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Programmed cell death and tissue remodeling in plants

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Abstract

The use of programmed cell death (PCD) to remodel plants at the cellular, tissue, and organ levels is particularly fascinating and occurs in such processes as tracheary element differentiation, lysigenous aerenchyma formation, development of functionally unisexual flowers from bisexual floral primordia, and leaf morphogenesis. The formation of complex leaf shape through the use of PCD is a rare event across vascular plants and occurs only in a few species of *Monstera* and related genera, and in the lace plant (*Aponogeton madagascariensis*). During early development, the lace plant leaf forms a pattern of equidistantly positioned perforations across the surface of the leaf, giving it a lattice-like appearance. Due to the accessibility and predictability of this process, the lace plant provides highly suitable material for the study of developmentally regulated PCD in plants. A sterile lace plant culture system has been successfully established, providing material free of micro-organisms for experimental study. The potential role of ethylene and caspase-like activity in developmentally regulated PCD in the lace plant is currently under investigation, with preliminary results indicating that both may play a role in the cell death pathway.

Key words: Lace plant, leaf morphogenesis, perforations, programmed cell death, remodeling.

Introduction to plant programmed cell death (PCD)

PCD is a genetically encoded active process, whereby cells organize their own destruction. Crucial to the development and survival of organisms, PCD is an essential process in both animals and plants. In plants, PCD serves numerous roles in normal development (reviewed by van Doorn and Woltering, 2005) and may be grouped on the basis of cytological features into apoptosis-like cell death, senescence-associated cell death, and PCD involving the early disruption of the vacuole (Fukuda, 2000). Apoptosis-like cell death in plants is rapid, beginning with the degradation of the nucleus and subsequent incomplete breakdown of cellular organelles (Fukuda, 2000). Cell death during senescence, however, proceeds very slowly, with chloroplasts degraded initially, followed by the disruption of the nucleus and vacuole at the end of cell death (Smart, 1994; Fukuda, 2000). The third cytological variant of plant PCD is characterized by the early disruption of the central vacuole, leading to the release of lytic enzymes into the cytoplasm (Fukuda, 2000). This variant of PCD occurs at a speed that is intermediate between that of apoptosis-like cell death and senescence-associated cell death in plants (Fukuda, 2000).

There are two broad categories of PCD in plants, namely developmentally regulated PCD and environmentally induced PCD. Developmental uses of PCD include xylem differentiation (Mittler and Lam, 1995; Fukuda, 2000), deletion of embryonic suspensors (Giuliani *et al*., 2002), formation of functionally unisexual flowers from bisexual floral primordia (Calderon-Urrea and Dellaporta, 1999; Caporali *et al*., 2003), root cap shedding (Wang *et al*., 1996), anther dehiscence (Bonner and Dickinson, 1989), and leaf morphogenesis (Gunawardena *et al*., 2004, 2005, 2006, 2007). Developmentally regulated PCD occurs at a predictable time and location, and is induced by internal factors. In contrast, environmentally induced PCD, such as the development of lysigenous aerenchyma triggered by hypoxic stress (Gunawardena *et al*., 2001a) and the hypersensitive response (HR) in leaves triggered by pathogen invasion (Heath, 2000), is initiated in response to external abiotic or biotic signals.

A particularly fascinating use of PCD occurs during the remodeling of cells, tissues, or organs, as part of the normal developmental progression of the plant life cycle. In

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the context of plant cell death, the process of remodeling serves to redefine a structure by altering its physical characteristics. Examples of PCD-enabled remodeling in plants include tracheary element differentiation at the cellular level (Fukuda, 1997), lysigenous aerenchyma formation at the tissue level (Drew *et al*., 1979; Gunawardena *et al*., 2001*a*, *b*), and abortion of stamen primordia in female flowers (Cheng *et al*., 1983; Dellaporta and Calderron-Urrea, 1994; Calderon-Urrea and Dellaporta, 1999) as well as leaf morphogenesis at the organ level (Gunawardena *et al*., 2004, 2005, 2006, 2007). Such PCD-enabled remodeling is a prerequisite for normal plant development and is essential for these developmental processes to occur. Analogous to the process of remodeling in plants at the tissue level, for example, is vertebrate limb bud development, whereby if PCD fails during formation of the digits, digits remain joined by soft tissue. Likewise, if the PCD responsible for remodeling during normal plant development does not occur, this lack of cell death paradoxically can have deleterious consequences for the entire organism. The main objective of this paper is to review a few select examples of PCD-enabled remodeling in plants at the cellular, tissue, and organ levels, with an emphasis on research involving the lace plant—a model system for the study of developmentally induced PCD.

