Arunika N. Gunawardena · Paul F. McCabe
Editors

Plant Programmed Cell Death

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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

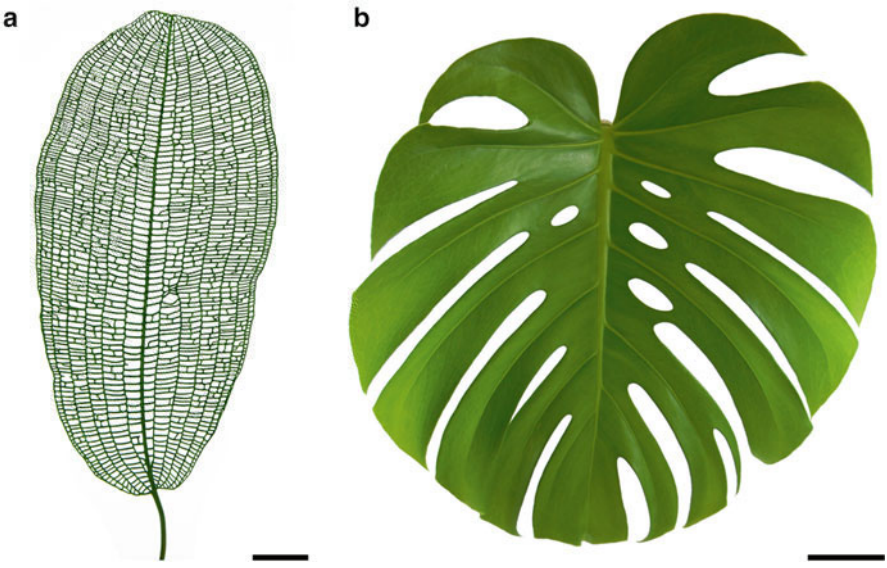

Fig. 1.1 Programmed cell death (PCD) during leaf morphogenesis only occurs in vascular plants of the Aponogetonaceae and Araceae families. (**a**) The aquatic lace plant (*Aponogeton madagascariensis var. major*) has a highly perforated leaf lamina compared to that of (**b**) *Monstera deliciosa* (Araceae).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

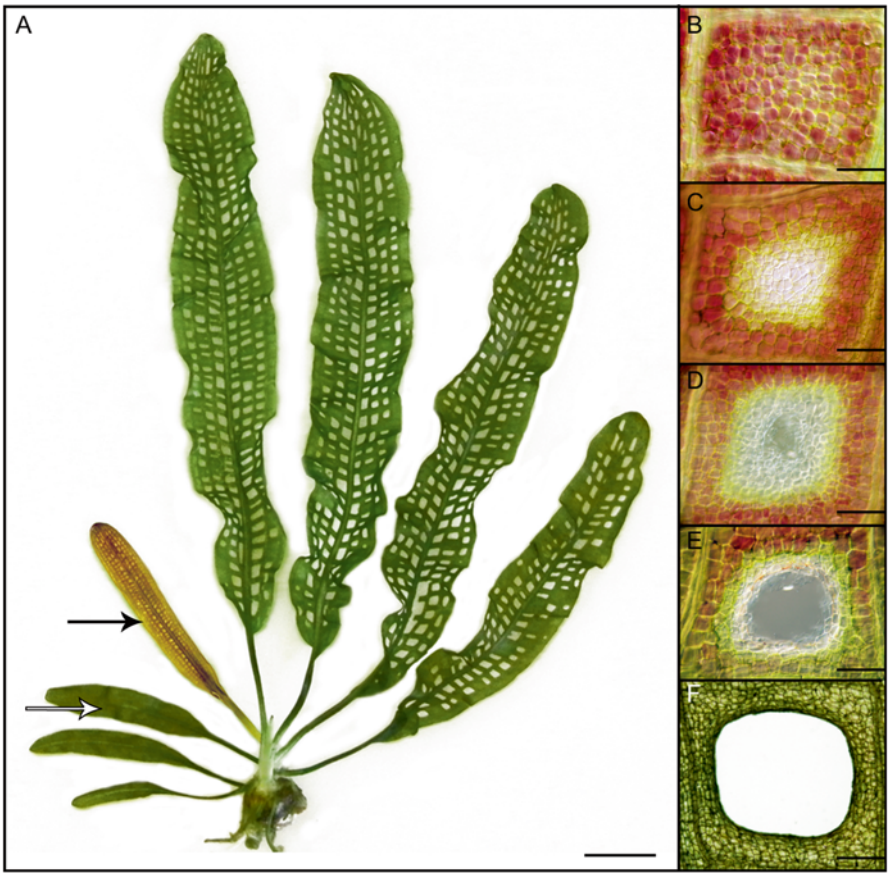

Fig. 1.2 (**a**) The lace plant is a tractable model system for studying PCD primarily due to the spatiotemporal predictability of the process. The first 3–4 leaves of the plant are juvenile and do not form perforations (*white arrow*). The subsequent leaves to develop are adult leaves and emerge from 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

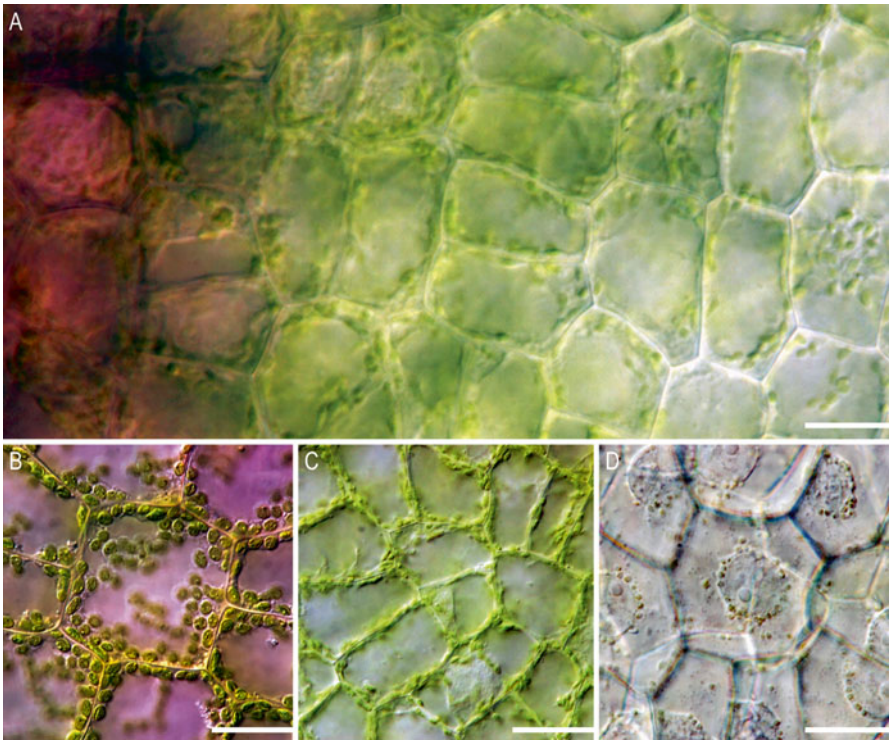

Fig. 1.4 (**a**) Gradient of cell death in a window stage leaf of lace plant (*left* to *right*: N, E, and L or non, early, and late PCD, respectively). (**b**) NPCD stage cells have anthocyanin (in the mesophyll), as well as chlorophyll pigmentation, and do not undergo cellular death during leaf morphogenesis. (**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

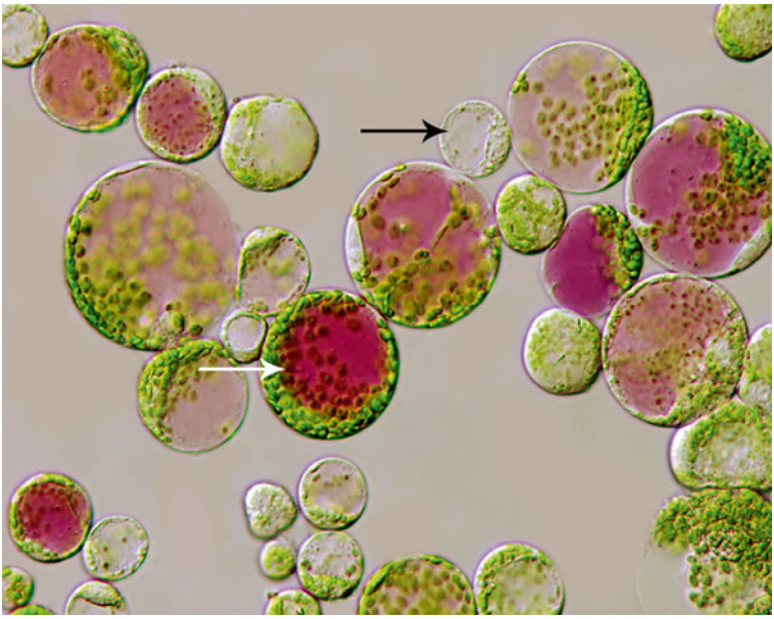

Fig. 1.6 Isolated protoplasts from a window stage leaf. Note that the larger, anthocyanin-laden cells (*white arrow*) are NPCD mesophyll cells and the smaller clear cells (*black arrow*) are epidermal cells. Scale bar: 10 μm

exposing them to 2 mg L^{-1} of the cytokinin 6-benzylaminopurine (BAP) and 1 mg L^{-1} of the auxin 1-naphthaleneacetic acid (NAA). Additionally, they were able to regenerate whole plants from this callus using this treatment under light conditions, which exhibited the same heteroblastic series of leaf development described above. Although this method yielded viable callus, flowering is extremely rare with the current culture technique. Therefore, highly abundant sterile corm tissue was used as alternate explant material. Recently, regeneration of whole plants has been accomplished utilizing callus derived from corm tissues (Fig. 1.5b, c), which are highly abundant in sterile lace plant cultures (unpublished data).

1.5.2.5 Induction of Cell Death

Protoplasts are plant cells with their cell walls removed (Fig. 1.6). They are commonly used in basic cell biology research and somatic hybridization experiments. Methods were developed for protoplast isolation in lace plant in order to facilitate rapid in vitro investigations. Different leaf explants of various ages, in combination with several carbohydrate sources and enzyme incubation times, had significant effects on the yield. Optimum results were obtained from window stage leaves using an enzymatic mixture containing 2 % w/v cellulose, 0.5 % pectolyase Y-23 that was dissolved in a 5 mM MES, and 600 mM sorbitol solution with a pH of 5.5 [103].

Following the establishment of the protocol, an in vitro study was carried out in which environmentally induced PCD was triggered in protoplasts of cells from early mature stage in order to ensure that cells already undergoing PCD were not present. Environmentally induced death was triggered via exposure to a heat shock treatment at 55 °C for 20 min. The study drew comparisons between environmentally and developmentally regulated PCD – two broad categories of PCD [27]. The study by Lord and Gunawardena [103] was the first to compare and contrast these two classes of cell death within a single system. Similarities between the two modes of cellular death included the blebbing of the PM, increased numbers of transvacuolar strands and vesicles, Brownian motion in the vacuole, nuclear condensation, and TUNEL positive nuclei [103]. Additionally, the mitochondria were implicated in the environmentally induced system as heat shock and cyclosporine A-treated protoplasts were able to retain their membrane potential and maintain viability longer when compared to the HS treatment alone [103].

A recent study by Dauphinee et al. [94] investigated induced cell death in lace plant leaf sections and whole leaves using a custom slide. A spectrum ranging from low to extreme levels of various stressors including heat, sodium chloride, hydrogen chloride, and sodium hydroxide was applied, and then NPCD cells were observed via live cell imaging. Video data was used to analyze the morphologies and determine the timeframe until death. Comparisons were then drawn to the established developmental PCD pathway described by Wertman et al. [93] and detailed above. In lace plant developmental PCD, the vacuole plays a central role, and there is a retraction of the PM at death (Fig. 1.7a). Interestingly, there are consistencies in vacuolar dynamics in all instances where death was induced, including extremely harsh stressors (e.g., 1 M NaOH, 12 M HCl) where the mean time for cell death is quite rapid (several minutes or less). There are also significant differences in morphologies among the treatments, most notably in cell volume reduction or retraction

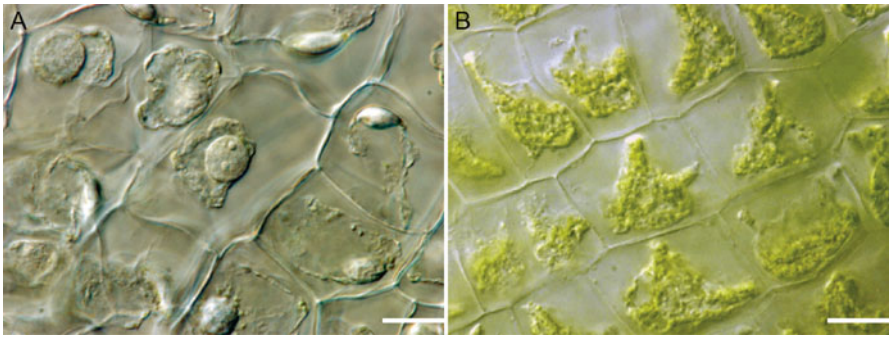

Fig. 1.7 Developmental and induced cell death in the lace plant system. (**a**) Cells which have died via developmentally regulated PCD (~48 h process) showing a neatly package cell corpse. (**b**) 2 M NaCl wet mount cells turn green and die within ~4 h of initial exposure. The cells also have a condensed morphology that is somewhat similar to that seen in developmental PCD, but there is less cytoplasmic clearing in comparison, and the process is considerably faster. Scale bars: (**a**) = 10 μm, (**b**) = 20 μm

of the PM at death. Stressors that induce cell death without PM retraction include a 65 °C heat shock, 400 mM NaCl, 3 mM HCl, 30 mM NaOH, and 1 M NaOH. The treatments that do initiate PM retraction are a 55 °C heat shock, 12 M HCL, and 2 M NaCl (Fig. 1.7b). Although a cell death morphology with a reduction in cell volume and PM retraction is typically considered a sign of PCD, the data suggests that the type of environmental stress will significantly alter the dynamics of cell death and thus the final morphology [94]. A live cell imaging comparison between developmental PCD and induced cell death (2 M NaCl) can be seen in Online Supplementary Video 1.2. The current authors believe that understanding morphological variability in response to various cell death induction signals, along with biochemical and molecular, will contribute to the debates surrounding cell death classifications.

1.5.2.6 Current Work

Despite the numerous protocols listed above, the lace plant model system can still be considered in the development phase. There are several research projects underway that will contribute to the toolkit and illustrate its future viability as a model system. A protocol for *A. tumefaciens*-mediated genetic transformation is being carried out using callus tissues, as well as isolated shoot apical meristems (Fig. 1.8; Online Supplementary Material Video 1.3) in order to achieve whole plant transformants that express genes of interest to better understand the mechanisms of PCD. Additionally, transient transformation techniques are being developed using leaf sheath tissues (Fig. 1.9), which are nearly transparent and have cells that are ideal for live cell imaging as they are only two cell layers thick (Online Supplementary Material Video 1.4). Fluorescent proteins tagged to organelles are of particular

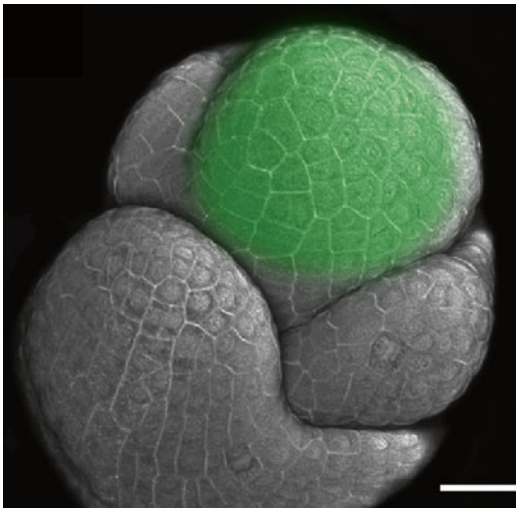

Fig. 1.8 Shoot apical meristem (SAM; *green*) and early leaf primordial of the lace plant. Maximum projection image from 30 Z-stacks acquired via laser scanning confocal microscopy. Scale bar: 30 μm. Image background was removed using Adobe Photoshop CC

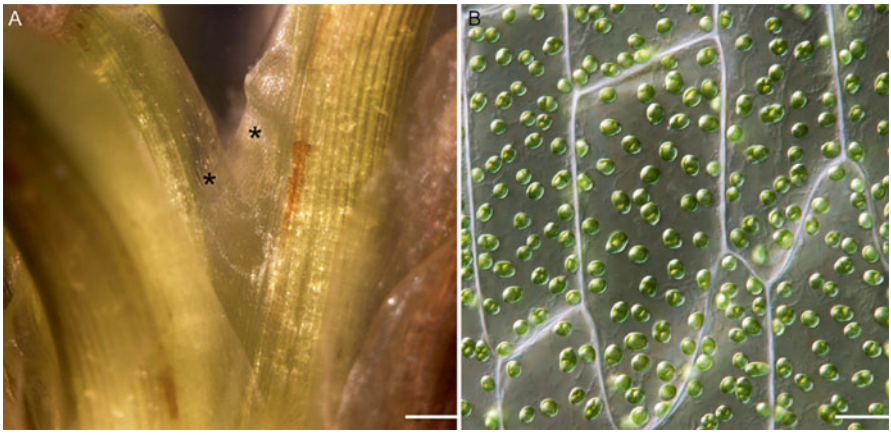

Fig. 1.9 Lace plant leaf sheaths. (**a**) Leaf sheaths (*asterisks*) are nearly transparent tissues flanking both sides of the petiole base. (**b**) Cells within the apical region of a mature leaf sheath contain many chloroplasts with starch granules. Scale bars: (**a**) = 2 mm, (**b**) = 15 μm

interest as they would facilitate cellular observations and circumvent the use of stains. Preliminary results acquired in collaboration with Dr. Keiko Yoshioka (University of Toronto, Canada) suggest that the lace plant is amenable to *A. tumefaciens* transformation and that callus is most effective for stable transformation; however, protocols are still being developed to increase transformation efficiency (unpublished).

