Arunika N. Gunawardena · Paul F. McCabe
*Editors*

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# Preface

Why a book about plant programmed cell death? For us, one of the central reasons is that programmed cell death (PCD) is a fundamental process that, while utterly destructive on a cellular level, plays an indispensable role in plant development and defense. Indeed, it is a crucial cellular event that occurs throughout a plant's life cycle from the death of the embryonic suspensor to leaf and floral organ senescence. Plant PCD, however, has only been recognised as an organised, genetically controlled cellular process in the past 20 years, but after a slow start, publications are now beginning to exponentially increase as PCD becomes a mainstream research topic. While the number of research groups grows rapidly, there is at the same time a lack of content that provides a comprehensive overview, and which summarizes recent findings, in this fascinating new area of cell death. With this in mind, we therefore accepted the invitation of Eric Stannard, the Editor of Botany, Springer Science, USA, to write a book on "plant programmed cell death." We invited a broad range of internationally recognized PCD experts to contribute chapters for this book. There are 11 chapters in total, covering the most recent research findings in the area of plant PCD at the molecular, biochemical, and cellular levels. We hope this book will be an invaluable guide for graduate students, upper-level undergraduate students, and researchers who are entering the field of cell death research for the first time. Established researchers will also find this work indispensable as an up-to-date review of PCD topics.

We would like to thank all the authors for their help and patience in completing their chapters and for ultimately contributing to a book that provides researchers with a valuable and timely resource into the topic of cell death. We are grateful for the encouragement that we have received from many colleagues; without them, we would not have completed this book.

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# Chapter 1
# An Overview of Programmed Cell Death Research: From Canonical to Emerging Model Species

**Adrian N. Dauphinee and Arunika N. Gunawardena**

## 1.1 Cellular Death

### *1.1.1 The Significance and Diversity of Cell Death*

All living cells will eventually die, but the way in which they reach that fate, whether by environmental stressors or as part of development has profound implications on surrounding cells and tissues. Programmed cell death (PCD) is an active intracellular-mediated form of destruction that is critical for the development and survival of metazoans and plants, and more recently has been discovered in fungi and a diverse range of microbial organisms including yeast and bacteria [1, 2]. Bayles [2] draws parallels between bacterial and eukaryotic processes and, due to their functional conservation, proposes that mechanisms involved in bacterial cell death may have contributed the "evolutionary nuts and bolts" for eukaryotic PCD. It should be noted that the existence of PCD in bacteria has been debated and that further investigation is required in order to understand the evolution of eukaryotic PCD [2, 3]. PCD is best understood in multicellular eukaryotes, where it operates during the specialization and homeostasis of cells and tissues and provides defense toward destructive environmental stimuli. Therefore, dysfunction of the cellular death machinery can result in alterations to the organization of the body plan or cause disease and have serious fitness effects on an individual.

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### *1.1.2 Classes of Metazoan Cellular Death*

Cellular death is best understood in animals and was initially categorized into three major types primarily based on morphology: apoptosis (type I), autophagy or autophagic cell death (type II), and necrosis (type III; [4, 5]). Apoptosis is the most commonly observed form of PCD [5]. Kerr et al. [6] coined this usage of the term apoptosis, which is a Greek derivation for the dropping or falling off of petals or leaves from flowers or trees, respectively. Kerr et al. [6] observed that apoptosis was an active phenomenon with a complementary, although opposing role to mitosis in the homeostasis of animal cell populations. The morphological features of this process include: a reduction of cellular volume, chromatin condensation, nuclear fragmentation, no (or few) modifications to the ultrastructure of cytoplasmic organelles, plasma membrane (PM) blebbing, and an intact PM until advanced stages [7]. Cellular contents are packaged into apoptotic bodies, which are then engulfed and digested by phagocytes, and thus, an immune response is not triggered [5]. The biochemical pathways and molecular interactions that carry out apoptosis are now well understood.

In order to facilitate comparisons of cell death mechanisms among the kingdoms, the vertebrate apoptotic regimes known as the intrinsic and extrinsic pathways will be discussed in brief. In both of these apoptotic pathways, the irreversible destruction of the cell is executed by cysteine-aspartic proteases (caspases). The differences are in the activation of these executioner caspases. In the intrinsic pathway, Bcl-2 family proteins are key pro- and anti-apoptotic regulators that affect the release of the mitochondrial intermembrane space (IMS) protein cytochrome *c* (cyt *c*). Cyt *c* goes on to trigger intermediates (which vary among animals) prior to caspase activation. In the extrinsic pathway, caspase activation occurs after the formation of a death-inducing signaling complex (DISC), which follows the ligation of a death receptor found on the cell surface [8, 9]. Additionally, it should be pointed out that among animal systems, there is diversity in the numbers and types of caspases present, as well as a myriad of induction signals and pro/anti-apoptotic regulatory proteins involved. It appears as though evolution has fine-tuned apoptosis in various species leading to the wide diversity of pathways which have been found and reviewed in detail elsewhere [5, 10, 11]. It should also be noted that there are caspase-independent forms of apoptosis that have been recently reviewed, along with the role of autophagy in cell death (see [12]).

Type II cellular death, or autophagy (self-eating), is the other classically defined form of PCD that occurs in animals, but it is not as well defined or understood in comparison to apoptosis [5]. Similarities exist in that (1) neither triggers an inflammatory response in tissues and (2), like apoptosis, autophagy can exhibit DNA laddering and caspase activation; however, both of these features occur late, if at all [13–15]. Autophagic cell death is primarily distinguishable from apoptosis by morphology, the presence of autophagosomes, and involvement of autophagy proteins [5, 16]. Autophagosomes are double membrane lytic vesicles that contain cytosol or organelles that are still morphologically intact. Interestingly, autophagy can inhibit

and/or precede apoptosis [12]. Whether or not the autophagy itself actually contributes to cellular death has been debated; however, many researchers believe that this is the cells' last-ditch effort to stay alive, suggesting that the term autophagic cell death may be a misnomer [13, 16–18].