Tracheary element differentiation

A well-characterized developmental use of PCD is the differentiation of tracheary elements, which are the supporting and water-conducting tissues of vascular plants. The transdifferentiation of isolated *Zinnia elegans* mesophyll cells has been used to analyse tracheary element differentiation since this system lends itself well to the study of the sequence of events that occur during this process. In *Zinnia* cell cultures, individual mesophyll cells have been shown to transdifferentiate directly into tracheary elements without cell division in the presence of phytohormones (Fukuda and Komamine, 1980; Fukuda, 1997). After completion of secondary wall synthesis, a rapid collapse of the large central vacuole releases lytic enzymes such as nucleases and proteases, leading to the complete degradation of cellular contents and producing hollow dead conduits (Groover *et al*., 1997; Ito and Fukuda, 2001; Obara *et al*., 2001; Obara and Fukuda, 2004). Vessel elements are produced when the end wall is completely removed, while only partial degradation of lateral walls occurs during vessel element and tracheid differentiation (O'Brien, 1970; Fukuda *et al*., 2000). In addition, *Zinnia* has been proven to be a particularly good model system for the study of tracheary element differentiation because of the high frequency of differentiation that occurs in *Zinnia* cultures and because the process can be observed in single cells (Chasan, 1994; Fukuda, 1994, 1996).

Lysigenous aerenchyma formation

Another prominent example of PCD-enabled remodeling in plants is the formation of aerenchyma, which occurs either by lysigeny or by schizogeny (reviewed by Evans, 2004; Seago *et al*., 2005). Lysigenous aerenchyma forms via the death of cells, whereas schizogenous aerenchyma develops through cell separation. During lysigenous aerenchyma formation, interconnected gas spaces are formed by the controlled collapse and death of certain root cortical cells, while adjacent cells remain alive. The formation of aerenchyma enables a wide range of species to survive and grow in poorly aerated environments (Jackson, 1989), since a network of interconnected intercellular spaces is created that facilitates gas diffusion (Armstrong, 1979; Drew *et al*., 1979). Previous studies have shown that aerenchyma formation in maize roots can be triggered by hypoxic environmental conditions, as well as the plant hormone ethylene (Drew *et al*., 1979; Jackson *et al*., 1985; Gunawardena *et al*., 2001*a*, *b*). Aerenchyma formation occurs at a predictable time and location; the cell death process begins in the cortical cells when they are <0.5 d old and within 10 mm of the root tip. The formation of aerenchyma is initiated in the midcortex of maize roots and then progresses radially and tangentially into the surrounding cells (Campbell and Drew, 1983; Gunawardena *et al*., 2001*a*, *b*). Aerenchyma formation displays key characteristics of PCD, including chromatin condensation, oligonucleosomal DNA fragmentation, and the formation of membrane bodies enclosing organelles (Gunawardena *et al*., 2001*a*). Unlike xylem differentiation, where at least the lateral walls remain, cell wall degradation in ethylene-induced aerenchyma formation in maize roots is complete (Gunawardena *et al*., 2001*b*).