Molecular studies on the genetic regulation of PCD during perforation formation in the lace plant were challenging since nothing was known about its genome and there were no molecular data on the species prior to this work. Despite these challenges, several approaches have been successfully used to elucidate some candidate genes involved in lace plant PCD. These approaches include degenerate primers, expressed sequence tag (EST), cDNA Amplified fragment length polymorphism (cDNA AFLP) and Quantitative PCR. The EST database revealed some of the genes expressed in lace plant leaves undergoing developmental PCD. cDNA AFLP analysis carried out in collaboration with Dr. Frank Van Breusegem (Ghent University, Belgium) was used to identify transcripts from some of the differentially expressed genes during lace plant PCD. This technique also provided data on the transcript expression patterns of different clusters of genes during lace plant leaf development. Degenerate primers and quantitative PCR were used to study the transcript expression patterns of the genes involved in ethylene signaling and VPEs throughout the entire process of lace plant leaf development. Moreover, the expression pattern of these genes were compared in PCD versus NPCD cells, which were separated using state of the art laser capture microscopy. In brief, the results of this work point towards the involvement of two ethylene receptors and two VPEs in the regulation of lace plant PCD and ethylene effects the expression levels of VPEs (Rantong et al., unpublished). These results will provide a foundation for further studies on genetic regulation of PCD in the lace plant and the techniques used in this

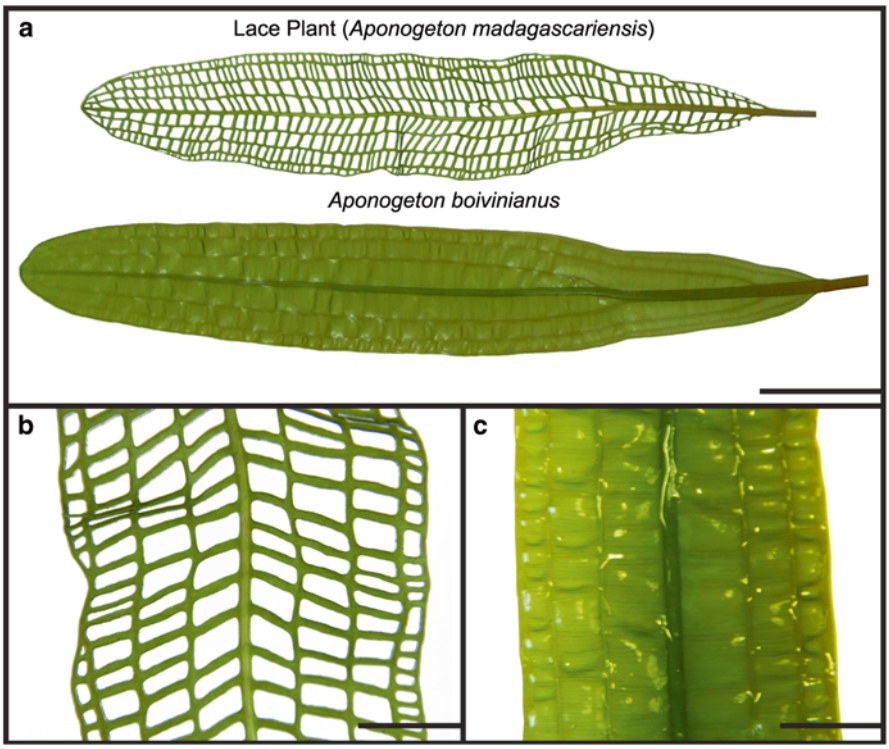

Fig. 1.10 Lace plant (*Aponogeton madagascariensis*) and *Aponogeton boivinianus*. (**a**) The mature leaf morphologies of lace plant and *A. boivinianus* are similar in framework; however, lace plant leaves have holes which are produced via developmentally regulated PCD, whereas *A. boivinianus* has a bullate leaf morphology and does not undergo PCD during leaf morphogenesis. (**b**) Central region of a mature lace plant leaf. (**c**) Central region of a mature *A. boivinianus* leaf. Scale bars: (**a**) = 3 cm, (**b**) and (**c**) = 1 cm. Image backgrounds were removed using Adobe Photoshop CC

research provide a potential guide for researchers looking to work on a non-model species with no available molecular data.

There are two lace plant projects in collaboration with Dr. Christian Lacroix (University of Prince Edward Island). The first involves anthocyanins, which in epidermal cells are known to play a phytoprotective role in land plants [104], but their function in lace plant mesophyll cells is unknown, therefore the role of anthocyanin and ROS in lace plant PCD using whole plants and detached leaves are currently under investigation. The other study focused on the early developmental morphologies of the lace plant and *Aponogeton boivinianus*. *A. boivnianus* is a close relative of the lace plant, but unlike the lace plant it does not employ PCD during leaf development (Fig. 1.10). The shoot apical meristems and the entire sequence of early leaf development were investigated in both species using confocal microscopy, as well as SEM using both fresh, hydrated specimens and conventional sample preparations [105]. Studying a close relative that does not produce holes via

PCD, but is otherwise quite similar in terms of the framework of its leaves, may provide a new avenue for research and a system to better understand the regulatory mechanism of lace plant PCD.

1.5.3 Stages 3 and 4: Establishment and Maintenance, Future Perspectives

The perforated leaves of lace plant have an unusual and fascinating morphology that provide a unique opportunity to advance the understanding of plant PCD, particularly the spatial and temporal controls of developmentally regulated PCD. Is the suite of characters involved in preexisting PCD, for example, that are used in the HR response simply co-opted for a new use in leaf morphogenesis? Or are the mechanisms of PCD execution assembled piecemeal during the evolution of leaf development in this group of plants? Lace plant has great potential for advancing understanding of the cellular processes of plant PCD and their role in morphogenesis; however, in order for the lace plant to become a model organism, more genetic data is required, as well as an increase in its use in research labs. The amenability of the lace plant to molecular techniques and *A. tumefaciens*-mediated transformation are major achievements in this aspect that will likely play significant roles in the development of the system.

Another future project to establish the organism as a model system is through the development of suspension cultures from callus and/or isolated protoplasts. The aforementioned techniques will facilitate a greater understanding, and the manipulation of the mechanisms behind PCD, which is especially intriguing in the lace plant where the morphological consequences of genes can be readily assessed on the cellular, tissue, and organismal levels. The protocols described above support the potential of lace plant to become not only a model organism for PCD study but also a representative freshwater aquatic species. Additionally, the plant may prove useful for teaching purposes as laboratory exercises have already been published and implemented due to the aesthetics of the plant and effortless live cell imaging in a laboratory setting [106].

Considering the strengths of the lace plant model system and the PCD research field, a few potential short- and long-term applications will be discussed. Firstly, the current work mentioned above may have an immediate impact on our understanding of plant PCD. Secondly, plant PCD study may reveal regulatory mechanisms that can be manipulated in order to enhance horticultural or agricultural practices. This notion is not only limited to senescence-associated losses but also to embryogenesis and aerenchyma formation among other developmental processes that employ PCD. More distant hopes for PCD research relate to the potential contribution toward the development of biomedical applications using compounds involved in the regulation of plant cell death. Such crossover studies are not out of reach, for example, it has been shown that overexpression of an anti-apoptotic mammalian Bcl-2 family protein as well as a *C. elegans* homolog (ced-9) has the ability to

reduce UV, HR, and paraquat-induced cell death in tobacco [107]. This suggests that there are functional similarities between animal and plant cell death and therefore lend to the possibility of gaining a deeper understanding of cell death by expressing plant genes of interest in animal systems and vice versa.

1.5.3.1 Roadblocks

While reviewing the importance of plant model organisms, Mandoli and Olmstead [47] discussed the tendency of researchers not to publish negative results and how this may limit the development of model systems, as other research groups may succumb to the same hazards. Therefore, they suggested that researchers developing a model system take the time to write a review and discuss troubles incurred in hopes to promote the use of the model system. Throughout the last decade, there have been numerous obstacles in the development of protocols, one of which was presented during RNA extraction. No kits or protocols from developed systems worked for lace plant leaf tissues until the midrib of the leaves was removed. Microscopic observations revealed laticifers were associated with lace plant leaf veins. Complex secondary products such as phenolic compounds and polysaccharides are known to make RNA extraction difficult in plants [108]. The lace plant midrib, containing laticifers [82, 109], likely contained secondary compounds that lead to the hindrance or inhibition of quality RNA extraction from lace plant leaves. Therefore, all RNA extractions were carried out without the midrib of the leaves, and good-quality RNA was obtained thenceforth.

Continuous long-term (12–72 h) live cell imaging of this unique PCD process is also very challenging, particularly due to the fact that intact leaves continue growing throughout the observation period. In order to accommodate experiments using whole organs such as leaves, custom slides or containers are required. Acquiring appropriate materials and developing such a slide took numerous trials. While developing the slides used for lace plant leaves, various plastics of different thicknesses were tested using a compound light microscope until one was found that allowed for good resolution. Along with an idealized slide, an autofocusing system would be useful in combination with a mechanical stage in order to compensate mechanical drift and leaf growth, respectively. Without such attachments on a microscope, manual adjustments and therefore a considerable amount of labor are necessary to carry out such experiments.

Perhaps the most challenging roadblock in the past thirteen years has been the reliable induction of inflorescences. Obtaining viable seeds remains an issue both in aquarium and the axenic cultures. Various light settings, temperatures, and hormone treatments have been tested. Recently however, there has been progress with culturing technique and aquarium plants have produced viable seeds. The challenge now will be to produce new axenic cultures from the seeds. The promotion of flowering in the plant and the ability to collect viable seeds will enhance the system, provide new options for plant transformation and reduce our reliance on clonal propagation for steady plant production.

1.6 Conclusions

While there remain many questions surrounding the evolution, regulation, and classification of PCD, a great deal of experimental data has been acquired through the use of model and non-model organisms from all kingdoms of life. With an increasing number of genetic sequences readily available, comparative genetic studies can be carried out in a wide range of species that may not possess all the criteria to be an obvious choice, but have interesting or unique developmental processes that warrant investigation. Although there are downsides to using non-model species, specifically the time investments required for the optimization of protocols, which may already be available in model species, it is still necessary for researchers to branch out and explore a greater diversity of life forms. In plant PCD specifically, we believe that broadening research to more species or systems will contribute in the resolution of cell death classification, as there are only a limited number of well-characterized developmentally regulated PCD systems to date. One such system amenable for PCD study is the unique emerging lace plant model. Further elucidation of the biochemical and molecular pathways involved in the induction and execution of plant PCD will be carried out using established and emerging models and through the use of various systems the field is expected to advance significantly.

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Chapter 2
Mitochondrion and Chloroplast Regulation of Plant Programmed Cell Death

Theresa J. Reape, Niall P. Brogan, and Paul F. McCabe

2.1 Introduction

Programmed cell death (PCD) is a sequence of events that lead to the controlled and organised destruction of the cell [1]. PCD is a fundamental process in plants, controlling the elimination of cells during development, defence (the hypersensitive response) and stress responses (see Kacprzyk et al. [2] for comprehensive review). The decision as to whether a plant cell activates PCD, or not, is determined by information it receives from a number of sources, including its environment, for example, cell survival signals, developmental cues, pathogen recognition, stress signals or internal information such as developmental history, cellular damage and metabolic state [3]. Different modes of PCD occur in plant cells; one type is strongly characterised by condensation of the protoplast away from the cell wall. This distinctive morphology has been visualised in cells which have died following stress, during developmental programming or during the hypersensitive response [4]. In terms of a stress response, cells can often survive a mild stress, while higher levels of stress induce the cells to initiate a PCD programme resulting in corpse cells with a condensed protoplast [5]. Increasing the stress to an even higher level leads, of course, to death, but cells do not display this distinctive morphology and are deemed to have died via necrosis [5] (see Fig. 2.1). By monitoring the level of stress applied, an abiotic stress such as heat shock can cause plant cells to die via PCD, and following heat shock, the classic condensed protoplast morphology has been observed in many species such as *Arabidopsis*, carrot, tobacco, soybean, *Zea mays*, *Quercus robur*, *Medicago truncatula* and lace plant [6–11]. Following biotic stresses such as the hypersensitive response (HR), cells at the site of pathogen invasion undergo PCD, isolating the pathogen and preventing further spread [12, 13].

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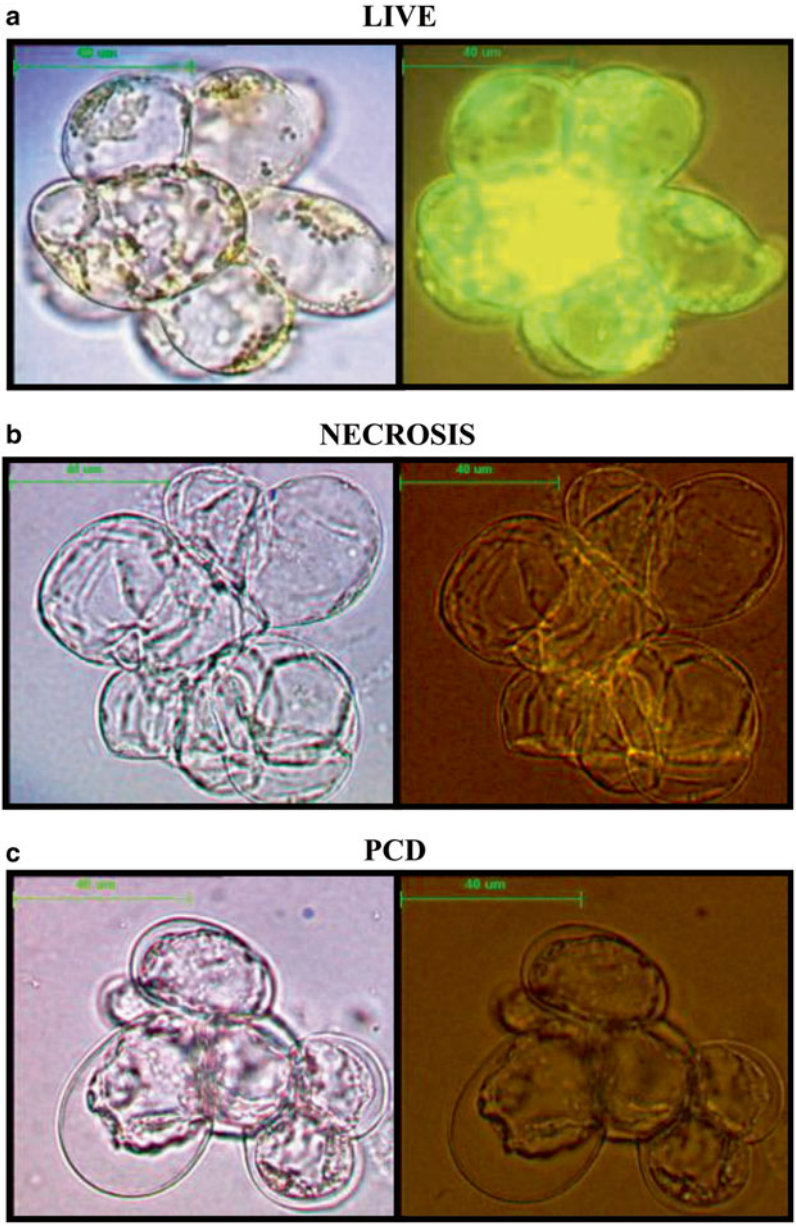

Fig. 2.1 Cell types present following a 10-min 53 °C heat treatment of *Arabidopsis thaliana* suspension cells. (**a**) Cells that are alive have the ability to cleave FDA and fluoresce under light at a wavelength of 490 nm. (**b**) Necrotic cells cannot cleave FDA and so do not fluoresce and show no evidence of protoplast condensation. (**c**) In cells that undergo PCD, the protoplast retracts from the cell wall. These cells cannot cleave FDA and so do not fluoresce. As this is a moderate stress, the majority of the cells die via PCD. Adapted from Diamond, M.; Reape, T. J.; Rocha, O.; Doyle, S. M.; Kacprzyk, J.; Doohan, F. M.; and P. F. McCabe (2013). The *Fusarium* mycotoxin deoxynivalenol can inhibit plant apoptosis-like programmed cell death. *PLoS ONE* 8: e69542. DOI:10.1371/journal.pone.0069542, with permission from the authors

When HR cell death is induced in oats with the host-selective toxin victorin, cell shrinkage is found to be associated with death [14], and during and following this shrinkage, the plasma membrane remains intact. HR elicitors also induce this condensed morphology in soybean and tobacco [12, 15]. This mode of PCD has also been observed in many examples of normal plant tissue development such as leaf morphogenesis [16], senescence [17], embryogenesis [6], tapetum development [18] and premature death of anther tissues during cytoplasmic male sterility [19].