The third type of cellular death is necrosis (Type III), which is typically caused by severe injury to the cell and can be viewed as a chaotic form of death with swelling and bursting of the cell prior to the trigger of an immune response within an animal body. Necrotic death is usually initiated by an early rupture of the plasma membrane from a given stressor that triggers sudden physical damage to the plasma membrane, decreased energy levels, or loss of function of ion channels [19]. There is evidence suggesting that necrosis is also a form of PCD; however, this notion is still contested. Intermediate classifications such as necroptosis or aponecrosis [19, 20] have been used to define categories of cellular death, but these terms are not yet widely accepted [5]. There has been extensive research on animal cell death over the last four decades, and many aspects of the signaling and regulatory pathways of these cell death types have been discovered and are detailed elsewhere [5, 12, 19, 20]. It should be pointed out that as biochemical and molecular evidence accumulates, there appears to be overlapping mechanisms involved in apoptosis and autophagy [12, 21]; however, the ability of autophagy to cause cell death is in doubt [22]. In light of advancements in the field, the Nomenclature Committee on Cell Death (NCCD) recently proposed classifications based on well-defined subroutines that allow for their detection [4]. These subroutines are extrinsic and intrinsic apoptosis, regulated necrosis, autophagic cell death, and, finally, mitotic catastrophe [4]. However, for the purposes of this chapter, henceforth we will draw comparisons between cell death in plants and the characteristics of the three classical forms of animal cell death outlined above.

### *1.1.3 Cell Death Processes in Plants*

Plant cells, like those of animals, can be destroyed via PCD or necrosis, the latter being originally defined as an uncontrolled, accidental death. PCD is critical for proper development and survival of plants [23] and is activated during a myriad of life processes [24]. Putative examples of PCD in higher plants include the deletion of the embryonic suspensor, aleurone degeneration (in monocots), tracheary element and trichome development, shedding of root cap cells, aerenchyma formation, leaf morphogenesis, abortion of floral organs, megaspore development, and the hypersensitive response (HR) [25–28]. Through the study of these processes in various systems, similarities to animal apoptosis have been found including a reduction of cellular volume, nuclear condensation, nDNA fragmentation, release of cyt *c*, and the involvement of caspase-like proteases such as vacuolar processing enzymes (VPEs) and metacaspases [28–31]. Despite these similarities, there are marked differences between plant and animal PCD, some of which can be attributed to plant-specific properties such as cell walls, large central vacuoles, and chloroplasts. There

are no apoptotic bodies in plants, which is likely due to the fact that plant cells have rigid cell walls and do not require a systematic avoidance of an immune response [32]. The plant vacuole is a large hydrolytic compartment contributing up to 90 % of a cell's volume, and it plays a major role in the degradation of cellular constituents. Chloroplasts are suspected of playing key regulatory roles in cellular death signaling primarily due to their role in energy production and ability to produce reactive oxygen species (ROS) [33].

Studies of plant PCD have, in general, drawn heavily upon comparisons to animal cell death pathways [34]. However, the sessile life of the plant could very well have played a role in the evolution of different pathways for PCD. It has also been hypothesized that a "Red Queen" arms race between plants and pathogens in the HR may have contributed to the evolution of plant PCD [26]. Although the evolution and mechanisms of plant PCD pathways remain unclear, certain components have been elucidated and are summarized in recent reviews [28, 35]. Despite limited biochemical and molecular evidence, the categorization of the cellular death types found thus far is underway.

### *1.1.4 Classification of Plant PCD*

Van Doorn et al. [32] proposed that there be two groups of PCD in plants based on morphology: vacuolar cell death and necrosis. In brief, vacuolar cell death is a cellular death employing autophagy-like processes along with the release of hydrolases from lytic vacuoles to produce a cell corpse that is largely cleared. In contrast, necrotic death can be differentiated, as there is an early rupture of the PM, shrinkage of the protoplast, swelling of various organelles, a lack of autophagy, and a cell corpse that is more or less unprocessed. Additionally, the authors argued that apoptosis or apoptosis-like terminology should not be applied to plant PCD systems since the formation of apoptotic bodies is not observed following blebbing of the plasma membrane, which, as discussed earlier, is a primary morphological indicator of type I cell death (or apoptosis) in animals. Moreover, it is also noted by van Doorn et al. [32] that cases exist of either mixed or "atypical" cell death types that do not neatly fall into the proposed categories. The authors emphasize that their classification is not meant to be static and should be changed if necessary as more evidence is gathered.

Soon after the classification by van Doorn et al. [32], van Doorn [15] updated the groupings and suggested the replacement terms of autolytic and non-autolytic PCD for vacuolar and necrosis, respectively. Autolytic PCD is described as cell death with rapid clearance of the cytoplasm following tonoplast rupture, whereas non-autolytic is PCD that may or may not feature tonoplast rupture. In contrast to autolytic PCD, rapid destruction of the cytoplasm does not occur. It is argued that these two classes of PCD in plants subsume Type II (autophagy) and Type III (necrosis) cellular death in animals, respectively, and it is maintained that there is no evidence for Type I cell death (apoptosis) in plants. In the time since these publications, some

authors have cautioned the use of these proposed classifications [35–37], and revision of the current classifications continues.

The notion that the term apoptosis cannot be applied within plant PCD classification remains a point of contention. Reape and McCabe [37] came to the defense of "apoptotic-like PCD" (AL-PCD) classification and present evidence that there are cases of plasma membrane retraction that occurs in a programmed manner within plants. The result is a condensed cell morphology that is not due to the rupture of the PM. Under the classification of van Doorn et al. [32], this "AL-PCD" would be described as necrotic cellular death. The current authors understand Reape and McCabe's application of the terminology since a condensed cell morphology with shrinkage of an intact PM is a feature of lace plant PCD. However, we also believe that it may be a misnomer, as there are marked differences both morphologically and biochemically between apoptosis in animals and all forms of PCD that have been discovered in plants thus far. Further complications arise in that apoptosis-like cell death modalities are also described in animals [38], protozoans [39], yeast [40], and prokaryotic [41] systems which, in brief, bear some similarities to apoptosis but are regulated independently of caspases. This led the current authors to ask the following question: to what degree must cell death processes resemble each other before they should be considered alike? We believe this is a topical issue that extends beyond the plant PCD research community. Additionally, it should be mentioned that the application of an apoptosis-like cell death nomenclature in all kingdoms of life may raise concerns as it implies that there is an ancestral unicellular form of the PCD modalities seen in animals and plants, which has yet to be proven. Wang and Bayles [42] discuss functional conservation of underlying mechanisms between bacterial cell death and plant PCD and propose that the process is evolutionarily conserved among kingdoms of life. The current authors believe that further elucidation of cell death pathways is a necessary step in clarifying the evolutionary origins of PCD.