Formation of functionally unisexual flowers

Several patterns of unisexuality exist in the plant kingdom, with monoecious species bearing unisexual flowers of both sexes on the same plant, in contrast to dioecious species which bear unisexual flowers on different individuals. Sex determination in flowers of maize, a monoecious species, involves the selective abortion of either the female or male organ primordia within a bisexual floral meristem (Cheng *et al*., 1983). Early in floral development in maize, both ear and tassel flowers (called florets in grasses) are bisexual, and the transition to the unisexual state is marked by the arrest and abortion of one of the organ primordia—either the pistil primordia in the tassel or the stamen primordia in the ear (Dellaporta and Calderon-Urrea, 1994). In the tassel, the elimination of the pistil primordia occurs through a cell death programme that involves cellular vacuolation and the degradation of organelles, while adjacent stamen initials continue to divide and differentiate until they reach sexual maturity (Cheng *et al*., 1983; Dellaporta

and Calderon-Urrea, 1994). Conversely, the abortion of stamen primordia in female flowers proceeds through a similar cell death process, which is initiated near the apex of the primordium and propagated basipetally (Cheng *et al*., 1983; Dellaporta and Calderron-Urrea, 1994; Calderon-Urrea and Dellaporta, 1999). PCD is, therefore, essential in remodeling bisexual flowers into unisexual flowers in maize, with the transition involving extensive changes in the morphology of the flower.

Leaf morphogenesis

The production of complex leaf shape during leaf morphogenesis represents a unique role for developmentally regulated PCD. The remodeling of leaf blades through the death of discrete subpopulations of cells is an extremely rare event across vascular plants. It occurs only in a handful of monocotyledonous species, such as in *Monstera obliqua*, *M. deliciosa* and related aroids (Araceae), and the distantly related *Aponogeton madagascariensis* (Aponogetonaceae; Serguéeff, 1907; Gunawardena *et al*., 2004, 2005). Early in development, the leaf blades of *Monstera* form distinctive perforations through the death of discrete patches of cells. These minute pinprick-size perforations extend about 10 000-fold in area as the leaf expands to form conspicuous holes in the mature leaf (Gunawardena *et al*., 2005). The dying cells at each perforation site exhibit numerous hallmarks of PCD, including DNA degradation, chromatin and cytoplasm condensation, and disruption of vacuoles (Gunawardena *et al*., 2005). Organelles remain intact until late in the process, with no evidence of cell wall degradation. All cells within each perforation site undergo PCD simultaneously. Neighbouring protoderm and ground meristem cells are unaffected, and exposed ground meristem cells at the rim of the perforation transdifferentiate as epidermal cells (Gunawardena *et al*., 2005). The remodeling of *Monstera* leaf shape using PCD is striking and, although the functions of the leaf perforations in this species are unknown, several hypotheses have been put forth. First, perforations may serve as camouflage by disrupting the leaf outline and/or giving the appearance of previous grazing damage, thus deterring herbivores by signalling the presence of induced chemical and/or physical defences. Alternatively, perforations may serve to increase the ratio between leaf perimeter and surface area, thus improving leaf tissue temperature balance by increasing the heat transfer (Madison, 1977; Brown and Lawton, 1991).

Of all species in the Aponogeton family, only the lace plant uses PCD to remodel its leaves, forming a lattice pattern of equidistantly positioned perforations at a set time in development (Serguéeff, 1907; Gunawardena *et al*., 2004). Lace plants grow as submerged aquatics that give rise to leaves at the apex of a short spherical corm. Centrally located cells within each perforation site initiate PCD, with cell death extending to more peripheral cells as perforation formation progresses, stopping approximately five cell layers from the veins. Mesophyll cells at the perforation border transdifferentiate as epidermal cells and adopt a narrow, elongate shape (Gunawardena *et al*., 2004). Additionally, cell wall degradation is a key event during the formation of perforations in lace plant leaves, and walls of perforation border cells are modified by suberin deposition late in development (Gunawardena *et al*., 2007). Similar to *Monstera*, the morphogenesis of the unusual perforate leaves of the lace plant has been shown to display many hallmarks of PCD (Gunawardena *et al*., 2004). In lace plants, PCD is first detected by the loss of the anthocyanin-induced red coloration in young leaves and an alteration of cytoplasmic streaming. This could be a direct result of the rupture of the tonoplast and the acidification of the cytoplasm. At the same time, nuclei in cells in the perforation zone become TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) positive, which is an indicator of DNA fragmentation. Other characteristics of PCD such as invagination of membranes, abundant vesicles, shrinkage of the cytoplasm, and the late degradation of organelles have been observed during ultrastructural analysis by transmission electron microscopy (Gunawardena *et al*., 2004).