The plant cell is constantly sensing signals from its internal and external environment and must act on those signals. How does the plant cell coordinate these signals and make this ultimate decision to undergo PCD? We know that the mitochondrion plays a central role in control of plant PCD (reviewed recently by Diamond and McCabe [20]), but our understanding of its role as executioner is far from complete. We can manipulate in vitro models and study the contributions of individual organelles, proteins and genes to PCD; however, in the context of the whole plant, it is unlikely that any organelle or molecule will act alone during PCD or indeed behave in a uniform fashion in different parts of the plant. The orchestration of PCD throughout a plant is without doubt extremely complex and reliant on numerous factors. However, we can progress our understanding by studying these less complicated in vitro models, and recently, further insight into the role of the chloroplast in plant PCD has been explored in the *Arabidopsis* suspension cell heat shock model [21, 22]. In this review, we discuss how the mitochondrion can act on environmental cues and orchestrate plant PCD and examine what we know to date of the involvement of the chloroplast in this process.

2.2 The Mitochondrion and PCD

Mitochondria were first shown to be components of PCD regulation in mammalian cells [23, 24]. Since then mitochondria have proved to have a central regulatory role in integrating environmental signals which determine whether a cell will live or die. The mitochondrion does this by coordinating death signals that lead to the initiation of cell death and by releasing molecules that drive the destruction of the cell (see reviews by Diamond and McCabe [20], Green and Reed [25], Jones [26], Bras et al. [27]).

2.2.1 The Mitochondrion and Apoptosis

Many studies investigating the role of mitochondria in plant PCD have focused on possible similarities with mammalian apoptosis, as the mitochondrion is a central component during this form of PCD. Mitochondria coordinate death signals in the induction phase, which involves the perception of the death-inducing stimulus by the cell and initiation of the death programme and then triggers the cell death

programme through the release of pro-apoptotic molecules during the effector phase, where the cell commits irrevocably to death [28]. The release of these pro-apoptotic molecules or mitochondrial factors is of critical importance to the progression of apoptosis. However, the mechanism by which the pro-apoptotic molecules are released from mitochondria is still under investigation. When mitochondria perceive stimuli, for example, death signals within the cell trigger alterations in the inner mitochondrial membrane (IMM), resulting in a loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and opening of the mitochondrial permeability transition pore (PTP). This loss of the mitochondrial transmembrane potential allows for the release of two main groups of pro-apoptotic proteins from the intermembrane space (IMS) into the cytosol [29]. The first group of proteins consists of cytochrome *c* (cyt *c*), SMAC/DIABLO and the serine protease Omi/HtrA2 [30–34]. Cyt *c* is a soluble protein found in the intermembrane space loosely associated with the MM [35]. Under normal conditions, cyt *c* is a necessary component of the electron transport chain in mitochondrial respiration [24, 36]. However upon its release, cyt *c* forms a complex with procaspase-9, dATP and the apoptosis protease-activating factor (Apaf-1), which leads to the formation of the apoptosome. This apoptosome can then activate downstream effector caspases which regulate apoptosis. Caspases are amongst the most specific of proteases, with an absolute requirement for cleavage of aspartic acid, consistent with the observation that apoptosis is not accompanied by indiscriminate protein digestion. The binding of cyt *c* and Apaf-1 increases the affinity of Apaf-1 for dATP and induces a conformational change that exposes the caspase recruitment domain (CARD) of Apaf-1 [27]. This CARD interacts with procaspase-9 creating a holoenzyme with the ability to activate caspase-3 and caspase-7, leading to the cleavage of procaspase-2, procaspase-6, procaspase-8 and procaspase-10 resulting in a proteolytic cascade eventually causing cellular destruction [37, 38]. However, regulatory mechanisms do exist both to prevent the unnecessary activation of apoptosis and also to prevent the inhibition of apoptosis where cell death is necessary. The activation of caspases can be negatively regulated by inhibitor of apoptosis proteins (IAPs). These IAPs bind and inhibit caspase-3, caspase-7 and/or caspase-9; however, upon apoptosis induction, these IAPs can also be inhibited through the antagonising effects of the mitochondrial proteins SMAC/DIABLO and Omi/HtrA2 [39].

Further regulation checks are also in place for the release of pro-apoptotic molecules, in the form of B cell lymphoma 2 (Bcl-2) family of proteins, which contain both pro- and anti-apoptotic members [40]. The anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-XL contain four Bcl-2 homology regions (BH1234). The pro-apoptotic members consist of multidomain proteins containing three Bcl-2 homology regions (BH123) such as Bax and Bak and single-domain BH3-only proteins including Bid and Bad. Under normal homeostatic conditions, Bak is localised in the outer mitochondrial membrane (OMM), while Bax is localised to the cytosol. However, once apoptosis is induced, the activation of Bax by the cleaved Bid results in homooligomerization within the OMM forming a molecular opening that facilitates the release of pro-apoptotic molecules from mitochondria and induction of cell death. On the other hand, it is believed that the anti-apoptotic molecules are normally

localised in the OMM, so they can interact, bind and inhibit the pro-apoptotic Bcl-2 members to prevent mitochondrial membrane permeabilisation (MMP) and the release of pro-apoptotic molecules from the mitochondria into the cytosol. The pro-apoptotic function of BH3-only molecules is completed in two distinct ways, (1) activation of the BH123 proteins to induce MMP, that is achieved by translocation of Bax to the OMM, or (2) binding to BH1234 molecules so they cannot interact with pro-apoptotic Bcl-2 family proteins [41], thus indirectly facilitating apoptosis.

The extrinsic pathway is mediated by molecules known as "death receptor" molecules that are located on the cell surface; this "death receptor" pathway is activated from outside the cell by ligation of transmembrane death receptors such as Fas, TNF, TRAIL and DR3–6 receptors with their corresponding ligands. Upon activation, the adaptor molecules bind to the cytosolic ends of the death receptors to generate a signal, known as the death-inducing signalling complex (DISC), by recruiting the adaptor Fas-associated death domain (FADD) and procaspase-8 and procaspase-10, resulting in caspase-8 and caspase-10 activation, leading to cleavage of effectors caspase-3 and caspase-7. Through this pathway, the death receptors can directly activate caspases and initiate apoptosis without mitochondrial involvement, as active caspase-8 can activate the downstream effector caspases [42]. However, if the death receptors do not generate a signal sufficient enough to induce cell death, mitochondrion-dependent apoptotic pathways can amplify this weakened signal. In this scenario, caspase-8 can cleave Bid and initiate the release of cyt *c* from IMS, which leads to the initiation of apoptosis in a manner that does not seem to cause typical mitochondrial swelling [43].

2.2.2 The Mitochondrion and Plant PCD

2.2.2.1 Cytochrome *c*

Changes in mitochondrial morphology, loss of $\Delta\Psi_m$ and cyt *c* release from the mitochondria are the earliest markers of plant PCD following stress [14, 44–47]. Cyt *c* release from the mitochondria has been documented in numerous in vitro stress models of plant PCD [44–46, 48–50], during developmental models [19, 51, 52] and following activation of the HR [14, 53]. Unlike apoptosis, plant cells do not contain caspases [54], and therefore, cyt *c* release from plant mitochondria cannot directly result in an apoptotic-like proteolytic cascade of events leading to the demise of the cell [45, 49]. However, although plants lack canonical caspases, there is well-documented evidence for caspase-like activity during plant PCD [2, 4], and some of this activity has been shown to be associated with plant subtilisin-like proteases, saspases and phytaspases which hydrolyse a range of caspase substrates following the aspartate residue [55]. The addition of broken plant mitochondria to *Arabidopsis* nuclei in a cell-free system results in chromatin condensation, high molecular weight DNA cleavage and DNA laddering, whereas addition of purified cyt *c* has no effect [45]. Pharmacological studies and submitochondrial localisation

suggested that a Mg^{2+}-dependent nuclease which resides in the mitochondrion IMS is responsible for the high molecular weight DNA cleavage and chromatin condensation, but DNA laddering required addition of a cytosol extract in addition to the mitochondria [45]. Therefore, evidence points towards there being molecules, in addition to cyt *c*, which are released from plant mitochondria during PCD and are involved in some way in plant death-associated protease activity. However, as of yet, these molecules have not been identified.

2.2.2.2 Mechanisms of Release of Mitochondrial Proteins

How are cyt *c* and potential death-inducing molecules released from the mitochondria during stress response? One way in which MMP is achieved in mammalian cells is via a specific Bax/Bcl-2 controlled pore. As mentioned earlier, the Bcl-2 family of proteins is composed of pro- (Bid, Bad, Bak and Bax) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) [56]. Under normal conditions, mitochondrial outer membrane integrity is maintained through a balance between these pro- and anti-apoptotic proteins, and proteins such as cyt *c* are prevented from being released from the mitochondria. There is an endoplasmic reticulum-located Bax inhibitor-1 (BI-1) conserved in animals and plants, and overexpression of *Arabidopsis* AtBI-1 can downregulate mammalian Bax-induced PCD [57]. Murine Bax (BI-1) can induce cell death and initiate a hypersensitive-like response in tobacco when expressed from a viral vector [58], and the anti-apoptotic Bcl-xL, when expressed in tobacco, can confer resistance to death induced by known activators of cellular ROS [59]. However, no plant homologs of the Bcl-2 family have been identified to date, and although these animal proteins can operate in plants, there is no direct evidence for a Bcl-2 family-formed pore occurring during plant PCD.

Another mode of release of apoptotic factors from the mitochondria, mentioned above, is via the PTP, a polyprotein complex formed at contact sites between the IMM and OMM and thought to act through the interaction between the voltage-dependent anion channel (VDAC) on the outer membrane, the adenine nucleotide transporter (ANT) from the inner membrane and cyclophilin D (CypD) in the matrix and other proteins [25, 26]. The PTP can be formed following cellular stress (build up of Ca^{2+}, changes in phosphate and/or ATP levels, ROS production), resulting in loss of the inner $\Delta\Psi_{m,}$ osmotic swelling of the mitochondria, disruption of the OMM and subsequent release of IMS proteins. There is strong evidence for the PTP playing a role in plant MMP. Homologs exist in plants for the major constituents of the PTP complex, VDAC, ANT and CypD [46]. In plants, an early loss of $\Delta\Psi_m$, preceding cell shrinkage and DNA degradation, has been detected after induction of PCD in oat seedlings following treatment with victorin, a host-selective toxin produced by *Cochliobolus victoriae* [14]. Similar findings have also been reported for *Arabidopsis* protoplasts treated with ceramide, methyl jasmonate or ultraviolet-C overexposure ([46, 60, 61], respectively) and in *Arabidopsis* suspension cells treated with harpin and acetylsalicylic acid [48, 62]. PTP opening can be inhibited by cyclosporin A (CsA) which acts by displacing the binding of CypD to ANT [63],

and this has been used as a tool to investigate the physiological role of the PTP in animal cells. Similarly, CsA has been used in plant PCD models to pharmacologically provide evidence for the existence of the PTP. CsA has been shown to inhibit Ca^{2+}-induced swelling of potato mitochondria [64], betulinic acid-induced PCD in tracheary element cells of *Zinnia elegans* [51], nitric oxide-induced PCD in *Citrus sinensis* [65], ROS-induced PCD in *Arabidopsis* cells [66] and caspase-1-like activity and PCD and actin breakdown during leaf perforation formation in the lace plant [67, 68]. In addition, CsA has been shown to protect against loss of $\Delta\Psi_m$ and cyt *c* release after protoporphyrin IX treatment of *Arabidopsis* protoplasts [46], prevent mitochondrial swelling by ROS following methyl jasmonate treatment of *Arabidopsis* protoplasts [60] and reduce the rate of fusaric acid-induced cell death in tobacco cells [69].

2.2.2.3 Control of MMP During Plant PCD

Hexokinase, the enzyme which catalyses the initial step in intracellular glucose metabolism, may control MMP in plants. Studies have shown that mitochondria-associated hexokinases play an important role in the regulation of PCD in *Nicotiana benthamiana* [70]. Hexokinase can bind with high affinity to mitochondria at sites in the OMM through its interaction with VDAC [71]. This interaction between hexokinase and the mitochondria is maintained by the serine/threonine kinase Akt and has been shown to play an important role in the control of mammalian apoptosis in the presence or absence of Bax and Bak [72]. Kim et al. [70] used tobacco rattle virus-based virus-induced gene silencing (VIGS) to investigate the function of signalling genes in *N. benthamiana*, and this screen revealed that VIGS of a mitochondria-associated hexokinase gene Hxk1 caused the formation of necrotic lesions in leaves similar to those formed during the HR. When cells in the affected areas were examined, they displayed hallmark features of PCD—nuclear condensation and DNA fragmentation—and death was associated with loss of $\Delta\Psi_m$, cyt *c* release, activation of caspase-like activities and expression of genes known to be induced during HR. Overexpression of the mitochondria-associated *Arabidopsis* hexokinases HXK1 and HXK2 can protect against oxidative stress-induced PCD [70]. Similar findings have been reported in potato tubers, where mitochondrion-bound hexokinase is thought to be involved in antioxidant function [73]. Cell death induced by heterologous expression of rice VDAC (OsVDAC4) in the tobacco bright yellow cell-2 line (BY2) and in leaves of *N. benthamiana* is reduced by co-expression of *N. benthamiana* hexokinase (NtHxK3), implying that VDAC/hexokinase interactions can modulate PCD in plants [74]. As in mammalian apoptosis, it looks like this association of hexokinase with the mitochondria is important in maintaining mitochondrial integrity during plant PCD. Of considerable interest is the fact that dissociation of hexokinase from animal mitochondria in the presence of apoptotic stimuli but in the absence of Bax and Bak still results in cyt *c* release (though not as effective) which is not suppressed by Bcl-2 [72]. It is possible that while both animal and plant cells employ hexokinases to control MMP, animals may

have evolved a further level of control involving the Bcl-2 family of proteins. Plants on the other hand may have found this mechanism sufficient for PCD or indeed may have evolved further plant-specific controls that have not been identified yet.

2.3 Reactive Oxygen Species and Antioxidant Control of Plant PCD

Continual production of reactive oxygen species (ROS) in mitochondria and chloroplasts throughout the life cycle of the plant is a by-product of metabolic processes such as respiration and photosynthesis, in peroxisomes during photorespiration and by enzymes such as plasma membrane NADPH oxidases, cell wall peroxidases and apoplastic amine oxidases [66, 75]. ROS present in plant cells include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals formed via the transfer of one, two or three electrons to oxygen, respectively [75]. ROS are highly toxic, with the ability to oxidise and damage cell components such as membrane lipids, proteins, enzymes and nucleic acids, and to balance this in plant cells, antioxidant scavengers of ROS have evolved to alleviate the toxic effects of ROS within the cell [75, 76]. Antioxidants and antioxidant enzymes are capable of quenching ROS without themselves undergoing conversion to destructive radicals. The interaction between ROS and antioxidants provides an interface for metabolic and environmental signals that can modulate induction of the cell's acclimation to stress or, alternatively, activation of PCD [77]. Therefore, despite their potential toxicity, ROS are important signalling molecules in plant cells [78]. The ROS signalling network during PCD is complex given the presence of different kinds of ROS within the cell, different sites of production and their interaction with other molecules involved in PCD [77, 79]. Levels of ROS, antioxidants and antioxidant enzymes act in balance during cell signalling [80]. Antioxidants are crucial to a plant cell's defence against oxidative stress, and there is evidence for their involvement in the control of plant PCD [21, 81–83]. Although release of cyt *c* from the mitochondrial IMS may not directly activate cytoplasmic components of plant PCD [45, 49], its release results in disruption of electron transport leading to generation of toxic levels of ROS. Activation or suppression of PCD in *Arabidopsis* cells by antioxidants has been shown to be dependent on the type and cellular localisation of ROS [82].