Bozhkov and Lam [34] called for the development of clear morphological classification, which lead to the proposed vacuolar and necrotic cell death types put forth by van Doorn et al. [32]. It is noteworthy that the authors indicated that the classes were not intended to be static, but instead be modified as more evidence comes to light. Despite the ongoing confusion regarding the proper categorization of PCD in plants, there appears to be a consensus that more biochemical and molecular data will ameliorate the situation and allow for the establishment of well-defined criteria. In 2005, the NCCD advised for the continued use of the three morphological types of animal cell death, but encouraged the scientific community to clearly state the accompanying regulatory processes [7]. Although biochemical and molecular data is far too limited to accurately categorize plant cell death pathways, the pursuit of a morphological classification system for plant cell death is a worthwhile endeavor that appears to have already stimulated researchers to analyze their systems more closely and join in the ongoing debate. Additionally, it is the opinion of the present authors that more morphological data from canonical and noncanonical plant systems will help in classifying cell death modes in plants and help to determine if atypical forms of PCD do in fact exist, as van Doorn et al. [32] suggest.

## 1.2 Model Species

### *1.2.1 Common Criteria*

In general, a model organism is one which exemplifies a given biological feature or is representative of a similar characteristic found in an organism of interest while being easier to conduct experiments with [43]. Smaller model organisms ease laboratory constraints, and their propagation and maintenance is often more economically feasible. Larger species are also used as models, but they may have more direct anthropogenic applications and economic impacts. Short generation times and high fecundity can also significantly impact the success of an organism as a model. This is primarily due to the benefits of generating large populations with ease, thus facilitating laboratory stocks. This in turn facilitates generational studies and may also affect genetic diversity and the time required to isolate additional populations, including mutants, all of which are important requirements for becoming a model system.

Typically, understanding the morphology and development of a species is the first step in characterizing a new model species. Further analysis of metabolism and physiology may follow. Currently, with the ease of genomic sequencing, the genomes of many model organisms have been sequenced [44]. The first few organisms to be sequenced were popular model species such as *Arabidopsis thaliana* [45] and *Drosophila melanogaster* [46]. Both of these species were known to have relatively few chromosomes (5 and 4 chromosomes, respectively). Organisms with small genomes are generally less costly and easier to sequence, and the availability of genomic sequences can greatly increase understanding of any given biological process or species [47]. The complete genomic sequencing of several key model organisms, such as those discussed herewith, has now opened the door for comparative genomic [44, 48] and proteomic [49] studies to better understand biological processes in non-model organisms.

The amenability of a species to genetic transformation is another factor that will influence its practicality and success as a model [50]. Recalcitrant species that require extensive optimization of protocols before yielding the desired transformants can become labor intensive and very costly. Without the ability to insert foreign genes into an organism of interest, a research program with great potential may be significantly limited. There are a wide range of protocols available for the transformation of plants including: microinjection, electroporation, polyethylene glycol, particle bombardment, calcium alginate beads, viral vectors, or most importantly perhaps *Agrobacterium tumefaciens* or *A. rhizogenes* [51–53].

### *1.2.2 Benefits and Costs of Working Within Model Systems*

Model organisms are used intensively in research due to the tractable nature of the system and are typically outstanding examples in one or more aspects that facilitate scientific exploration [47]. As established protocols for aspects of developmental

and genomic studies accumulate within a model system, an increasing number of laboratories are likely to implement it in their research programs due to the decreased investment in protocol development. Research communities then form, which leads to a common dialogue that propels efficiency. Over time, as the niche becomes more crowded, there may be significant downside through competition with other laboratories in terms of the publication of data and funding opportunities [47].

Model systems with "high-connectivity" occur when data from several organisms are integrated on multiple levels ranging from development to DNA [44]. The more research that is carried out within a given species, the more likely it is that connections can be drawn to other systems [54]. It is commonplace to use simple unicellular model organisms that behave similarly on a cellular level to a species of interest. This allows researchers to quickly determine what treatments are promising in a basic system, which would then be applied to another, more complex model organism. The approach is beneficial in the development of biomedical treatments dating back to the 1960s, as there has been a high degree of connectivity between biological processes in intensely studied models ranging from prokaryotic cells, yeast, protozoans, *Caenorhabditis elegans* (nematode), *D. melanogaster* (fruit fly), *Xenopus laevis* (clawed frog), *Mus musculus* (mouse), primates to mammalian cell cultures [54]. Using various systems can streamline the experimental process prior to testing it on mammalian model organisms, which is typically the final stage before human trials. One example comes from Alzheimer's research where model systems ranging from yeast, *C. elegans*, *D. melanogaster*, mammals to human cell cultures have been used [46]. It should be noted that it can be difficult or nearly impossible to assess the behavioral effects of a treatment in certain species and therefore, a given study may yield little to no information concerning how it may influence humans [46]. Additionally, in many cases, experimental treatments will vary significantly among different model systems, and misleading results may arise; nonetheless, this strategy remains viable in the search for advancements in the understanding of biological processes.

It is important to include a diverse range of organisms from all branches of the tree of life. Representatives from as many lineages as possible should be considered in order to understand evolutionary links and the relationships between extant and extinct species. In light of the complexity and diversity of cell death modalities among taxonomic groups observed to date, whether it is a cellular, biochemical, or molecular pathway, it seems imperative to expand horizons to non-model species. Although high-connectivity models (as described above) are often very useful, a given treatment that has an effect in one organism may yield very different results in another.

