthesis (Fig. 3d), and appears to be due to elevated levels of ethylene³⁰. Aleurone cells, the single layer of cells which separates the underlying endosperm from the maternally derived pericarp, enhance the degradation of the endosperm during germination through their release of hydrolytic enzymes; the aleurone cells then die. Aleurone cell death in barley and wheat appears to be programmed and is inhibited by abscisic acid, but facilitated by GA (Refs 31 and 32).

Maize *dek* mutants, with their reduced endosperm size, are a potential source of endosperm PCD mutants. Some of these mutants might exhibit a small endosperm size because of inappropriate timing of endosperm PCD. Mutants of aleurone PCD might be identified in the *emb* class, as some of these mutants have an altered aleurone structure²⁹.

Roots

The maize root cap meristem produces cells that are displaced through the root cap and are shed into the soil. When the cell reaches the soil its cell wall disintegrates and its protoplast lyses. The resulting cellular contents contribute to the mucilaginous covering of the root tip³³. Little is known regarding the mechanism that causes root-tip cell death; however, the maize root cap can be excised and maintained in tissue culture³³, which should help facilitate physiological and biochemical studies of this cell-death process.

PCD appears to occur in the roots of maize during the formation of lysigenous aerenchyma. Aerenchyma is tissue containing intercellular spaces that aids in the transfer of O₂ from the stem to the root, particularly in flooded conditions. In maize, root aerenchyma forms by the PCD of cortical cells. This process appears to be regulated by ethylene and involves signal transduction messengers including G-proteins, cytosolic Ca²⁺ and protein phosphatases³⁴. In addition, a flooding-induced xyloglucan endotransglycosylase homolog in maize, which is ethylene responsive, has been shown to be associated with aerenchyma formation³⁵. It has been suggested that this protein³⁵ might be involved in the cell wall degradation required for aerenchyma production in maize³⁶.

Aerenchyma production is common and extensive in the roots of prairie grasses such as eastern gamagrass (*Tripsacum*). Maize–*Tripsacum* hybrids have been produced in attempts to transfer the apomictic trait of *Tripsacum* to maize³⁷. Coincidentally, the aerenchyma trait of *Tripsacum* was also transferred to maize, indicating that these two traits are linked in *Tripsacum*³⁸. Because the organization of maize and *Tripsacum* chromosomes is similar, it is likely that the gene or genes that are involved in aerenchyma production can be more precisely mapped and ultimately isolated.

Leaf and whole-plant senescence

Senescence is a type of PCD that represents the terminal stage of differentiation in plants. During senescence, nutrients are retrieved from organs that are no longer needed and are transported either to developing organs or to storage sites. In tobacco, senescence is delayed by cytokinin, which prolongs the photosynthetic lifespan of the leaves³⁹.

In leaves, initiation of senescence occurs in chloroplasts. These swell and exhibit an increasing number of plastoglobuli, indicative of the disassembly of the internal membranes. This is accompanied by a decrease in the expression of photosynthesis-related genes, and a decrease in the number of cytoplasmic ribosomes^{40,41}. Concomitantly, transcription of genes encoding nucleases and proteases increases. In maize, the level of expression of *see1* and *see2*, genes that encode a cysteine protease and a vacuolar processing enzyme, respectively, are enhanced during leaf senescence⁴². This could facilitate the degradation of proteins in senescing tissue. In addition, increases in the expression of genes encoding enzymes in certain metabolic pathways occurs. For instance, the level of expression of pyruvate orthophosphate dikinase increases during leaf senescence. The most likely role of this enzyme in senescing leaves is to enhance gluconeogenesis, providing energy for senescence in tissues that display a much reduced photosynthetic capability. The loss of structural integrity of mitochondrial and nuclear membranes occurs during the late stages of senescence⁴³. Final events include the rupture of the tonoplast and the subsequent release of hydrolytic enzymes into the cytoplasm of the cell, culminating in cell death^{40,41}.

'Stay green' maize varieties exhibit delayed senescence, even following ear removal or the prevention of pollination, which in some lines induce premature senescence of the whole plant. These varieties presumably contain alleles of genes that delay the onset of senescence and would be of interest for study at the molecular level⁴⁴. Two mutants that demonstrate delayed senescence are *indeterminate-1 (id-1)* and *perennialism-1 (pe-1)* (Ref. 18). By contrast, *premature senescence-1 (pre-1)* is a recessive mutant that starts to senesce early (Fig. 4a and 4b): although they follow the same pattern of senescence seen in wild-type plants, in the mutants senescence begins approximately two weeks prior to anthesis and the plants usually die within 3–4 weeks¹⁸. Genetic analysis of one maize line, B73, that undergoes premature senescence following ear removal, indicates that the early onset of senescence is controlled by a single dominant gene⁴⁵.

Beyond the mutants

The identification of a putative cell-death mutant, the isolation of the gene involved and the prediction of its general function based on sequence comparison falls far short of the goal of understanding the role that gene plays in cell death. Nevertheless, with the gene in hand, expression can be assessed, and the expressed polypeptide can be used to produce antibodies, permitting immunolocalization of the protein. It is also likely that the tentative identification of gene function will suggest other ultrastructural and biochemical analyses that can be performed further to define the role of the isolated gene.

It is now possible to use a PCR-based, reverse-genetic approach to screen a transposable element-mutagenized population of maize in search of mutations of any gene sequence that is known²⁴. Thus, as cell-death genes are cloned from other organisms or as genomic sequencing projects reveal genes potentially involved in cell death, maize plants with mutations in these genes can be identified.

Mutant phenotypes of genes that encode secondary messengers that are used in PCD signal transduction pathways might not always be easy to predict or identify from existing mutants. However, once a maize mutant for a step in a PCD signal transduction pathway is identified, it can be used in a second-site mutagenesis strategy to select for mutations in genes encoding proteins downstream in the PCD pathway. It is likely that this strategy could be exploited to identify PCD signal transduction mutants in maize. In addition, once a maize cell-death gene is isolated, it can be used in

interactive cloning strategies (e.g. yeast two-hybrid systems) to isolate genes that are critical to the functioning of this gene. Now that a few maize cell-death genes have been isolated (e.g. *lls1* and *ts2*) these powerful strategies should allow the isolation of other genes in cell-death pathways.

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