Disruption of mitochondrial electron transport has previously been suggested as the primordial mechanism by which the protomitochondrion killed its host [84]. Employing antimycin A to inhibit mitochondrial electron transport, in tobacco cells, results in induction of genes thought to be involved in senescence and defence responses. Interestingly, these same genes are induced by H_2O_2 and salicylic acid treatment which cause a rise in ROS and, when inhibited by antioxidant treatment, prevent the gene induction [85]. This work demonstrates that disruption of electron transport can certainly result in mitochondrial signals being transported to the nucleus,

possibly as a result of PTP opening, and results in alteration of gene expression. It may be that a ROS has at least two roles in plant PCD; it acts as a signalling molecule which leads to the opening of the PTP, which would lead to release of and the generation of more ROS, causing a feedback loop which amplifies the original PCD-inducing stress signal [86]. Cyt *c* release does not only lead to an increase in ROS, but it can interact with H_2O_2 and superoxide radicals to form hydroxyl radicals, which are amongst the most reactive and mutagenic molecules known [87], thereby escalating the damage caused by ROS. A study using rice protoplasts showed that overexpression of mitochondrial heat shock protein 70 inhibited MMP and cyt *c* release, preventing amplification of ROS and inhibiting PCD [88].

Blackstone and Kirkwood [87] hypothesise that the protomitochondrial electron transport chain, and ROS, may have played an important role in signalling between protomitochondria and host cell. These redox signalling mechanisms may have subsequently been adapted into primordial PCD mechanisms which were driven by ROS production and release of cyt *c* (causing increased ROS production). These authors further suggest that in animal cells, this release of cyt *c* could have been refined over time leading to the recruitment of caspases as the terminal effectors of apoptosis. As plants and animals share a common unicellular ancestor, it would not be surprising if plant cells also use ROS production and release of cyt *c* in cell death pathways.

2.4 The Chloroplast and PCD

In nature, plants need to continuously adapt to fluctuating environmental conditions including light. Light is a major factor in the control of growth, development and survival and therefore has a significant effect on a plant's response to biotic or abiotic stress, for example, progression of the hypersensitive response [89, 90] or the wound response [91].

As with the mitochondria, the chloroplast is also a major source of ROS in plant cells, and excess excitation energy (any light that the chloroplasts receive in excess of the amount required for photosynthesis in photosystem II (PSII) of chloroplasts) results in a decrease in photosynthetic efficiency and inhibition of plant growth [80]. Excess excitation energy increases with higher light intensity and also rises during stress responses, when photosynthesis is less efficient, causing more ROS production. Thus, the chloroplast, like the mitochondrion, is an organelle capable of sensing stress and initiating ROS signalling [92, 93]. It is not surprising, therefore, that the chloroplast plays a role in PCD. Indeed, illumination was found to be required for UV-induced PCD in *Arabidopsis* protoplasts and seedlings [94], cell death induced by fumonisin B_1 in *Arabidopsis* protoplasts is light dependent [95], and cyanide-induced PCD in guard cells of pea epidermal cells is enhanced by light [96]. Cell death induced by avirulent pathogen inoculation was found to occur in light-grown, but not dark-grown, *Arabidopsis* leaves [89, 97], and likewise a dark

treatment immediately following avirulent pathogen inoculation suppressed the HR in *Arabidopsis* leaves [90]. Seo et al. [98] found that the HR was accelerated by the loss of chloroplast function in tobacco.

Some insight into the involvement of chloroplasts in plant PCD emerged from a series of studies using *Arabidopsis* suspension cultures (ASC) as models for PCD [21]. Light-grown ASC contain functional chloroplasts [21]. However, dark-grown ASC do not, providing an excellent experimental system to study the role of the chloroplast in plant PCD. While total levels of cell death (i.e. PCD + necrosis) are similar in light- and dark-grown ASC after heat stress, dark-grown cells undergo significantly higher levels of PCD than light-grown cells, the latter undergoing more necrosis. Light-grown ASC lacking functional chloroplasts, due to norflurazon treatment, also responded to heat stress with higher levels of PCD compared to untreated light-grown cultures, suggesting chloroplast involvement. $^1O_2$ is the major ROS produced in photosystem II of chloroplasts, and in another study using light and dark ASC, mild photooxidation damage to cells using rose bengal (RB), a potent artificial $^1O_2$ sensitizer that accumulates inside chloroplasts, results in $^{1O2\text{-}mediated}$ PCD only in cells containing functional chloroplasts [22]. Similarly, studies on the leaf-variegated mutant *variegated2* show that $^1O_2$-mediated PCD is only activated in green leaf sectors of the plant containing fully developed chloroplasts but not in white sectors containing undifferentiated plastids [99]. The data in these papers suggest that $^1O_2$ can be a potent signal that initiates PCD but only when it is produced in the chloroplast. This is in agreement with the findings of Doyle and McCabe [82] that showed that antioxidants could suppress PCD but did not always do so, suggesting again that ROS per se does not induce PCD but rather the contexts—the location and type of ROS—are the important determinants.

Are chloroplasts a source of pro- or anti-PCD proteins? Another study linking the chloroplast and PCD came from the finding that palmitoleic acid induces cell death in eggplant cells and protoplasts, in a process involving cytochrome *f* (cyt *f*) [100]. Cyt *f* is a subunit of the cyt b_6f complex, an essential component of the major redox complex of the thylakoid membrane catalysing the transfer of electrons from photosystem II to photosystem I. Cyt *f* has also been shown to be released from the chloroplast into the cytosol following heat shock treatment of the unicellular green alga *Chlorella saccharophila*, accompanied by thylakoid membrane structure alterations, culminating in PCD [101]. Release of cyt *f* from chloroplasts has also been shown in senescent rice leaves prior to PCD [102], and these authors also demonstrate that cyt *f* can activate caspase-3-like activity in a cell-free system. Release of trace amounts of cyt *f* from the thylakoid membrane has also been observed in the *flu* mutant; however, in this case cyt *f* release was only observed after onset of cell death leading the authors to conclude that cyt *f* is not a trigger for $^1O_2$-mediated PCD but rather a marker for cellular collapse [99]. However, as in the case of cyt *c* release from the mitochondria, disruption of the cyt b_6f complex and release of cyt *f* will have a major impact on ROS production which will subsequently govern the type of death a cell undergoes. So, despite these interesting findings, it is not clear whether this loss of function of cyt *f* relates it to plant PCD or a more direct regulatory role exists as in the case of cyt *c* in apoptosis [101, 102].

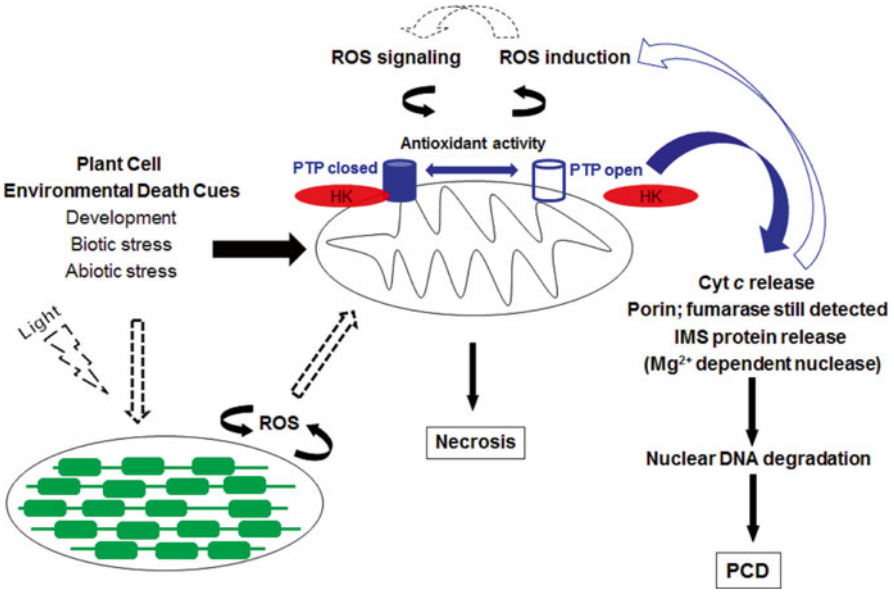

Fig. 2.2 Schematic representation of mitochondrial involvement in plant PCD. It is thought that the mitochondrion plays a role in integrating signals generated through developmental signals or stress, thus determining whether the cell activates its PCD pathway or not. Similar to animal cells, cyt *c* is released rapidly from the mitochondria in the early stages of plant PCD but, unlike the animal system, does not appear to be directly responsible for activating a caspase-driven cascade of events, which leads to PCD, but rather may serve to amplify the death process. Cyt *c* release will disrupt the electron transport chain resulting in generation of ROS. As well as a signalling molecule, which can lead to opening of the PTP and release of cyt *c*, more ROS can be generated in this way, causing a feedback loop which amplifies the original death signal. Early cyt *c* release from the IMS during PCD is not accompanied by loss of porin, an integral outer membrane protein, or fumarase, a mitochondrial matrix protein. The PTP in plants may be regulated through its interaction with mitochondria-associated hexokinase, which also has a role in regulating antioxidant activity which in turns regulates ROS signalling and release. We also know that a Mg^{2+}-dependent nuclease which also normally resides in the mitochondrial intermembrane space can be released, causing DNA degradation. When functional chloroplasts are present, they also sense and respond to environmental stress and produce excess ROS which also serves to amplify the response thereby influencing the severity of the stress, leading to increased levels of necrosis. Necrosis is known to occur under conditions of high stress, and the cell is thought to be unable to activate a PCD pathway. *HK* hexokinase, *IMS* intermembrane space, *PTP* permeability transition pore; *dashed arrows* represent signals that can occur in cells containing chloroplasts

While our understanding of the role of the chloroplast in PCD is increasing, it is far from complete. What is certain though is that the presence of chloroplasts, while not necessary for plant PCD, plays a significant role in the regulation of PCD and increases the complexity of ROS-mediated PCD pathways in cells containing functional chloroplasts (see Fig. 2.2 for schematic representation of mitochondrial and chloroplast involvement in PCD).

2.5 Mitochondrial and Chloroplast Crosstalk During PCD

2.5.1 Proximity of Organelles During PCD

Mitochondria and chloroplasts have an intimate relationship within the plant cell, and the environment of the mitochondria is dynamically influenced through metabolic interactions and redox exchange with the chloroplasts. Under normal conditions, plant mitochondria are localised around chloroplasts in an even distribution [46, 60, 103], and this is thought to be due to oxygen and carbon dioxide gradients which establish metabolite interchange between the two organelles [103]. During PCD, a different picture emerges and has been observed by several different laboratories employing different inducers of PCD in vitro: mitochondria begin to aggregate and later in the process show a more clumped/clustered morphology surrounding chloroplasts or aggregated within other areas of the cytoplasm [60, 104, 105]. Chloroplasts also show changes in their morphology and position from close to the plasma membrane to being evenly distributed throughout the cytoplasm [104]. In the case of cadmium-induced PCD, which increases ROS in *Arabidopsis* protoplasts, pretreatment with ascorbic acid or catalase prevents subsequent organelle changes and death [105]. Mitochondrial aggregation is also observed in developmentally regulated PCD in the lace plant [67]. Early stages of PCD in the lace plant, before loss of $\Delta\Psi_m$, are associated with an increase in observable transvacuolar strands, and mitochondria and chloroplasts can be seen moving along these transvacuolar strands in a seemingly orderly fashion, and later, chloroplasts could also be seen forming a ringlike structure around the nucleus [106] (see Fig. 2.3, kindly provided by A. Gunawardena; for more information and figures on this topic, see Chap. 1). It will be interesting to learn what the significance of these organelle distribution patterns is, but the close proximity of the chloroplast and mitochondria and their changes in morphology during PCD strongly support the involvement of the chloroplast in mitochondrial control of PCD.

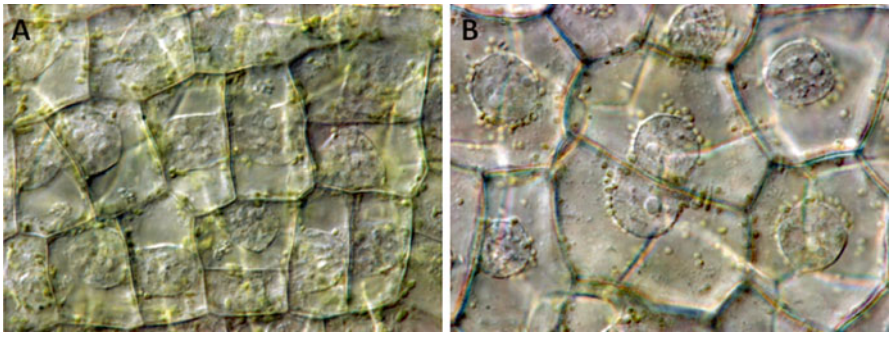

Fig. 2.3 Chloroplast distribution during PCD of the lace plant *A. madagascariensis*. (**a**) Regular chloroplast distribution in the cytoplasm during early PCD. (**b**). Perinuclear distribution of chloroplasts during late PCD (images are taken using differential interference contrast (DIC) optics and kindly provided by A. Gunawardena, Dalhousie University, Canada)

2.5.2 *Dynamic Location of Mitochondrial and Chloroplast Proteins Involved in PCD During Oxidative Stress*

2.5.2.1 Accelerated Cell Death 2 Protein

Some evidence for crosstalk between mitochondria and chloroplasts during PCD comes from a series of studies involving the chloroplast-localised protein, accelerated cell death 2 (ACD2). Overexpression of *Arabidopsis* ACD2, which encodes red chlorophyll catabolite reductase, can protect against disease symptoms and PCD in leaves caused by infection with virulent *Pseudomonas syringae* [107]. The *acd2* mutant undergoes excessive PCD during infection and displays spontaneous spreading cell death [108]. ACD2 localises to chloroplasts in mature leaves but in young seedlings localises to both chloroplasts (or plastids) and mitochondria [107]. During infection of leaves with *P. syringae* or protoporphyrin IX treatment, ACD2 also localises to both chloroplasts and mitochondria in leaves [46] and is thought to protect cells from pro-death mobile substrate molecules, some of which may originate in the chloroplast but have major effects on mitochondria by causing increases in ROS levels and ultimately cell death [109].

2.5.2.2 Antioxidative Enzymes

As we have discussed, the chloroplast, like the mitochondrion, is a major regulator of cellular redox homeostasis within the cell. While import of proteins into the mitochondria and chloroplasts is generally considered to be organelle specific, several key antioxidative enzymes are dual targeted to chloroplasts and mitochondria [110]. Dual targeting involves localisation of a single protein in more than one cellular compartment, and that protein is encoded by a single gene in the nucleus and translated in the cytosol as a single translation product but targeted to both mitochondria and chloroplasts [111–113]. It is now thought that dual targeting is not just an evolutionary solution to increase the number of cellular functions without increasing the number of genes, as in some cases both dual-targeted and specific protein isoforms exist in the same organelle, suggesting a regulatory mechanism for some processes [113]. Dual targeting could have important control implications for plant PCD in the context of mitochondria/chloroplast crosstalk. Of the approximately 100 proteins found to be dual targeted to date, most are involved in nucleotide metabolism [111, 114], but three of the four enzymes involved in the ascorbate-glutathione cycle, ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR) and glutathione reductase (GR), are dual targeted. The fourth enzyme involved in the cycle, glutathione-dependent dehydroascorbate reductase (DHAR), is not [110]. Interestingly, MDAR is a homolog of AIF [115]. AIF is a flavoprotein with NADH oxidase activity that (like cyt *c*) is normally found in the mitochondrial IMS but can translocate to the nucleus upon induction of apoptosis where it can bring about chromatin condensation and cleavage of DNA into large (~50 kb) fragments in a caspase-independent manner [116]. Although AIF has both apoptotic and anti-apoptotic activities [117, 118], indications in plant cells are that MDAR, being

involved in the ascorbate-glutathione cycle and crucial for ascorbate regeneration, has a protective role against oxidative stress in plants. In examining amino acid sequences of *Arabidopsis* MDAR and human AIF, Yamada et al. [119] found close homology between the oxidoreductase domains in both proteins, but not between the C terminal AIFM1 domains (DNA binding) which have the apoptotic function, leading these authors to conclude that higher plant MDARs do not have a similar pro-apoptotic function in PCD. In animal cells, DNA binding-defective AIF mutants remain capable of translocating to the nucleus but cannot induce cell death [120]. MDAR1 and DHAR overexpression in transgenic tobacco confers enhanced resistance to ozone, salt and polyethylene glycol stress, all known to increase levels of ROS ([121, 122], respectively). In a study looking at ascorbate metabolism in broccoli florets during postharvest senescence, where PCD occurs, gene expression of both the chloroplastic genes MDAR 1 and DHAR was found to be downregulated [123]. However, Locato et al. [81] found that during heat shock-induced PCD in tobacco BY-2 cells, mitochondrial MDAR activity decreases and becomes undetectable in cells undergoing PCD, while mitochondrial DHAR activity does not diminish, and the authors discuss the possibility of DHAR activity increase being involved in a regulatory feedback mechanism improving ascorbate regeneration from dehydroascorbate when ascorbate is depleted at the site of production.