## 1.3 The Models of PCD Research

### *1.3.1 Canonical Animal Systems*

Studies in animal development have suggested that certain mechanisms of apoptosis and autophagic PCD were conserved through evolution [10, 55] The first in-depth genetic understanding of how the process was regulated came from *C. elegans*, a

soil nematode and commonly used model organism in developmental biology due to its small size, short-generation time, high fecundity, availability of mutants, and a sequenced genome [25]. The hermaphroditic form has 1090 somatic cells of which precisely 131 undergo apoptotic PCD before maturity leaving the adult form with 959 cells. This system was utilized to understand the genetic regulation of apoptosis, which shares many commonalities throughout the animal kingdom including those in mammalian systems [25]. *D. melanogaster* is another model organism whose development is well understood and has been studied extensively in animal PCD research. The best known form of autophagic PCD occurs during larval salivary gland development in *D. melanogaster*, wherein dying cells exhibit autophagic vacuoles and several hallmarks of apoptosis including caspase activation (reviewed by [56]). Another key species in the understanding of animal PCD is the mouse, where apoptosis deletes excess cells during developmental processes such as: limb bud formation, formation of the sympathetic nervous system and the negative selection, or clonal deletion of autoreactive thymocytes (T-cell precursors; reviewed by [57]).

### *1.3.2 Other Animals*

More recently utilized model species in the study of developmental PCD of animals includes *Hydra magnipapillata* (freshwater polyp), *Schmidtea mediterranea* (planarian), and *Danio rerio* (zebrafish) [57]. *H. magnipapillata* is a small transparent cnidarian, which is a group of aquatic invertebrates that branched off early in the evolution of bilaterally symmetrical animals. Developmentally regulated PCD occurs during oogenesis and spermatogenesis in hydras. These animals are interesting models for cell death as they are among the simplest multicellular invertebrates and can be compared to the more complex and well-understood *C. elegans* and *D. melanogaster* invertebrate systems to better understand the evolution of this processes. Lasi et al. [58] identified 15 caspases and described members of the Bcl-2 family of pro- and anti-apoptotic regulatory proteins. The results of their functional studies suggested that PCD may be more complex in these basal animals than in *C. elegans* and *D. melanogaster. Schmidtea mediterranea* is a freshwater bilateral invertebrate which is a model system for regeneration and repair. Remarkably, these simple animals can regenerate from small body segments [59]. It is proposed that this tissue remodeling is possible via neoblast stem cell division in combination with apoptosis of differentiated cells [60]. The zebrafish has emerged as an excellent vertebrate model for studying apoptosis throughout early development during processes which include but are not limited to neuronal, tail bud, and fin development [61].

### *1.3.3 Unicellular Species*

Although PCD was originally thought to be limited to multicellular eukaryotes, it now appears as though forms of intracellular-mediated death exist in unicellular organisms. Whether or not PCD mechanisms from certain unicellular organisms

can be related to processes described in animals and plants is still under debate and discussed elsewhere [1, 42, 62, 63]. Nevertheless, cell death research is being carried out in a diverse range of unicellular model organisms including bacteria, protists, yeast, and green algae. *Escherichia coli* was one of the earliest prokaryotic model organisms and activates a proposed form of PCD through the *mazEF* toxin antitoxin module [64]. This is believed to be an altruistic mechanism that occurs under various stressful conditions, where the survival of a subpopulation of the colony is achieved through the release of signaling molecules and nutrients [64]. All major groups of eukaryotic protists, excluding *Rhizaria*, have unicellular species in which characteristics of apoptosis have been observed (reviewed by [1]).

Baker's yeast (*Saccharomyces cerevisiae*) is a model organism that has gained popularity due to the ease of which it can be handled in a laboratory setting and the fact it is a eukaryote sharing many characteristics of mammalian apoptosis. Similarities include DNA fragmentation, condensation of chromatin, increased vesiculation, membrane blebbing, the loss of mitochondrial membrane potential, oxidative stress, externalization of phosphatidylserine, the release of mitochondrial proteins, and an increase in metacaspase activity [1, 65]. Due to these similarities, this organism is often used in biomedical research. Moreover, this species may help to elucidate how different types of cellular death are regulated [65]. The green alga *Chlamydomonas reinhardtii* which is dubbed "green yeast" can be viewed in a similar fashion, as it is a unicellular plant sharing many characteristics of PCD in higher plants including cellular shrinkage, increased vacuolization, fragmentation of nDNA, and the external accumulation of phosphatidylserine in the outer plasma membrane [1].

### *1.3.4 Canonical Plant Models*

*Arabidopsis thaliana* is a small flowering plant of the mustard (Brassicaceae) family and is the most widely used botanical model organism and has contributed significantly to our understanding of plant PCD. The species meets all of the criteria for model organisms mentioned above and has been domesticated through laboratory use since the early 1900s with its widespread in the 1980s [66]. A large number of mutants are available, and it was the first plant to have its genome sequenced. Moreover, there is a wide range of established protocols including those for efficient *A. tumefaciens-mediated* transformation, although another model organism used in plant PCD research, *Nicotiana tabacum* (tobacco), was experimentally transformed before *A. thaliana*. Interestingly, a novel root hair assay has been developed [67] for scoring apoptotic-like PCD in *A. thaliana* and demonstrates that new systems can be developed within a well-established model. A significant amount of research has been carried out on the hypersensitive response (HR) in Arabidopsis, tobacco, and tomato. This form of cellular death has immense agronomic significance and occurs as part of the systemic acquired resistance (SAR) of plants when a foreign biotic pathogen invades a plant cell [68]. The compromised cell signals to surrounding

cells which causes the cells to undergo cellular death and prevent the spread of the infection.

Another model is Norway spruce (*Picea abies*), which is an economically important conifer, but due to its size and slow reproduction, it may appear to be an unwieldy candidate for developmental PCD research. Nonetheless, the species' large embryos were identified as being ideal for live cell imaging and provided a useful system for studying somatic embryogenesis during which two waves of PCD can be detected [69]. Culturing methods were established, and a time-course analysis was carried out to determine the stages of development [70]. The involvement of autophagy and metacaspases has been shown in this form of PCD. For example, the metacaspase mcII-Pa is activated in terminally differentiated cells, where it then translocates to the nuclei from the cytoplasm prior to the dismantling of the nuclear envelope and the fragmentation of DNA. This model recently became the first gymnosperm species to have its genome sequenced [71], which will facilitate further research on developmental PCD and allow for more connections with established models such as *A. thaliana*. Similarly, the poplar (*Populus trichocarpa*) genome has been sequenced, and xylogenesis has been heavily studied in this species as well as close relatives. Interestingly, the stems of a hybrid aspen (*P. tremula* × *P. tremuloides*) have xylem fibers, wherein cells clear all their cytoplasmic contents prior to the rupture of the tonoplast, which is the opposite order of cellular events in other xylem element formation systems [72]. Overall, it has become an excellent angiosperm model.