Although both the lace plant and *Monstera* use PCD to remodel leaves during development, perforation formation differs between these species in two important respects. First, PCD occurs sequentially within the lace plant perforation site, in contrast to *Monstera* where PCD occurs simultaneously in all cells targeted for destruction (Gunawardena *et al*., 2004, 2005). In the lace plant, cell death is initiated within the centre of the perforation site and is propagated outward, stopping about five cell layers from the vascular tissue. Therefore, cells at different stages of PCD are observed in the early stages of perforation formation. Unlike the lace plant, however, cells within the perforation site of *Monstera* undergo PCD simultaneously, sharply delineating the border between dying and healthy tissue. Secondly, cell wall degradation is a key event in lace plant PCD and is required for the opening of perforations in the leaves (Gunawardena *et al*., 2007), unlike in *Monstera* where no evidence of cell wall degradation is observed (Gunawardena *et al*., 2005).

The lace plant as a model system to study programmed cell death

Several key properties of the lace plant make it an advantageous model system for the study of developmentally regulated PCD in plants. The continuous process of perforation formation in lace plant leaves was subdivided into five developmental stages: pre-perforation (stage 1, longitudinally rolled leaves), 'window' formation (stage 2, distinct transparent regions, Fig. 1B), perforation formation

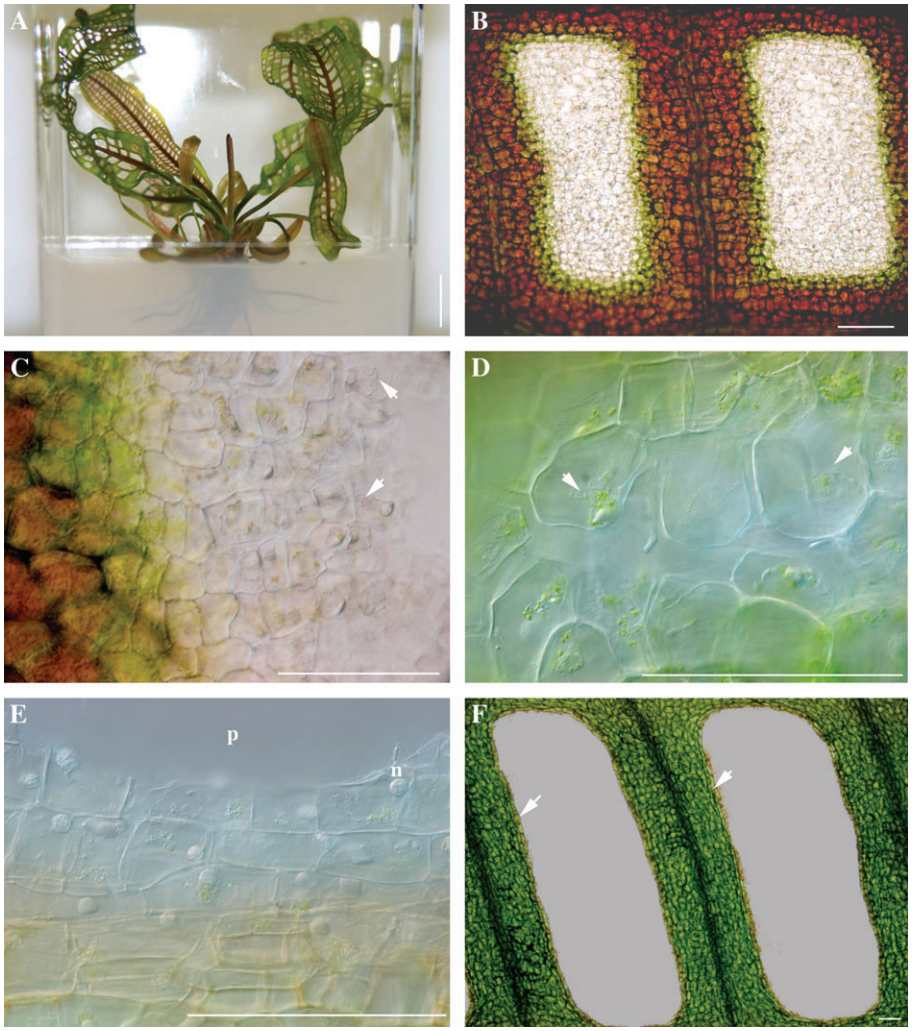

Fig. 1. Light micrographs of living lace plant leaves illustrating the stages of perforation development. (A) A lace plant grown in axenic culture in a magenta box. (B) Stage 2 ('window' formation) leaf showing distinct transparent regions between longitudinal and transverse veins as a result of loss of anthocyanin and chlorophyll, while showing anthocyanin in cells near the veins. (C and D) Stage 3 (perforation formation) leaf showing plasma membrane shrinkage and collapsed cytoplasm (arrows) in the dying cells. (E) Early stage 4 (perforation expansion) leaf showing the developing perforation (p) and adjacent cells at the perforation border undergoing programmed cell death. Note intact nuclei (n) in the dying cells and deposition of suberin (brown pigmentation) in cell walls along the perforation border. (F) Stage 5 (mature perforation) showing perforation formation arrested approximately five cells from the vascular tissue. Note deposition of suberin (arrows) within cell walls at the perforation border. Scale bars: A = 1.25 cm; B–F = 100 μm.