2.5.2.3 Anti-apoptotic Genes

Earlier in our discussion on release of mitochondrial proteins during PCD, we discussed the Bcl-2 family of proteins and the fact that although there are no plant homologs, these animal proteins have been shown to operate in plants [58]. Chen and Dickman [124] developed transgenic tobacco plants containing anti-apoptotic genes, animal Bcl-2 and Bcl-xL and *Caenorhabditis elegans* CED 9, and subcellular fractionation revealed that the anti-apoptotic proteins associated not only with mitochondria and nuclear fractions, as previously shown, but also with chloroplast membranes. This finding was not surprising as all three proteins have transmembrane binding domains. However, what is of significance in the context of PCD is that light is required for herbicidal (whose primary site of action is the chloroplast) treatment of tobacco plants, resulting in production of lethal levels of ROS and subsequent death with apoptotic-like features, while transgenic plants expressing the anti-apoptotic genes survive.

2.6 An Evolutionary Perspective

It is clear that there are many shared features amongst plant PCD and other eukaryotic cell death programmes. However, the extent to which these shared features arise from an ancient unicellular death programme is uncertain and remains controversial. Have redox signalling mechanisms between the mitochondrial symbiont and host cell

become subsequently adapted into primordial PCD mechanisms [84, 87]? Although we don't have the answer to this question, one point is clear and that is, as in animal apoptosis, the mitochondria appear to be key regulators of the cell death process. In the context of plant PCD, it is clear from the evidence discussed in this chapter that the chloroplast, the other endosymbiont-derived organelle, plays a role in PCD.

The theory that mitochondria and chloroplasts derive from bacteria taken into other cells as endosymbionts is particularly relevant to recent findings by Yang et al. [125] where plant orthologs to putative bacterial PCD proteins were found using a proteomic analysis of chloroplast envelope proteins. This plant *Cid*/*Lrg* ortholog *AtLrgB* may play a role in plant PCD in that a T-DNA insertion mutation of *AtLrgB* resulted in plants with interveinal chlorotic and premature necrotic leaves [125]. Interestingly, these *Cid*/*Lrg* bacterial proteins are thought to be evolutionarily linked with the Bcl-2 family of proteins [126]. The relevance of PCD in bacteria is a fairly recent consideration and connected to their ability to form biofilms [126]. The *Staphylococcus aureus cid* and *lrg* operons that control cell death and lysis are important in biofilm development [126], and these proteins have many similarities to the bacteriophage holin/antiholin family which regulate membrane permeability in lysis. The Bcl-2 proteins are able to functionally replace holins to promote lysis [127]. Thus, the finding of plant *Cid*/*Lrg* plant orthologs led the authors to hypothesise about the potential conservation of this membrane permeability control mechanism in animal, plants and bacteria and a similar evolutionary relationship between bacteria and chloroplasts [126], similar to that of bacteria and mitochondria and the control of PCD [84, 87].

2.7 Conclusions

The studies discussed in this review highlight the complexity involved when these two major plant organelles, the mitochondria and chloroplast, sense and respond to stress, which in turn determines whether the cell lives or dies via PCD or necrosis. Cell monitoring of fitness by major cellular organelles is probably a significant player in determining cell fate. When relating PCD research findings to the whole plant, one cannot consider results pertaining to mitochondria alone, as it is clear that when chloroplasts are present they are capable of mediating PCD under oxidative stress conditions.

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Chapter 3
Life Beyond Death: The Formation of Xylem Sap Conduits

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3.1 Introduction

The transition of plant life from water onto land required the development of systems to ensure the hydration of all the plant organs exposed to the dry atmosphere as well as the strengthening of plant stature against gravity. Xylem (Greek *xylon* for wood) is the tissue which ensures both water and mineral transport from the roots up to the leaves and mechanically reinforces the axis of plant organs. Xylem is composed of several cell types including (1) tracheary elements (TEs), the conducting and support cells; (2) fibres, the non-conducting mechanical support cells; and (3) an associated xylem parenchyma. To allow TEs to conduct the hydro-mineral sap, these cells are hollowed by programmed cell death (PCD) to turn them into functional conducting corpses. The central importance of PCD to establish the hydro-mineral sap conduits becomes visible throughout the evolutionary history of TEs in plants. A subset of mosses or bryophytes, which are the most primitive living land plants, developed simplified conduits which relied on cell death to form a hollow cell delimited only by its primary cell walls to transport water and minerals [1, 2]. The earliest vascular land plants or tracheophytes, such as *Aglaophyton* and *Horneophyton* of the Devonian and Silurian periods, similarly had only primitive conduits formed by cell death and delimited only by the primary cell walls and no secondary cell walls [3]. PCD thus represents the first process acquired by higher land plants to establish a functional hydro-mineral sap conduit. The present-day TEs possess additional structural properties and morphological features which

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enable a highly efficient sap conduction [2, 4, 5]. These properties include (1) reinforcements of the TE sides with secondary cell walls to maintain the TE cell lumen open, (2) modification of the primary cell wall in between secondary thickenings to enable lateral distribution of the sap [6, 7] and (3) formation of thinned and/or perforated ends to allow the sap to pass from one TE to the next [8–10]. Once fully formed, TEs assemble end to end and/or laterally to establish a continuous vascular network throughout the plant body.

The study of TE formation in whole plants is challenging for several reasons. Very few TEs differentiate at a given time and even fewer are at the same differentiation stage. Another difficulty is that genetic or pharmacological modifications of TE formation often lead to drastic pleiotropic effects such as reduced growth, higher susceptibility to infection and problems in sap conduction [11–13]. In order to circumvent the deleterious effects caused by any modifications of TE formation and to obtain a large quantity of TEs at the same differentiation stage, simplified in vitro systems using suspension cell cultures have been established. The most common systems include cell suspensions from freshly isolated mesophyll cells of *Zinnia elegans* [14] or cell lines derived from roots of *Arabidopsis thaliana* [15–17]. Such in vitro suspension cell cultures enable TEs to differentiate under strict hormonal control, semi-synchronously, quickly and in large quantities. TEs formed in vitro share the same morphological, biochemical and genetic characteristics as TEs in whole plants [14, 15, 18–20]. Pharmacological and genetic modifications of TEs in these in vitro simplified systems are restricted to the single-cell level without interfering with the sap conduction or the overall development and physiology of the plant [21, 22].

Ménard and Pesquet [7] recently issued a comprehensive review on the different aspects of TE differentiation and function. The present review focuses on the cellular processes associated with PCD in TEs, their coordination with the other events occurring during TE differentiation as well as the signalling processes controlling TE PCD in the simplified in vitro TE differentiation systems in both *Z. elegans* and *A. thaliana*.

3.2 Cell Biology of TE Death

Programmed cell death during plant development is generally associated with the removal of unwanted cells. During TE formation, however, PCD is the main functionalising event of the differentiation programme as it empties the cell protoplast to convert the cell lumen into the future sap conduit. The maturation of TEs includes differentiation events occurring before TE cell death (*pre-mortem*), such as the cellulose and xylan deposition in the secondary cell wall thickenings [23], as well as events occurring after cell death (*post-mortem*), such as the lignification of secondary cell walls of TEs [22] and the clearing of the TE protoplast remains [24]. Altogether the succession of these events results in hollow TE corpses with reinforced and water-proofed side walls suitable for sap conduction. PCD is therefore essential as it initiates the “physiological life” of TEs to serve as sap conducting

elements. The occurrence of TE PCD needs to be tightly coordinated with the full completion of *pre-mortem* processes and the initiation of *post-mortem* events. The major breakthrough in determining the chronological sequence of TE differentiation has relied on the combined use of real-time live-cell imaging and in vitro TE differentiating cell suspension cultures.

Contrary to observations made on fixed samples or in tissues with cells at different developmental stages, real-time live-cell imaging enables direct monitoring of the chronological progression of TE differentiation at the single cell level as well as to estimate the time length of each sequential event involved. Fukuda and Komamine, who had optimised the in vitro TE differentiation system in *Zinnia* and explored its potencies, produced the first real-time live-cell observations of TE differentiation [25]. In this study, TE secondary cell wall deposition was estimated to occur as quickly as 6 h in some cells. A pioneering study using "intermediate" (up to several hours) real-time live-cell imaging was performed later by Groover et al. [24] with bright-field microscopy to analyse the spatio-temporal relationship between secondary cell wall formation and TE PCD in *Zinnia* differentiating TEs. This breakthrough study identified the vacuole rupture as the central event triggering TE cell death. It also showed that (1) TE cell death occurs only once secondary cell wall synthesis is completed; (2) TE death is triggered by an implosion of the tonoplast; (3) the residual TE protoplast is rapidly removed *post-mortem* to clear the lumen of the TEs, within a few hours following death; and that (4) organelles like chloroplasts can remain visible in TEs even long after the vacuole rupture.

3.2.1 From Life to Death by TE Vacuole Rupture

TE PCD is triggered by the inward collapse and breakdown of the tonoplast. This rupture releases the vacuolar content into the TE cytoplasm which then initiates the *post-mortem* degradation of all cellular components (Fig. 3.1 and Supplementary Movie 3.1; [24]). This rapid inward rupture of the vacuole occurring during TE cell death is illustrated using short-term real-time imaging in *Arabidopsis* TE differentiating cell cultures in Supplementary Movie 3.1. Within 50 min, TE cells sequentially progress from an intact cell structure animated with cytoplasmic streaming (Fig. 3.1a, b) to a fragmented vacuole with an inward collapsed tonoplast in a slightly plasmolysed cell (Fig. 3.1c) and finally to a shrinking vestigial protoplast with no distinct inner organisation (Fig. 3.1d–f). The change in TE tonoplast integrity results from evenly distributed ruptures all around the vacuole (as indicated by the dotted lines in Fig. 3.1g, h). Differences in the vacuole uploading of fluorescein in dying TEs in *Zinnia* show that a change in membrane permeability precedes the vacuole implosion [26]. This tonoplast permeability change was estimated to last around 3 min using live-cell imaging in differentiating *Zinnia* cells [24] and could be mimicked using probenecid which is an inhibitor of organic anion transport [26]. Moreover, pharmacological inhibition of protein translation using cycloheximide in differentiating *Zinnia* TE cultures inhibited both TE cell death and tonoplast permeability changes, suggesting that the timing of vacuole rupture is actively controlled

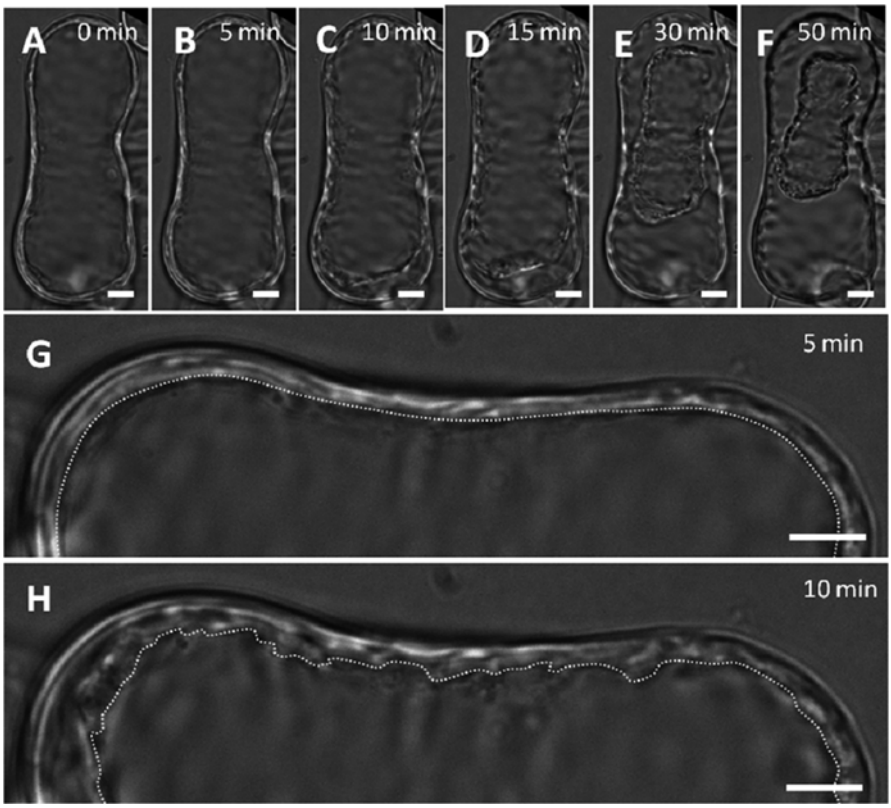

Fig. 3.1 The vacuole rupture is the terminal event of TE programmed cell death observed using real-time live-cell imaging in differentiating in vitro cells of *Arabidopsis*. (**a**–**f**) Photo montage of time points before, during and after the TE vacuole rupture using real-time live-cell imaging (full movie provided in Supplementary Movie 3.1), bars = 4 μm. Note that TE rapidly shifts from an intact structure (**a**–**b**) to tonoplast implosion (**c**), followed by a gradual *post-mortem* degradation of the protoplast (**d**–**f**). Close-up of tonoplast structure in TEs, highlighted by a *dotted white line*, prior to vacuole rupture (**g**) and after its rupture (**h**), bars = 4 μm. Note that the tonoplast evenly breaks all around the vacuole in (**h**)

by the production of specific proteins [26]. However, no gene/protein candidate(s) has yet been identified or demonstrated to be responsible of TE tonoplast permeability change and/or rupture.

3.2.2 Pre-mortem Cytological Changes Occurring Before TE Vacuole Rupture

The main *pre-mortem* cytological process is the deposition of the patterned secondary cell walls composed of cellulose and hemicelluloses. Long-term (2–3 days) real-time live-cell imaging using fluorescence microscopy revealed that *Arabidopsis*

TEs deposit cellulose and hemicellulose into the secondary cell walls during a period of 10–16 h, after which the TEs remain alive for another 2–6 h and finally commit cell death within less than 10 min [17, 27]. The PCD of TEs appears to occur in both the *Zinnia* and the *Arabidopsis* TE differentiation systems only once the cellulose and xylan deposition are completed [17, 24, 26]. One key element for the proper formation and organisation of TE secondary cell walls is microtubules which specifically restrict and delimit the sites of secondary wall deposition [28, 29]. In both *Zinnia* and *Arabidopsis* TE differentiating cell cultures, a tight cortical microtubule network builds up while the secondary cell wall deposition progresses and then disassembles once TE cell wall synthesis is completed [30]. The mechanism controlling microtubule disassembly in TEs once the secondary cell wall deposition is completed is not yet fully understood. A Ca^{2+} influx, which is known to destabilise microtubules in plant and animal systems, was specifically associated with the triggering of *Zinnia* TE cell death [31, 32]. The subcellular localisation of Ca^{2+} accumulation in *Zinnia* TEs using chlortetracycline revealed that membrane-associated Ca^{2+} accumulation shifted from a broad cytoplasmic distribution to vesicular structures in between secondary thickenings as TE secondary cell wall was being deposited [33]. Treatments of *Zinnia* cell culture with $CaCl_2$ and calcium ionophore A23187 induced premature cell death of TEs whereas incubation with Ca^{2+} chelators reduced cell death [31, 32]. Although changes in microtubule organisation have been shown to trigger PCD in animal systems [34], treatments with microtubule-stabilising or microtubule-destabilising drugs did not affect TE cell death in *Zinnia* or *Arabidopsis* TE suspension cell cultures [16, 35, 36].