Other well-characterized developmental PCD processes in plants include: endosperm development, which has been studied in species such as *Brachypodium distachyon* or purple false brome [73], maize [74], and wheat [75]. Aerenchyma formation has been studied in maize [76, 77] and rice [78]. Additionally, *Zinnia elegans* has been used to study the mechanisms that trigger transdifferentiation of isolated mesophyll cells into tracheary elements (TEs) and provides a highly efficient model for in vitro analysis (Fukuda and Komamine [79]). Secondary cell wall synthesis was found to be tightly correlated with PCD [80] which demonstrates that proteolysis of the extracellular matrix plays a central role in regulating this cell death process. Recently, *Z. elegans* cultures and *A. thaliana* whole plants were used to determine that lignification of TEs occurs postmortem [81]. The cross section of plant species mentioned thus far illuminates that plant models do not necessarily follow the same constraints as animals. With the exclusion of *A. thaliana* and *B. distachyon*, the majority are large in size; however, these larger species have been domesticated and have wide-ranging economic impacts.

## 1.4 Emerging Plant Models

The importance of emerging models in plant biology research was nicely reviewed by Mandoli and Olmstead [47]. They describe the pathway and potential downfalls that ultimately determine whether or not an emerging system will become an established model and also discuss the need for a variety of systems. In order to understand plant diversity from the organismal to molecular level, a wide range of models will be

needed [47]. Emerging models should be chosen wisely due to the costs associated with developing a new system such as the development/adaptation of protocols, domestication of the species, access to mutants, and the unavailability of genomic data. Although genetic data may be unavailable for many species, the advent of comparative genomics is likely to facilitate the use of non-model systems [44].

Mandoli and Olmstead [47] proposed that initiation and maintenance are the two phases in the construction of a model system. Although the two phases described the path to establishing a model, the current authors suggest that the two-phase pathway be expanded into four stages: identification, development, establishment, and maintenance. The identification step is critical and should be considered carefully as the species of interest may exhibit a biological process that is either outstanding or has the potential to contribute significantly to the literature. Next is the development phase where protocol establishment occurs, which would typically begin with the culturing and propagation of the organism. During this phase, the limitations of the organism are revealed, but if it proves to be a tractable system, further development will lead to its establishment as a model. Once established, a model organism accumulates genomic data and its use becomes widespread. Maintenance of a model is then determined by its popularity, which in all likelihood would be dictated by the economic climate, the niches available within the system, and competition from other established or emerging models. The aim of the following section is to provide a review of the development, as well as future perspectives, of lace plant PCD research while applying the proposed four stages that result in an established model organism.

## 1.5 Road Map to Success as a Model System: Lace Plant

Lace plant [*Aponogeton madagascariensis* (Mirb.) H. Bruggen] is a member of the Cape Pond-weed family, the Aponogetonaceae, which consists of a single genus of aquatic monocots believed to have 43 species [82]. Lace plants are endemic to the Comoros Islands and Madagascar, and naturalized in Mauritius. It is a submerged freshwater species found in stagnant and running waters, including rapids and torrents [82]. The plant has a spherical corm-bearing roots and helically arranged leaves and inflorescences with white or violet flowers on two spikes, which are exposed after the peduncle grows, allowing the spathe to emerge from the water [82]. The leaves have a distinct perforated leaf morphology which has led to its cultivation as an aquarium ornamental for over a century, although its cultivation is difficult and the plant rarely flowers [82, 83]. Lace plant is the only aquatic vascular plant known to produce holes during leaf morphogenesis. Outside of the *Aponogetonaceae*, only a few genera of the Araceae family are known to contain species with leaf perforations [84–86]. Both the Aponogetonaceae and the Araceae belong to the monocot order Alismatales, but it is unknown whether the formation of perforations during leaf morphogenesis in the two families has a common evolutionary origin. The function of perforations in leaf lamina remains unknown; however, several hypotheses have emerged. First, the perforations may aid in the regulation of heat transfer by

increasing the leaf perimeter to surface ratio. Alternatively, the holes may reduce herbivory, either by providing camouflage or by signaling to a herbivore that the leaf blade has already been compromised by a predator [85].

### *1.5.1 Stage 1: Identification*

The few publications concerning lace plant prior to the twenty-first century were descriptive studies involving the habitat, morphology, and classification of the species. Over a decade ago, research interests in PCD and leaf morphogenesis using the lace plant (Fig. 1.1) and *Monstera spp.* led to a study that characterized perforation formation during leaf development and demonstrated stage-specific DNA fragmentation indicative of PCD [84, 87]. PCD in the lace plant occurs in a spatiotemporally predictable manner between longitudinal and transverse veins and results in a lattice-like pattern in the mature adult leaves of the plant [87]. Typically, the first 3–4 leaves to form are juvenile and have a simple, non-perforated morphology and are smaller in comparison (Fig. 1.2a). Due to the dramatic change between the juvenile and adult leaf morphs, the plant can be said to develop in a metamorphic heteroblasty series [88, 89]. The availability of non-perforated, juvenile leaves may provide potential experimental controls or tissues to investigate inducers of