(stage 3, cytoplasm and cell wall degradation, Fig. 1C, D), perforation expansion (stage 4, beginning of suberin deposition at the perforation border, Fig. 1E), and mature perforation (stage 5, Fig. 1F; Gunawardena *et al*., 2004). Perforations therefore are formed at a set time in development and are localized between the intersections of longitudinal and transverse veins across the surface of the leaf. Since cell death occurs at a predictable stage of leaf development and at a precise location in relation to the completely formed leaf vein system, the lace plant provides ideal material for the study of PCD. In addition, lace plant leaves are only four cell layers thick and are transparent, facilitating whole-mount experimental procedures, time-lapse, and live-cell imaging of living leaves (Gunawardena *et al*., 2004). Whereas the cytological study of PCD is facilitated by the timing and location of perforation formation in lace plant leaves, *Monstera* is unlikely ever to be as tractable as a system for studying developmentally regulated PCD in plants. This is because perforation formation occurs early in development, when the leaves are rolled tightly in the bud and cannot be readily observed in living material (Gunawardena *et al*., unpublished).

In lace plants, the perforation site is characterized by a gradient of cells at different stages of PCD at any one time during the process of perforation formation in maturing leaves. This pattern of cell death resembles developmental PCD in some other well-characterized model systems.

For example, the starchy endosperm tissue in maize undergoes progressive cell death from tip to base during seed development (Young *et al*., 1997). Likewise, PCD during the abortion of stamen primordia in female flowers in maize is initiated near the apex of the primordium and is propagated basipetally (Cheng *et al*., 1983; Dellaporta and Calderon-Urrea, 1994; Calderon-Urrea and Dellaporta, 1999). The tepals of iris flowers are also an example of PCD that starts at the apex and moves basipetally (van Doorn *et al*., 2003). Based on the consistency of these spatial and temporal patterns, developmentally regulated PCD appears to be tightly regulated.

The lace plant grows as a submerged aquatic, and one major limitation of carrying out physiological experiments on submerged aquatic plants, in general, is the unknown role of associated micro-organisms in developmentally regulated PCD. Since lace plant leaves are actively perforating early in their development, it is assumed their immediate environment has high concentrations of nucleic acid, protein, and carbohydrate products of cellular degradation, which may promote the growth of micro-organisms (Gunawardena *et al*., unpublished). In addition, microscopic observations indicate the presence of a considerable flora and fauna in aquarium culture. To circumvent the problem of associated micro-organisms, Gunawardena *et al*. (2006) have successfully established axenic cultures of lace plant grown in magenta boxes (Fig. 1A), providing a system free of micro-organisms for experimental study. The process of perforation formation in leaves of subcultured plants did not differ substantially from that described for aquarium-grown lace plants, except for a slightly reduced level of cell wall degradation during perforation formation (Gunawardena *et al*., 2006).