During the active deposition phase of TE secondary cell walls, the TE cytoplasm accumulates small vacuolar structures in the cytoplasmic spaces in between secondary thickenings (Fig. 3.2a; [24, 37]). These specific structures have been suggested to be autophagosomal/lysosomal structures [38, 39]. Autophagosomes are specific subcellular organelles which are responsible of the degradation of damaged and/or superfluous cytoplasmic components [40]. Although the 13 classical autophagy-associated genes are not upregulated during TE formation in cell culture [23], autophagy has been associated with TE differentiation based on the activation state of the small GTP-binding protein RabG3b in *Arabidopsis* and poplar calli [38, 39]. Constitutive activation of RabG3b stimulated both TE formation and autophagosome formation whereas its inactivation or silencing led to the opposite effect [38]. These authors also proposed that autophagy positively regulates TE PCD, but the exact role of autophagy in this process remains to be functionally demonstrated. Several other organelles are also implicated in the control of TE PCD. Before the vacuolar rupture, the mitochondria lose their defined internal structure as well as the integrity of their external membrane (Fig. 3.2b; [41]). These changes were shown to be associated with the depolarisation of the mitochondrial membranes and the release of cytochrome *c* in *Zinnia* TE cell cultures [41]. Pharmacological agents (betulinic acid, cyclosporin A) were used by Yu et al. [41] to further show that TE PCD correlates positively with changes in the mitochondrial membrane potential but not with the cytochrome *c* release from the mitochondria. Cytochrome *c* release, contrary to the animal apoptotic cell death, is therefore not causally related to TE

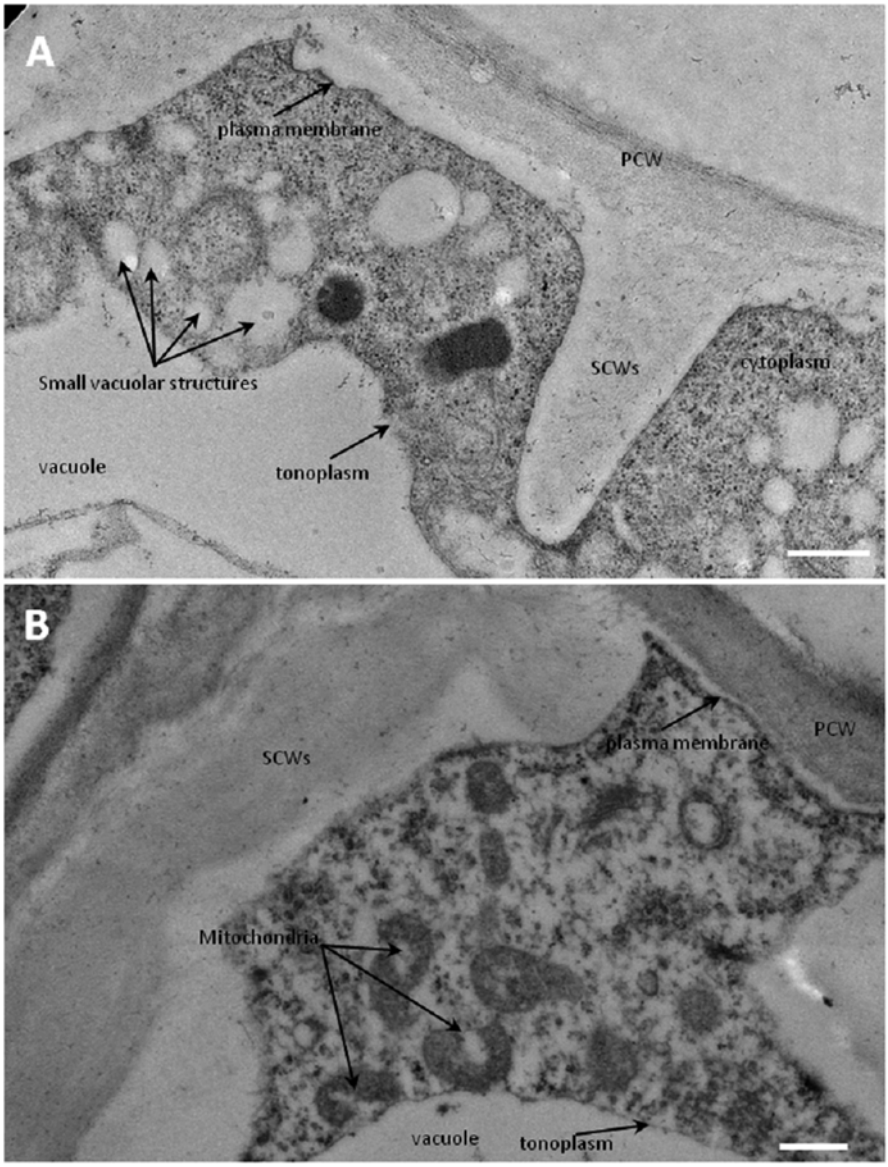

Fig. 3.2 Transmission electron microscopy images illustrating the cytological changes prior to vacuole rupture in *Arabidopsis* TE differentiating cell cultures. (**a**) Accumulation of small vacuolar structures in the cytoplasm between secondary cell wall thickenings similar to what was reported by Groover et al. [24] in isolated *Zinnia* TEs. (**b**) Disorganised mitochondria similar to what was reported by Yu et al. [41] in isolated *Zinnia* TEs. Bars = 500 nm

PCD. The combined observations of the *pre-mortem* cytological changes occurring during TE formation are schematised in Fig. 3.3a–c.

3.2.3 *Post-mortem Cytological Changes Occurring After TE Vacuole Rupture*

Live-cell imaging in combination with fluorescence microscopy has been essential in defining the *post-mortem* events occurring after TE vacuole rupture. Short-term (up to 30 min) real-time live-cell imaging combined with fluorescent staining of the nuclei in *Zinnia* TEs revealed that the nucleus is the first organelle to disappear within 10 min after the tonoplast rupture [42]. The 10 min timing of nuclear degradation after the vacuole burst was confirmed using stable transgenic cell lines constitutively expressing translational fusion of histone2A and green fluorescent protein (GFP) with real-time live-cell imaging in *Arabidopsis* differentiating TEs (Fig. 3.4a–d and Supplementary Movie 3.2; [17, 26]). Supplementary Movie 3.2 illustrates this rapid nuclear degradation occurring during TE cell death using short-term real-time imaging in *Arabidopsis* TE differentiating cell cultures [17]. Within 15 min, TE development progresses from an intact cell structure with a nucleus (Fig. 3.4a) to a protoplast with no nucleus (Fig. 3.4b) which gradually retracts and degrades itself (Fig. 3.4c, d). This nuclear DNA fragmentation was further investigated using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay in whole plant and in differentiating cell cultures. Multiple studies confirmed that the fragmentation of TE nuclei occurs once the SCW has been deposited [22, 24, 43]. Figure 3.4f, g and Supplementary Movie 3.3 illustrate nuclei labelled in *Zinnia* TEs using the TUNEL assay. The degradation profiles of TE genomic DNA do not however exhibit a clear fragmentation through internucleosomal cleavage as observed in other types of PCD but rather a random degradation pattern [24, 44]. Chromatin condensation, which is another indicator of PCD, does not seem to occur regularly either [24], but it is clear that the nucleus can have a lobed structure before the vacuolar bursting [45]. In TEs, nuclear degradation is a consequence of PCD rather than the triggering mechanism like in many other plant and animal systems [46]. The active removal of the TE residual protoplast occurs *post-mortem* in the hours following the vacuole rupture (Figs. 3.1c–f and 3.2b–d; [17, 24, 41, 47]). The other cytoplasmic organelles are not degraded as quickly as nuclei, for instance, the endoplasmic reticulum (ER) is maintained intact up to 40 min after TE vacuole rupture [48]. The degradation of the other organelles include the disorganisation and rupture of mitochondria [41], the swelling of the Golgi apparatus [24, 37] and the breakdown of the ER into inflated vesicular structures [37, 49]. A schematic representation of the sequential cytological *post-mortem* changes occurring during TE formation is represented in Fig. 3.3d–f.

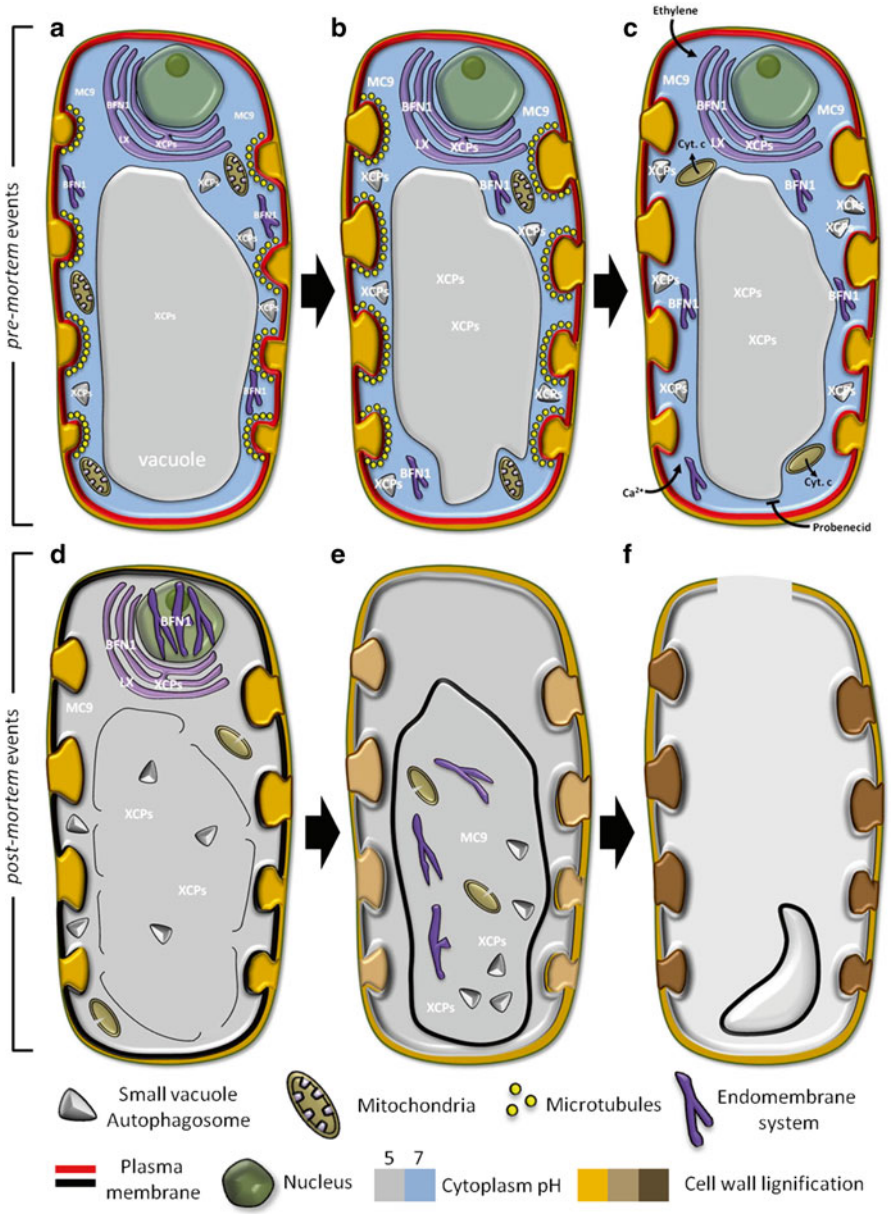

Fig. 3.3 Schematic representation of the *pre-* and *post-mortem* events during TE PCD. (**a**–**c**) *Pre-mortem* events include: (**a**) active secondary cell wall formation delimited by a scaffold of microtubules. This stage is characterised by intact mitochondria and the accumulation of small vacuolar structures in the cytoplasm between secondary thickenings. (**b**) Completed secondary cell wall deposition still delimited by microtubules. This stage is characterised by intact mitochondria and the accumulation of small vacuolar structures. Note that hydrolytic enzymes (MC9, XCPs, LX and BFN1) accumulate in concert with secondary cell wall formation (shown by increasing font size). (**c**) TE prior death with altered tonoplast permeability, rupture of mitochondria external membrane

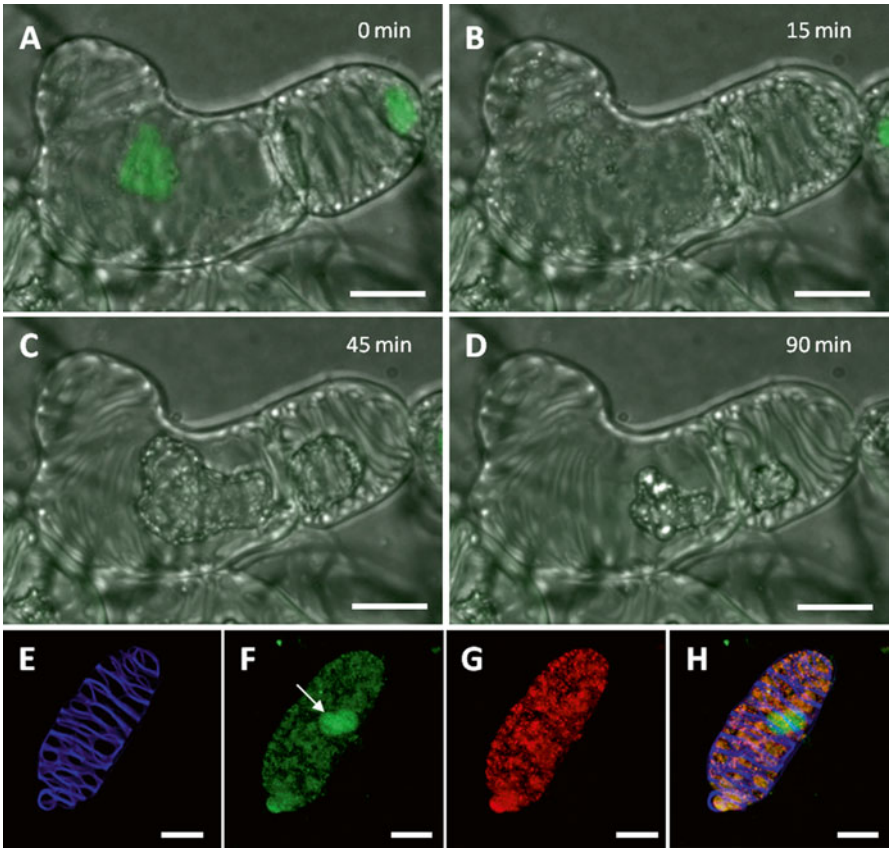

Fig. 3.4 *Post-mortem* degradation of nuclei in *Arabidopsis* and *Zinnia* TE differentiating cells. (**a**–**d**) Real-time live-cell imaging of nucleus degradation, labelled by histone2a-GFP [17], in differentiating in vitro TEs of *Arabidopsis*; bars = 6 μm. Note that TE rapidly shifts from an intact structure with a nucleus (**a**) to a protoplast without nucleus (**b**), followed by a gradual *post-mortem* degradation of the protoplast (**c**–**d**). (**e**–**h**) TUNEL assay performed on *Zinnia* TEs observed using confocal microscopy with TE secondary cell wall stained by calcofluor (**e**), FITC-labelled genomic DNA breaks (**f**), chlorophyll autofluorescence (**g**) as well as merged images of E to G (H); bars = 7 μm. *Arrow* indicates TUNEL-positive nucleus in *Zinnia* TE

Fig. 3.3 (continued) and their release of cytochrome *c* (cyt. *c*). Progression from this stage onwards is regulated by calcium intake, tonoplast permeability (affected by probenecid treatment) and ethylene. (**d**–**f**) *Post-mortem* events include: (**d**) the inward collapse of the tonoplast which alters the cell pH. Note that BFN1 is localised in ER-derived structures which surround the nucleus. (**e**) Complete degradation of the nucleus and disorganisation of the ER in the retracting protoplast. Note that secondary cell wall thickenings are starting to lignify. (**f**) TE with residual vestigial protoplast and lignified secondary cell wall ready for sap conduction

3.3 Genes/Enzymes Associated with TE *Post-mortem* Protoplast Degradation

The optimal vascular function of TEs requires a complete removal of the protoplast once the cell has died. This process represents one of the *post-mortem* events occurring during TE formation to ensure that no remnants of the protoplast interfere with or occlude the sap flow and/or the lateral distribution. The complete clearing of TEs lasts from less than 10 min to more than 1 h depending on the in vitro TE experimental system used [17, 26, 42, 24]. The clearing process is achieved by a set of hydrolases including among others the *A. thaliana* xylem cysteine proteases 1 and 2 (XCP1 and XCP2), bifunctional nuclease 1/endonuclease 1 (BFN1/ENDO1) and metacaspase 9 (MC9). These genes/proteins are misleadingly referred to as "TE PCD markers" even though they affect the speed of TE clearing rather than TE cell death per se [47, 50–52]: they should therefore be called "TE autolysis markers". Interestingly, all these genes are co-regulated with secondary cell wall synthesis genes such as cellulose synthases in both *Zinnia* and *Arabidopsis* (Fig. 3.5). They are also transcriptionally controlled by the master transcription

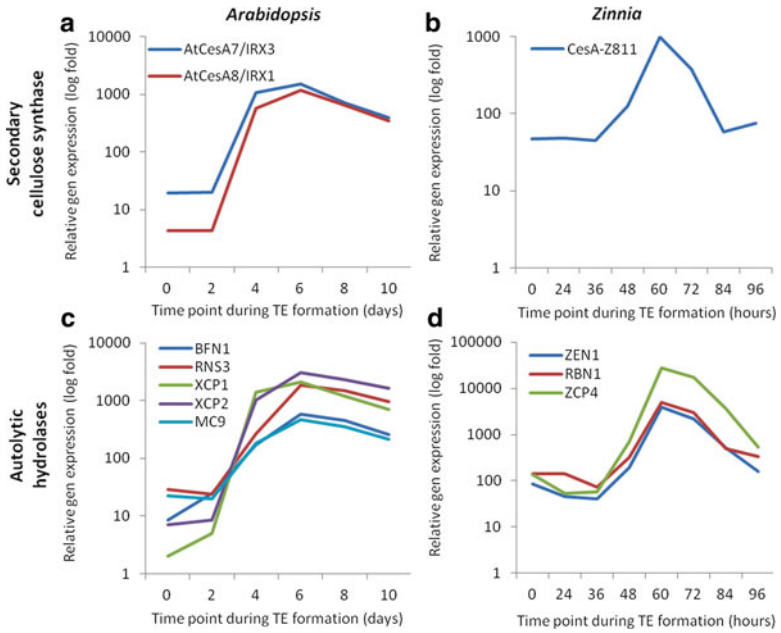

Fig. 3.5 Microarray expression profiles of genes associated with TE *post-mortem* autolysis compared to secondary cellulose synthase genes. In silico gene expression is presented from microarray experiments performed on TE differentiating cells of *Arabidopsis thaliana* (**a**, **c**), from Kubo et al. [15], and *Zinnia elegans* (**b**, **d**), from Demura et al. [18]. Secondary cellulose synthases are presented in (**a**, **b**) and autolytic genes in (**c**, **d**). Homologous autolytic genes include RNS3, BFN1, XCP1 and XCP2 in *Arabidopsis* and RBN1, ZEN1 and ZCP4 in *Zinnia*

factors vascular-related NAC-domain 6 and 7 which trigger the expression of the secondary cell wall synthesis genes as well (VND6/At5g62380 [53] and VND7/At1g71930 [54]).