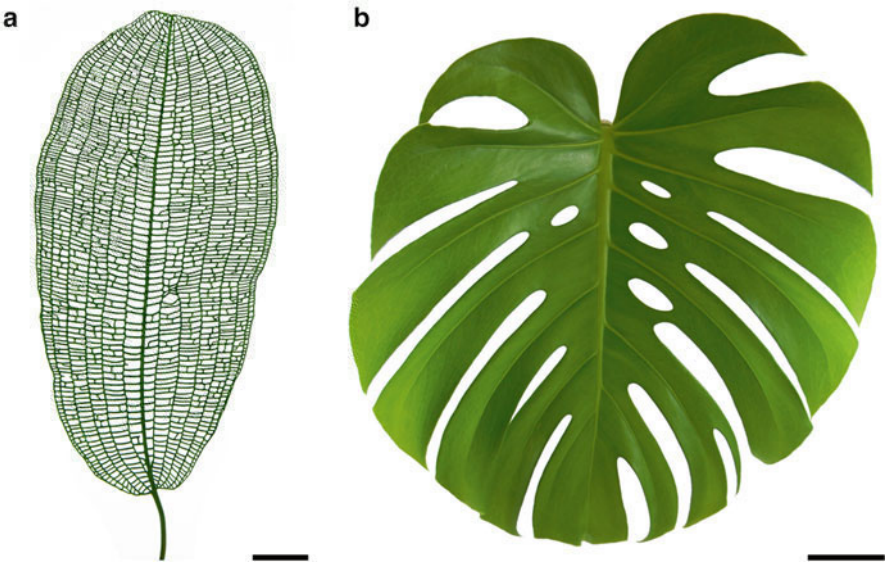


**Fig. 1.1** Programmed cell death (PCD) during leaf morphogenesis only occurs in vascular plants of the Aponogetonaceae and Araceae families. (**a**) The aquatic lace plant (*Aponogeton madagascariensis var. major*) has a highly perforated leaf lamina compared to that of (**b**) *Monstera deliciosa* (Araceae). Scale bars: (**a**) = 2 cm, (**b**) = 5 cm. Image backgrounds were removed using Adobe Photoshop CC (Adobe Systems Inc., San Jose, CA, USA)

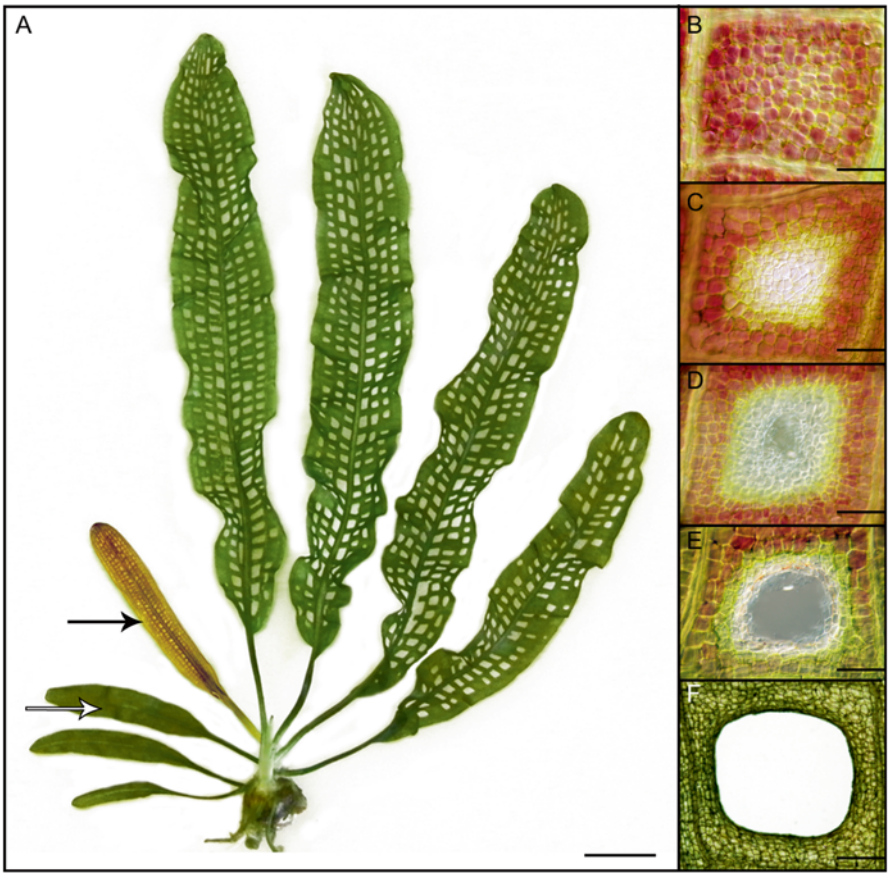


**Fig. 1.2** (**a**) The lace plant is a tractable model system for studying PCD primarily due to the spatiotemporal predictability of the process. The first 3–4 leaves of the plant are juvenile and do not form perforations (*white arrow*). The subsequent leaves to develop are adult leaves and emerge from the corm with a red pigmentation from anthocyanin (*black arrow*). (**b**) There are no visible signs that PCD will occur in pre-perforation stage areoles (space between longitudinal and transverse veins). (**c**) During the window stage of development, a gradient of PCD is visible as cells in the late stages of death have lost nearly all of their pigmentation. Cells in the early stages of death are green, but have lost their anthocyanin, which is still present in the cells that will survive through maturity. (**d**) The perforation formation stage occurs when a physical tear is visible in the areole. (**e**) Cell death then radiates outward, and the hole widens significantly before the perforation expansion stage. (**f**) PCD halts 4–5 cell layers from the veins by the mature stage. Scale bars: (**a**) = 3 cm, (**b**) = 50 μm, (**c**–**d**) = 75 μm, (**e**) = 100 μm, (**f**) = 300 μm. Image (**a**) background was removed using Adobe Photoshop CC

PCD. Interestingly, the transition between the juvenile and adult phases can be recognized at early stages of development as immature adult leaves emerge from the corm with a red pigmentation due to anthocyanins.

There are several visual cues that indicate the development of perforations and progression of adult leaf development. Based on macroscopic and microscopic

observations, Gunawardena et al. [87] identified five stages during this process: pre-perforation, window, perforation formation, perforation expansion, and mature. Pre-perforation leaves are those which have newly emerged from the corm, are tightly furled, and have an abundance of anthocyanins. Based on light and scanning electron microscopy observations, there are no morphological indications that PCD will occur in these leaves (Fig. 1.2b). PCD visibly occurs during the window stage (Fig. 1.2c), which exhibits distinct coloration within areoles (framed by longitudinal and transverse veins) due to cells at detectable stages of PCD which are discussed below. Next during perforation formation (Fig. 1.2d), a hole forms at the center of the areole and the zone of cell death extends outwards. The size of the perforation significantly increases by the perforation expansion stage, but halts 4–5 cell layers from the veins (Fig. 1.2e). At the mature stage of development (Fig. 1.2f), perforation formation is complete, and there are no longer any cells undergoing PCD. Additionally, mesophyll cells at the perforation border transdifferentiate to epidermal cells. The aforementioned developmental progression illustrates the temporal and spatial predictability of PCD and was a crucial stepping stone to identifying lace plant as a potential model system.