In summary, the accessibility and predictability of perforation formation in lace plant leaves, combined with the ability to propagate this plant in sterile conditions, provide an attractive model system for the study of developmentally regulated PCD in plants.

Current work

Numerous signals and factors are thought to play an important role in PCD during perforation formation in lace plant leaves, with preliminary evidence pointing to caspase-like proteases and ethylene as potential mediators of the cell death signalling pathway (Gunawardena *et al*., unpublished). Ethylene has been shown to promote PCD in a number of systems, and one of the earliest examples of PCD recognized in plants, aerenchyma formation in hypoxic maize roots, requires ethylene (Jackson *et al*., 1985; He *et al*., 1996; Gunawardena *et al*., 2001*a*). Alternatively, inhibitors of ethylene biosynthesis or ethylene perception have been demonstrated to suppress PCD. For example, de Jong *et al*. (2002) demonstrated that aminoethoxyvinylglycine (AVG), a competitive inhibitor of ethylene biosynthesis, blocks camptothecin-induced hydrogen peroxide production and PCD in tomato suspension cells. To understand the role of ethylene in PCD during perforation formation in lace plant leaves, a series of pharmacological experiments using silver nitrate, an inhibitor of ethylene perception, were carried out by Gunawardena *et al*. in 2006. Although suppression of perforation formation was incomplete, leaves formed fewer perforations compared with controls. The length of treated leaves, however, also decreased and since there was a positive correlation between leaf length and perforation number for control and treatment plants, these experiments were inconclusive. Current experimental work using a wide range of concentrations of AVG to suppress perforation formation in lace plant leaves supports the contention that ethylene is a significant mediator of developmentally regulated PCD (Gunawardena *et al*., unpublished). The substantially reduced number of perforations in leaves of subcultured whole plants and in detached leaves treated with AVG suggests that ethylene may be an initial trigger in the cell death signalling pathway in lace plants. Likewise, preliminary results using a caspase-1 inhibitor, acyl-Tyr-Val-Ala-L-aspartic acid aldehyde (Ac-YVAD-CHO), on whole lace plants and detached leaves points to the involvement of caspase-like proteases in PCD during the remodeling of lace plant leaves. There is a wealth of data available, whereby inhibitors of caspases have been shown to block chemically induced PCD (Sun *et al*., 1999; de Jong *et al*., 2000; Mlejnek and Prochazka, 2002), HR-induced PCD (del Pozo and Lam, 1998; Hatsugai *et al*., 2004), developmentally induced PCD (Korthout *et al*., 2000; Bozhkov *et al*., 2004), and UV-induced PCD (Danon *et al*., 2004), strongly indicating that caspase-like activity is involved in the induction of PCD in plants. The interconnection between ethylene and caspase-like proteases in developmentally regulated PCD in lace plant is currently under study.

Conclusions and future work

The lace plant, by virtue of its highly accessible and predictable leaf perforations, provides an ideal model system for the study of developmentally regulated PCD in plants. Although progress has been made in the developmental and physiological analysis of the lace plant, much remains to be learned about the mechanisms that govern the cell death pathway in this system. The formation of perforations at precise locations between the longitudinal and transverse veins of lace plant leaves suggests that signals responsible for initiating PCD may originate in the vascular tissues. A similar hypothesis with regards to the origin of differentiation signals has been put forth to explain the placement of specialized cell types in the leaves of numerous higher plants (Nelson and Dengler, 1997). The lace plant system seems promising in the pursuit of

identifying the intracellular movement of putative death-activating or death-suppressing molecules in leaves. Similar to aerenchyma formation, the question of how a discrete subpopulation of cells is designated to undergo cell death, leaving adjacent cells intact, warrants future investigation. The leaves of the lace plant show a unique developmental pattern, in which two previously indistinguishable cells embark on opposite trajectories and have completely different fates: one to survive and the other to commit suicide. Identifying genes that are differentially expressed between tissue undergoing PCD and adjacent control tissue in the lace plant system may aid in providing further understanding of developmentally regulated PCD in plants.

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