3.3.1 TE Nuclear Degradation and BFN1/ENDO1

The main enzyme responsible for the degradation of TE nucleus is BFN1/ENDO1 in *Arabidopsis* or its homolog with 54 % similarity, the endonuclease 1 (ZEN1) in *Zinnia* [55]. The expression of these nuclease genes is co-regulated with secondary cell wall cellulose synthase genes in TE differentiating cell suspension cultures of *Zinnia* [18, 20] and *Arabidopsis* [15, 17] (Fig. 3.5). BFN1/ENDO1 (AT1G11190) and ZEN1 (AB003131) are S1-like nucleases capable of degrading RNA as well as single- and double-stranded DNA [56]. The optimal nucleolytic activity of BFN1/ENDO1 and ZEN1 is dependent on the presence of divalent cations such as Ca^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} [56]. Although believed to fulfil the same role in both species, BFN1/ENDO1 and ZEN1 exhibit distinct biochemical features: BFN1/ENDO1 is activated in neutral pH ~7 by Ca^{2+} and Mn^{2+} but inhibited by Zn^{2+} [56], whereas ZEN1 is activated in acidic pH ~5 by Zn^{2+} and is insensitive to Ca^{2+} and Mg^{2+} (Fig. 3.6b; [57, 58]). *BFN1/ENDO1* is also expressed in cells undergoing other types of cell death processes such as leaf senescence, fruit abscission and the

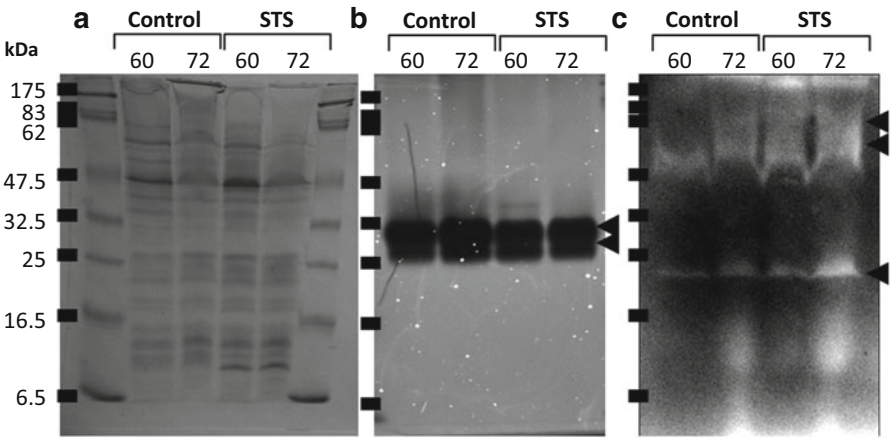

Fig. 3.6 Enzymatic activity gel characterising the autolytic apparatus (nucleases and proteases) present in differentiating *Zinnia* TEs prior to vacuole rupture. TE autolytic hydrolases accumulate during the secondary cell wall deposition in *Zinnia* cell culture which appear between 60 and 72 h of culture [25]. Autolytic activities of total soluble proteins at these time points (presented by Coomassie staining) in (**a**) reveal that both nuclease activities (cf. [58]) in (**b**) and protease activities (cf. [71]) in (**c**) are present. *Arrow* heads indicate different nucleolytic and proteolytic activities. Note that treatment with STS does not affect the autolytic enzymes present in TEs

autolytic activity in the lateral root cap cells [59–61]. Both BFN1/ENDO1 and ZEN1 present a peptide signal and no C-terminal ER-retention sequence suggesting that these nucleases are located beyond the ER in the endomembrane secretory system. BFN1 is more precisely localised in specific filamentous compartments derived from the ER (Fig. 3.3a–d; [62]). These specific compartments have a neutral pH and do not colocalise with acidic vesicles labelled with LysoTracker (Fig. 3.3a–d; [62]). As PCD progresses, BFN1 is relocalised to ER filaments surrounding the nucleus where it is believed to contribute to the degradation of the nuclear DNA (Fig. 3.3a–d; [62]). The functional analysis of ZEN1 in differentiating TE cell cultures of *Zinnia* confirmed the role of ZEN1 in controlling the rate of *post-mortem* nuclear degradation but is not implicated in the vacuole rupture itself [58].

3.3.2 RNA Degradation by Ribonucleases

The ribonucleases—ribonuclease 1 in *Zinnia* (Rbn1, AAC49325) and its 46 % homologous ribonuclease 3 in *Arabidopsis* (RNS3, AT1G26820)—have been associated with TE *post-mortem* autolysis on the basis of gene expression profiles and enzymatic activity during TE formation [15, 18, 63]. The expression of these ribonuclease genes is also co-regulated with secondary wall cellulose synthase genes in cell suspension culture of *Zinnia* [20, 64] and *Arabidopsis* [15, 17] (Fig. 3.5). These TE-specific ribonucleases present a peptide signal and no C-terminal ER-retention sequence suggesting that these enzymes are localised in the endomembrane secretory system (Fig. 3.3). Both Rbn1 and RNS3 are T2/S-like endoribonucleases capable of fully hydrolyzing RNAs and are associated with several types of cell death and senescence processes based on their expression patterns [65, 66]. RNS3 presents a very narrow pH optimum around 6 [67]. The tomato homolog of RNS3, the ribonuclease LX, was shown to accumulate in the ER of maturing TEs [66] and to participate in leaf senescence, but no apparent function has been yet defined in xylem differentiation [68].

3.3.3 Proteolysis of the TE Cytoplasmic Content

One of the first evidence of the *post-mortem* degradation of TE protoplasts is the reduction of cytoplasmic density observed by electron microscopy, suggesting that the cytoplasmic content is rapidly being hydrolysed [24, 47]. Both intracellular and extracellular activities of cysteine and serine proteases have been detected during TE formation in *Zinnia* cell cultures [53, 69–71] and in *Arabidopsis* xylem [72]. The cysteine proteases associated with TE autolysis have an optimal acid pH ~4.5 [70–73], which is obtained by the release of the acidic contents of the vacuole into the cytoplasm once the tonoplast ruptures [48]. These proteases include cysteine proteases such as XCP1 (AT4G35350) and XCP2 (AT1G20850) in *Arabidopsis*

[72] or ZcP4 (AB091070) and p48h-17 (U19267) in *Zinnia* [18, 64, 70], putative serine proteases such as Xylem Serine Protease 1 (XSP1–AT4G00230) in *Arabidopsis* [72] or SP (AB091070) in *Zinnia* [64, 70, 71] and a phytepsin aspartate protease in *Hordeum vulgare* (X56136) [74]. *XCP1* and *XCP2* are specifically expressed in TEs [51] and co-regulated with secondary cell wall synthesis genes in both *Arabidopsis* [15, 23, 75] and *Zinnia* TE cell cultures [18, 20] (Fig. 3.5). XCP1 and XCP2 both present an N-terminal peptide signal targeting them to the secretory pathway. Constitutive 35S-driven over-expression in undifferentiated cells results in vacuolar accumulation of XCP1 [51], while immunolocalisation of XCP1 and XCP2 in *Arabidopsis* TEs shows first a localisation of these proteins close to ER followed by an accumulation in the central vacuole as TE PCD progresses (Fig. 3.3a–e; [50]). Interestingly, a homologous cysteine protease in *Trifolium repens* was shown to accumulate only in the ER and not in the vacuole [76]. Both genetic over-expression and knockout analyses clearly demonstrated that the XCP proteins are not involved in triggering TE PCD but are implicated in the *post-mortem* clearing of TEs [50, 51]. However, it is also clear that additional proteases participate in the *post-mortem* TE clearing process as the single and double loss-of-function mutants *xcp1* and *xcp2* in *Arabidopsis* only show a slight delay [50]. The asparaginyl-specific cysteine protease vacuolar processing enzymes (VPEs) which are associated with other types of developmental PCDs [77] could be candidates. Indeed, α- and γ-VPEs (AT2G25940 and AT4G32940) are expressed during the active deposition of TE secondary cell walls together with cellulose synthase genes in *Arabidopsis* TE differentiating cell cultures and in whole plants [15, 23, 78] as well as during poplar xylem formation [79]. Treatments of *Zinnia* TEs with caspase-1 inhibitor Ac-YVAD-CMK—considered to target VPEs in plants—reduced TE differentiation [80]. A quadruple VPE knockout mutant of *Arabidopsis* did not seem to have any defects in vegetative growth [81], which makes it unlikely that VPEs play a central role in TE autolysis. Other potential protease candidates include the ER-resident cysteine endopeptidases CEP1, CEP2 and CEP3 that are highly similar to XCP1/2 and are all expressed in the plant vascular tissues [82]. It has been recently shown that CEP1 (AT5G50260) is located in the ER during tapetum development and is then relocated during the later stages of tapetum development to the vacuole [83]. These authors speculated that the vacuolar localisation of CEPs activated the enzymes, due to the acidic content of the vacuole, thus accelerating the speed of autolysis. Although single CEP mutants show no obvious xylem defects [83, 84], investigations in double and triple mutants need to be performed to clarify the intervention of CEPs during TE autolysis.

3.3.4 Proteolysis by Cytoplasmic MC9

Caspases are a family of cysteine proteases responsible of both early signalling and execution of animal cell PCD. Caspase-like activities have frequently been observed in various plant tissues and even in differentiating TE cell cultures of *Zinnia* [80].

Treatments with caspase inhibitor Z-Asp-CH2-DCB in *Zinnia* differentiating TEs supported the functional role of caspase-like activities in *post-mortem* degradation of TEs [80]. Plants do not have canonical caspases per se but structurally related proteases named metacaspases (MC) which have distinct enzymatic activities [85]. In *Arabidopsis*, these form a small family of nine members [47], but only MC9 (AT5G04200) is specifically expressed in TEs. MC9 is also co-regulated with the TE secondary cell wall cellulose synthase genes in both *Arabidopsis* and poplar (Fig. 3.5; [15, 75, 79]). MC9 has a pH optimum of 5.5 [85] and presents a cytoplasmic localisation [47, 85]. Functional analyses showed that, just like XCP1 and 2, MC9 is involved in the TE content clearing process [47]. It is also plausible that MC9 is involved in processing other cysteine proteases in plant PCD [47], hence operating in analogy to the caspase cascades during animal PCD.

3.4 TE Cell Death Signalling

In order to coordinate the execution of cell death with the secondary cell wall formation, environmental cues and the *post-mortem* processes, a tight signalling mechanism of TE PCD in both time and space is paramount. Several molecular signals have been associated with TE cell death like Ca^{2+} intake (Fig. 3.5c; [32]) and nitric oxide release [86]. However, in both cases the associated drug treatments used (scavenger for NO [86] and chelators, ionophores for Ca^{2+} [32]) did not uncouple TE differentiation from TE cell death. The role of reactive oxygen species (ROS), classically associated with PCD, is not clear either. ROS have been shown to accumulate during TE differentiation but appears to be associated with *post-mortem* lignification rather than cell death itself (Fig. 3.7; [22, 24, 87]). An interesting signalling mechanism was proposed by Groover and Jones [32] to coordinate PCD with the completion of TE secondary cell wall deposition through the accumulation of an extracellular serine protease. While TEs develop their secondary cell walls, this serine protease hydrolyses the extracellular matrix which, together with an influx of calcium, can trigger the vacuolar collapse causing the cell death. To prove the significance of this mechanism, further studies are needed, but it is a fascinating idea that the timing of PCD could be determined by the accumulation levels of molecule(s) reflecting the thickness of the secondary cell wall.

3.4.1 Ethylene: Dual Function to Induce and Kill TEs

Ethylene, a gaseous hormone, represents a molecular signal associated with many types of cell death and senescence processes in plants [88]. Ethylene is produced in a triphasic manner during TE formation in *Zinnia* cell cultures with characteristic peaks of accumulation during the initiation, secondary cell wall formation and after TE death—generally referred to as phase II, III and IV of *Zinnia* TE formation

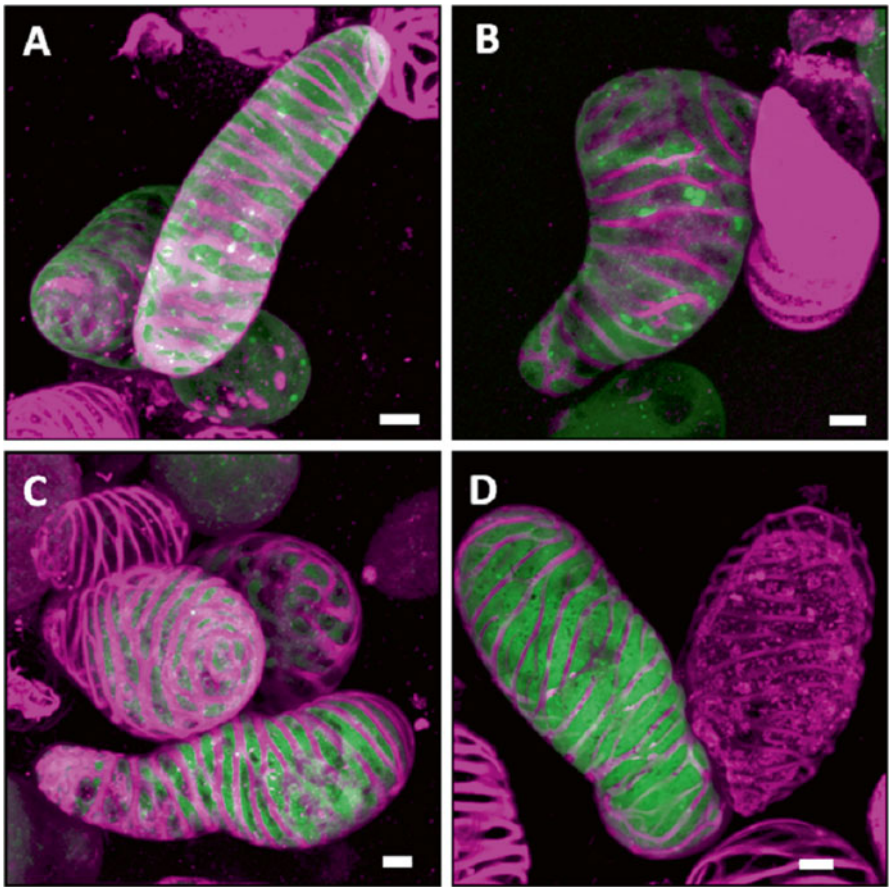

Fig. 3.7 Confocal laser scanning microscopy projections of reactive oxygen species (ROS) accumulation in differentiating TEs of *Arabidopsis* stained by dichloro-dihydro-fluorescein diacetate (DCFH-DA; *green*) and counterstained with LysoTracker Red (Fuschia, acidotropic dye also colouring plant cell walls) at 4 days (**a**), 5 days (**b**), 6 days (**c**) and 8 days (**d**) of culture. ROS signal (*green*) is concentrated in spherical structures at early time points (4 and 5 days) and then becomes more homogenous and intense at later time points (6 and 8 days). Bars = 10 μm

(Fig. 3.8a; [89]). Ethylene is first required to trigger TE differentiation in the *Zinnia* differentiation system as treatments with ethylene synthesis inhibitor aminoethoxyvinylglycine (AVG) abolish TE formation [89]. Ethylene is then required to trigger TE PCD in the *Zinnia* TEs as treatment with ethylene perception inhibitor silver thiosulfate (STS) prevents TE *post-mortem* processes (inhibition of lignification and protoplast autolysis) without interfering with the *pre-mortem* secondary cell wall deposition (Fig. 3.8b, c; [22]). The role of ethylene produced by living parenchyma cells after TE cell death remains unclear; but its role supposedly regulates the coordination between TEs in formation, differentiated TEs and their associated parenchyma cells [89].