## *1.5.2 Stage 2: Development*

### 1.5.2.1 Propagation

Lace plants can be maintained in aquariums, but it is notoriously difficult, and the culture conditions of various *Aponogeton* species are known to affect leaf development [82]. In order to further establish the lace plant system, Gunawardena et al. [90] developed a protocol for clonal propagation of lace plant corms via sterile cultures. Sterile cultures are maintained in magenta G47 containers (Fig. 1.3) at 24 °C on a 12 h light/dark cycle. The plants are embedded in solid Murashige and Skoog (MS) media containing 1 % agar and submerged in liquid MS [90]. It is important to note that some *Aponogeton* species are known to exhibit morphological variability when cultured [82]; however, lace plants grown in magenta boxes follow the same developmental patterns, but produce slightly smaller leaves than aquarium-grown specimens [90]. The establishment of axenic cultures was a significant advancement as it provides a reliable source of tissues for further study and allows for pharmacological experimentation.

### 1.5.2.2 Live Cell Imaging and a Unique Gradient

The aquatic nature of the plant produces leaves that are nearly transparent and only 4–5 cell layers thick, making them ideal for live cell imaging [27, 87, 91–94]. Wright et al. [91] carried out a detailed study of the chloroplast and showed that the size and number of chloroplasts decreases throughout lace plant PCD similar to leaf senescence. Interestingly, the chloroplasts can also be seen dividing throughout

**Fig. 1.3** A lace plant one month after tissue culturing being grown under aseptic conditions in a magenta box containing approximately 50 ml of solid Murashige and Skoog (MS) media with 1 % agar and 200 ml of liquid MS media. Note the window stage leaf wherein PCD is actively occurring (*central, red leaf*). Scale bar = 2 cm. Image backgrounds were removed using Adobe Photoshop CC

PCD and accumulating around the nucleus in a ring-like formation [91]. The clustering of chloroplasts around the nucleus also occurs in tobacco suspension cells under osmotic stress [95]. The observation of chloroplast ring formation around the nucleus is particularly intriguing as the function of this process during cellular death is unknown. Due to the distinctive behavior of chloroplasts during lace plant PCD, it has been hypothesized that the chloroplast may have a critical role in the PCD signaling pathway. Chloroplasts have been implicated in PCD signaling pathways in other systems due to their critical roles in energy production and their ability to produce ROS [96]. Wright et al. [91] also witnessed an increase in transvacuolar strands in the early stages of lace plant PCD and a cessation of mitochondrial streaming within the final minutes prior to the death of the cells. Additionally, a time-course analysis revealed that the event of tonoplast collapse to the shrinkage of the PM occurs within 15–20 min.

Further cellular observations of the window stage leaves revealed that there is a visible gradient of cell death within each areole (Fig. 1.4a). Lord et al. [92] identified three distinct cell phases along this gradient: non, early, and late PCD (Fig. 1.4b–d; N-, E-, and LPCD, respectively). NPCD cells are those which retain their chlorophyll and anthocyanin pigmentation (which is in mesophyll cells) throughout perforation formation and persist throughout maturity. These cells maintain regular functions, unlike EPCD stage cells which are green due to chlorophyll pigmentation but have lost their anthocyanin and are fated to die. LPCD cells are on the brink of death and are distinguishable as being nearly transparent, with little to no chlorophyll pigmentation left. The fascinating gradient of PCD in the lace plant window stage is extremely accessible and can be seen and within a single field of view. This

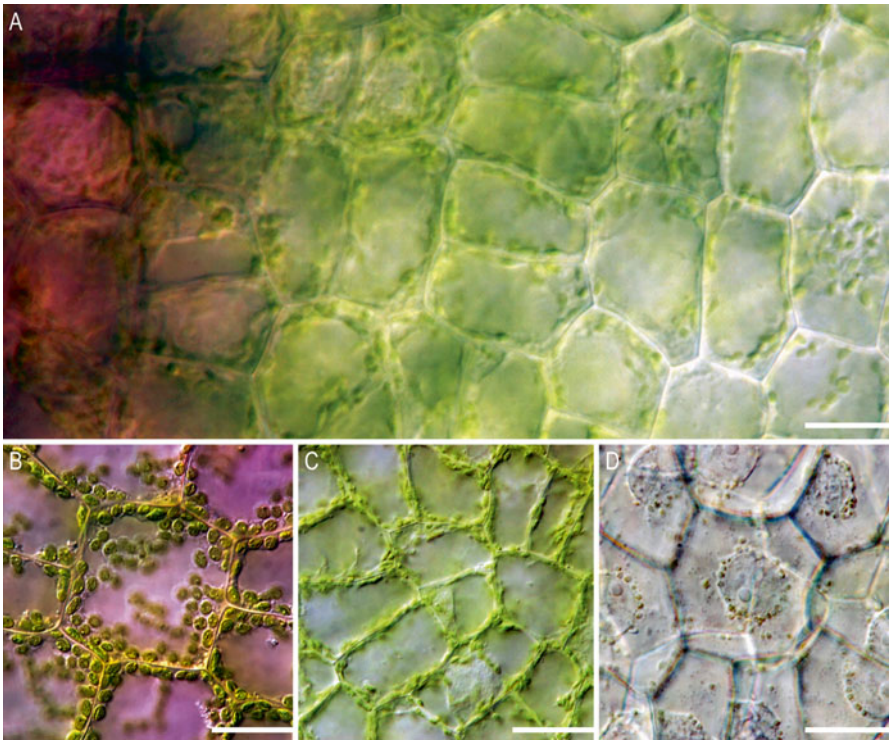


**Fig. 1.4** (**a**) Gradient of cell death in a window stage leaf of lace plant (*left* to *right*: N, E, and L or non, early, and late PCD, respectively). (**b**) NPCD stage cells have anthocyanin (in the mesophyll), as well as chlorophyll pigmentation, and do not undergo cellular death during leaf morphogenesis. (**c**) EPCD stage cells have lost anthocyanin pigmentation and are slated for destruction, but are in the early phases of the process. (**d**) LPCD stage cells are in the later stage of death and nearly transparent due to a reduction in chloroplasts and chlorophyll. Interestingly, perinuclear accumulation of chloroplasts can be seen during this stage. Scale bars: (**a**) = 30 μm, (**b**) = 40 μm, (**c**) = 50 μm, (**d**) = 40 μm

provides a distinct advantage over many other systems as cellular observations can be made simultaneously among control cells (NPCD) and those in the early and later phases of developmental cell death (Online Supplementary Material Video 1.1).

The dramatic cessation of mitochondrial streaming witnessed by Wright et al. [91] led to an investigation of the mitochondrial dynamics throughout lace plant PCD. To achieve this goal, Lord et al. [92] used the cell death gradient of the window stage. The temporal gradient of EPCD and LPCD allowed for the quick comparison of organelle dynamics within a single field of view with NPCD acting as the control. Individual mitochondria can be seen streaming actively in NPCD cells using CMXRos, which is a red-fluorescent dye that accumulates in mitochondria and is dependent on membrane potential. EPCD stage cells differ from NPCD in that there is observable organelle aggregation, while in LPCD stage cells, there is a cessation of streaming followed by the loss of mitochondrial membrane potential

($\Delta\Psi m$), as evidenced by the lack of CMXRos staining [92]. Moreover, the application of the mitochondrial permeability transition pore (PTP) inhibitor, cyclosporine A, causes the mitochondria to retain the dynamics of NPCD stage cells throughout leaf morphogenesis. Similar to the chloroplasts, mitochondria also play a role in the signaling pathway of lace plant PCD.

More recently, the chronological order of cellular events throughout lace plant developmental PCD was described using the unique gradient of PCD in combination with compound light, scanning electron, and laser scanning confocal microscopy techniques [93]. The degradation of anthocyanin pigmentation and an increase in vesiculation (which continues to the very late stages of PCD) are among the first visible cues indicating that cells are fated to die. Following this, chlorophyll degradation begins along with actin microfilament bundling (visualized using Alexa Fluor 488 phalloidin staining) and an increase in organelle movement on transvacuolar strands. The aggregation of mitochondria and the perinuclear accumulation of mitochondria and chloroplasts follow. Later in the cell death process, TUNEL positive nuclei (nDNA fragmentation), the breakdown of the actin cytoskeleton, and early changes in the cell wall were observed. Prior to cell death, there is a visible swelling of the vacuole followed by the tonoplast collapse. The cessation of vacuolar aggregate movement, nuclear shrinkage, and the complete loss of mitochondrial membrane potential occur in the time between tonoplast and plasma membrane collapse. The visible dissolution of the cell wall takes place after the condensation of the cell [93]. A previous study concerning the cell wall revealed that cell wall degradation begins early in the cell death process and the walls are significantly weakened by the time perforations form, thereby facilitating the mechanical rupture [97].

In order to accurately assess the timing of certain cellular events throughout lace plant PCD, a custom slide was created to allow the observation of whole leaves. The custom slide was a thin polyethylene rectangle mimicking a common glass slide, with a groove that could accommodate the width and depth of the midrib carved out using an awl. This allowed for a whole leaf mount and for the leaf blade to lie flat on either side of the groove, thereby reducing negative focal plane distortions within a single field of view. Long-term live cell imaging experiments lasting greater than 72 h were then carried out in whole leaves. Throughout the observation period, the leaves transitioned from window stage to the perforation expansion stage [93]. It should be noted that previous experiments had been carried out in detached leaves grown in petri dishes with MS medium, and development patterns were assessed over several days after which perforations form, indicating that early window stage leaves exhibit normal growth (unpublished data). Furthermore, long-term experiments that captured continuous video as the perforation developed revealed that during lace plant leaf morphogenesis, the point of chlorophyll reduction to the collapse of the PM takes approximately 48 h [93]. This imaging protocol for viewing perforation development when used for future in vivo studies in combination with pharmacological whole plant experiments will help to reveal the pathways of PCD in lace plant.

#### 1.5.2.3 Pharmacological Experiments

Sterile lace plant cultures grown in Magenta boxes are ideal for pharmacological experiments. Pharmacological experiments in the lace plant, thus far, have employed inhibitors or promoters of signaling molecules implicated in other PCD regimes. During these studies, whole plants are exposed to a given treatment, and subsequent leaf development is compared to those that develop at the same time in control plants. The first signaling molecule found to play a role in lace plant PCD was calcium [98], where the use of the calcium channel blocker ruthenium red resulted in the production of leaves with significantly fewer perforations compared to controls. Similar results were found by inhibiting biosynthesis of the phytohormone ethylene using aminoethoxyvinylglycine [99]. Additionally, an ethylene precursor (aminocyclopropane-1-carboxylic acid) was used in combination with AVG and reversed the effect. Whole plant experiments were also carried out using cyclosporine A, which also inhibited perforation formation and further implicated the mitochondria in lace plant PCD [92]. More recently, caspase-like protease activity has also been shown to play a role in the pathway [100]. The authors believe that the experimental results listed above illuminate the suitability of the lace plant model system for in vivo investigations of developmentally regulated PCD in plants.

#### 1.5.2.4 Callus Induction and Whole Plant Regeneration

Protocols for the induction of callus (Fig. 1.5a) can greatly benefit a plant research program as callus is comprised of undifferentiated cells, which is considered a source for generating whole plants, as well as a viable option for plant transformation [99]. Explants used for the development of these protocols in lace plant include roots, corms with and without meristems, leaves, isolated protoplasts, and immature inflorescences. In order to initiate callus, a variety of combinations and concentrations of exogenous cytokinins and auxins were tested. Carter and Gunawardena [101] were able to successfully induce callus from young inflorescences by


**Fig. 1.5** Callus induction from corm and whole plant regeneration. (**a**) Lace plant green (*white arrow*) and clear friable (*black arrow*) callus tissues capable of somatic embryogenesis. (**b**) Shoot development can be observed within 4–6 weeks. (**c**) Whole plants and multiple shoot systems typically develop within 8–12 weeks. Scale bars = 1 cm. Image backgrounds were removed using Adobe Photoshop CC