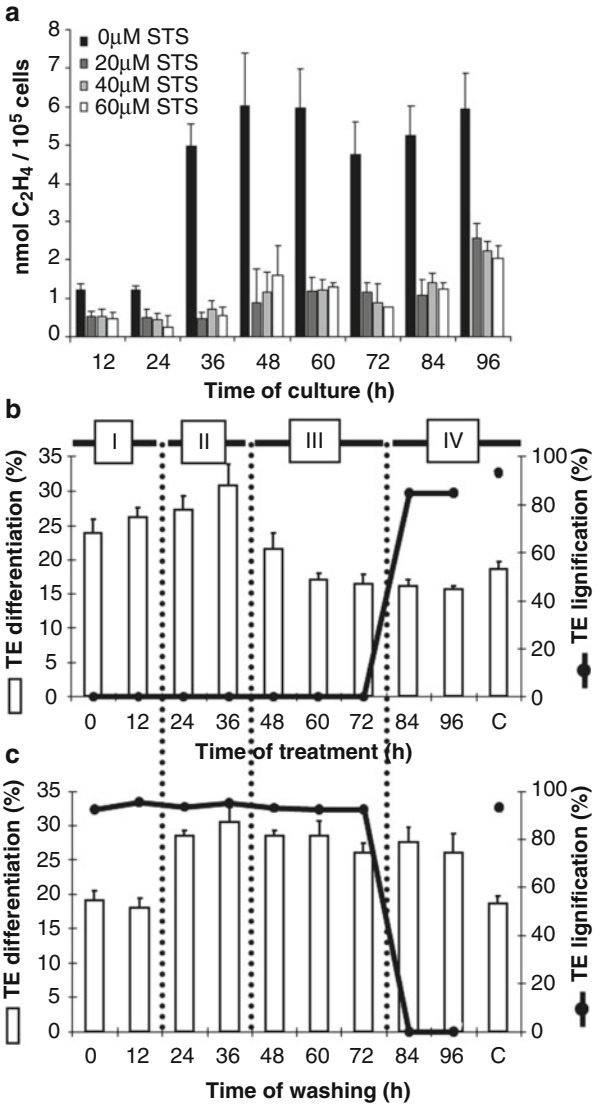

Fig. 3.8 Ethylene production and role during TE formation in *Zinnia* differentiating cell cultures. (**a**) Measurements of ethylene production in the presence of silver thiosulfate (STS) during TE formation according to Pesquet and Tuominen [89]. Note that ethylene accumulation in *Zinnia* TE differentiating cells is under a positive feedback control. (**b**) Effect of the delayed addition of STS during TE cell differentiation. (**c**) Effect of delayed removal by washing of STS during TE differentiation. Note that both TE differentiation efficiency and TE lignification are presented (three replicates of ~300 cells); bars represent standard deviation. TE differentiation is subdivided into four successive stages including dedifferentiation (I), initiation (II), secondary cell wall formation (III) and after TE death (IV) [89]

The delayed application (Fig. 3.8b) and removal (Fig. 3.8c) of STS during TE differentiation in *Zinnia* clearly confirms the dual role of ethylene in (1) increasing the TE differentiation efficiency in phase II and (2) controlling the TE *post-mortem* lignification in phase IV. Moreover, the STS treatment extends the *pre-mortem* phase III showing a longer gene expression of both secondary cell wall synthesis genes and autolytic hydrolases [22]. The STS treatment in *Zinnia* TEs presents no positive TUNEL labelling (Supplementary Movie 3.3; [22]), further confirming that TE *post-mortem* processes have not been triggered. However, both nucleolytic and proteolytic activities associated with TE *post-mortem* autolysis can be detected (Fig. 3.6), thereby confirming that ethylene is implicated in the signalling of TE cell death rather than TE autolysis. Genetic confirmation of ethylene intervention during TE PCD is difficult to achieve in whole plants: gain- and loss-of-function mutants affected in ethylene production or signalling do not show visible defects in TE cell death but only in TE precursor cell division [90, 91]. However, ethylene is produced in late maturing xylem [92] which supports the role of ethylene in the PCD of xylem cells in whole plants. It is nevertheless possible that the xylem tissue in the whole plant may somehow compensate defective ethylene signalling by activating an alternative pathway—which could not be possible in single-cell in vitro cultures.

3.5 Conclusion

The cell death of TEs is an extraordinary phenomenon as it is one of the few cell death processes in plants that initiates the functional "life" of the cells by enabling TEs to transport the hydro-mineral sap. The use of in vitro TE differentiating cell suspension cultures and real-time live cell imaging has provided breakthroughs in understanding the cell biology and physiology of TE cell death. Such discoveries include the elucidation of the central role of vacuole in triggering cell death, the timing and morphological characteristic of the cellular changes as well as the molecular and hormonal control of TE PCD. Great advances have also been made in understanding the TE clearing mechanism in which hydrolytic enzymes synthesised *pre-mortem* are activated rapidly *post-mortem*. A safety system, based on specific pH requirement of the enzymes, subcellular compartmentalisation and possibly proteolytic activation, prevents premature activation of the hydrolytic programme. Integrity of the vacuole is central in controlling this safety mechanism even though the importance of other organelles has to be emphasised as well. Increasing evidence supports the localisation of crucial hydrolytic enzymes in various ER-derived compartments rather than in the vacuole but also a translocation of enzymes between these various organelles. Therefore, we need to revisit the role of the vacuole as the main storage organelle for the *post-mortem* hydrolytic enzymes and also to increase our understanding of the function of the other components of the endomembrane system in the regulation of TE autolysis.

Another challenge for the future is to define the identity of the initial signals triggering TE PCD. It is widely accepted that cell death is carried out by the vacuolar

rupture, yet important signalling events and crucial cellular changes which precede the tonoplast implosion are likely to trigger the TE cell death. Studies on other developmental PCD in plants suggest that processes such as ethylene production, cytoskeletal modifications, pH changes, a burst of ROS and autophagy occur before vacuolar rupture and contribute to the outcome of the cell death. These processes need to be characterised in detail during TE cell death. The in vitro TE differentiation cell culture systems represent a perfect tool to unravel these lines of investigations.

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Chapter 4
Programmed Cell Death in Plant Immunity: Cellular Reorganization, Signaling, and Cell Cycle Dependence in Cultured Cells as a Model System

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4.1 Programmed Cell Death and Hypersensitive Responses in Plant Immunity Against Pathogen Attack

Programmed cell death (PCD) is a genetically regulated process of cellular suicide and is well known to play a fundamental role in a wide variety of developmental and physiological functions in multicellular organisms [1–3]. In plants, PCD plays a critical role in the control of developmental processes such as xylogenesis, embryogenesis, pollen maturation, seed development, seed germination, and leaf senescence, as well as various stress responses including innate immunity against pathogen attack [4]. Reproductive development in angiosperms involves PCD in a variety of cells in reproductive organs, such as reproductive primordium abortion, style transmitting tissue, nonfunctional megaspores, synergids, antipodals,

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endosperm, anther tapetum, and abortive pollen in male sterility [4–7]. Evidence to date suggests plants lack homologs of most animal apoptosis-related genes and have evolved several specific mechanisms for PCD.

Plants also lack immune systems based on antibodies or phagocytosis. Instead, they have evolved multiple active defense responses including reorganization of the cell wall and production of pathogenesis-related (PR) proteins and antimicrobial secondary metabolites called phytoalexins. Initial cellular responses also include the production of reactive oxygen species (ROS) mediated by enzymes such as NADPH oxidases (Nox), as well as plasma membrane ion fluxes and increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$; [8]). This dynamic cellular reorganization is triggered at the site of infection and often accompanies localized PCD, known as the hypersensitive response (HR), which is effective in preventing the spread of pathogens [9–11].

Notable differences of plant and animal cells are the presence of the cell wall, the plastids/chloroplasts, and the vacuole, which all play crucial roles in the regulation of plant immunity and PCD. Execution of PCD takes place with different morphological features from typical animal cell death programs such as apoptosis. Cellular morphological changes in animal cells undergoing apoptosis include cell shrinkage and nuclear fragmentation and are followed by the fragmentation of cells and formation of apoptotic bodies, which are then phagocytosed. Although the similarities and differences between PCD in plants and animals have been discussed extensively [12–14], the mechanisms for execution and regulation of plant PCD, including HR, still remain to be elucidated.

The execution of cell death in a regulated fashion accompanies dynamic reorganization of the cellular architecture. Cell biological aspects of immune responses accompanying PCD have been studied in various experimental systems using a combination of plants and microbes. A major experimental approach had been the immunostaining of plant tissues infected with microbes [15–17]. However, it should be noted that the deformation of endomembrane systems, by chemical fixation, occurs with this technique. In vivo imaging based on various fluorescent probes such as GFP has allowed time-sequential observations of the endomembrane systems in plant-microbe interactions [18, 19]. Cellular morphological changes are often governed by cytoskeletons such as actin microfilaments (MFs) and microtubules (MTs) as well as the vacuole.

Reduction of growth accompanying cell cycle arrest is induced by various kinds of abiotic stresses such as oxidative damage mediated by menadione [20] or $KMnO_4$, hypoosmotic stress [21], flooding stress [22], and DNA damage [23, 24]. Such growth reduction is also seen during immune responses against pathogens. Treatment with pathogen/microbe/damage-associated molecular patterns (PAMP/MAMP/DAMPs) induces both defense responses and growth inhibition [25]. Pathogen-derived molecules called elicitors also induce downregulation of some cell cycle-related genes along with the induction of defense-related genes [26–28], suggesting that the downregulation of cell cycle-related genes may be involved in growth inhibition. No homologs of p53, involved in cell cycle regulation in animals, have been found in plants [29]. Although the molecular mechanisms for

immunity-related growth inhibition are largely understood, the molecular mechanisms of stress-induced cell cycle arrest are mostly unknown.

Plant cell cultures are useful simple model systems to monitor cellular events. Defense responses including intracellular reorganization and gene expression upon fungal infection are basically similar between cultured cells and *in planta* [30]. Treatment of cultured cells with purified signal molecules from pathogens (PAMP/MAMP/DAMPs) can mimic most defense responses, including the expression of defense-related genes, as effectively as microbial infections [31–33].

Here, we give an overview on the spatiotemporal dynamic changes of PCD triggered by signals from pathogens and compare the interrelationships among the reorganization of the cellular architecture, cell cycle, and signaling including ROS production, MAPK activation, and Ca^{2+} rise during innate immunity in cultured cells.

4.2 Effects of the Pathogenic Signal-Induced Reorganization of Cellular Architecture

Execution of cell death in a regulated fashion is accompanied by dynamic reorganization of cellular architecture. In plants, cellular morphological changes are governed by the cytoskeleton, including MFs and MTs [34] and the vacuole [35]. Rapid development of live cell imaging techniques has recently revealed novel aspects on the dynamics of these intracellular structures. Transgenic tobacco BY-2 cell lines expressing the GFP-markers for vacuolar membranes (VM; [36]) and cytoskeletons [37, 38] have been effective in monitoring dynamic changes in the cellular architecture during cryptogein-induced defense responses in vivo [39, 40].

4.2.1 Vacuole-Mediated Programmed Cell Death in Innate Immunity

The plant vacuole occupies most of the cell volume and contains many hydrolytic enzymes for digestive processes, similar to lysosomes in animal cells. The vacuole performs various functions essential for plant growth, development, and adaptation to both abiotic and biotic stresses [41]. In these processes, vacuoles show dynamic morphological changes including VM rearrangement [42].

Disintegration or collapse of the VM has been suggested to trigger the final step of PCD in several cell types [35, 43, 44]. At the final stage of cryptogein-induced PCD in tobacco BY-2 cells, disintegration of the VM is followed by the irreversible loss of plasma membrane integrity and cell shrinkage ([39]; Fig. 4.1). Tobacco mosaic virus-induced hypersensitive cell death in tobacco leaves involves vacuolar rupture, in which a vacuole-localized protease called vacuolar processing enzyme

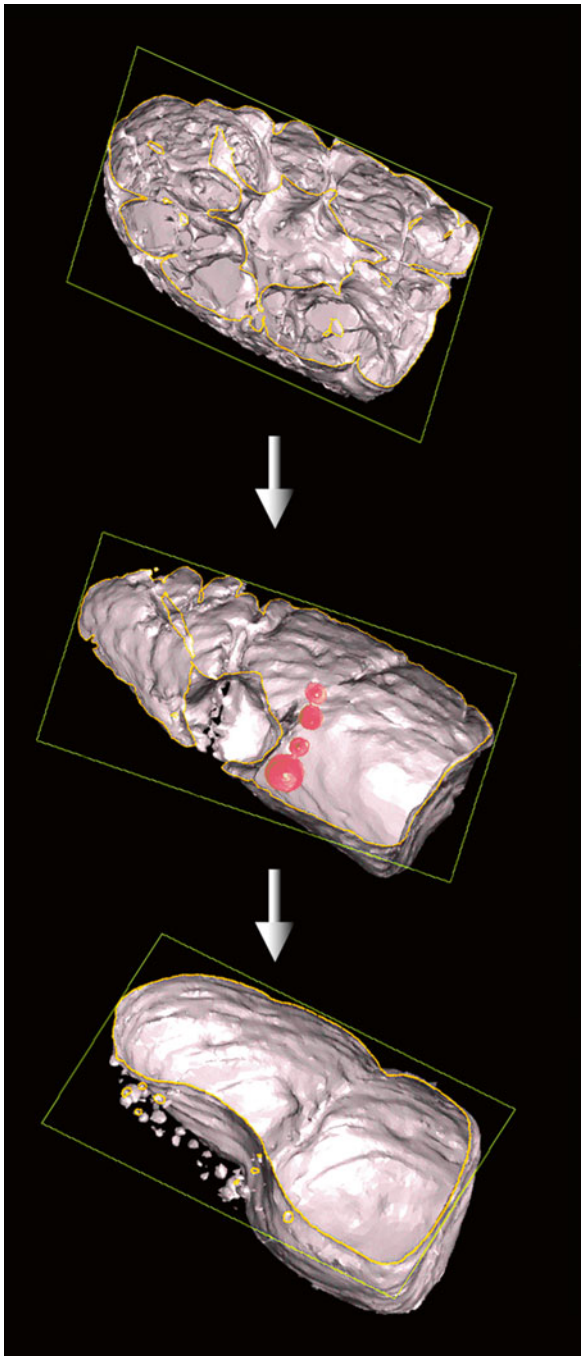

Fig. 4.1 Rearrangement of the vacuolar membrane structures during cryptogein-induced cell death in S-phase-synchronized tobacco BY-2 cells. Representative three-dimensional surface models reconstructed from serial sections of confocal microscopic images are shown. Experimental details are as described in Higaki et al. [39]. Many transvacuolar strands radially oriented from cell nuclei gradually decreased, and bulb-like structures (*pink*) appeared after the cryptogein treatment (from *top* to *middle*). Thereafter, the internal vacuolar membrane structures including bulb-like structures disappear before the disintegration of vacuolar membranes and execution of cell death (from *middle* to *bottom*)

(VPE) exhibiting caspase-1-like activity is involved [45, 46]. Disintegration of the VM and/or vacuolar collapse needs to be strictly regulated to accomplish PCD at appropriate timing. Another mechanism involving fusion of the VM with the plasma membrane, resulting in the discharge of vacuolar antibacterial proteins to the outside of the cells where bacteria proliferate, has also been proposed for biotrophic bacteria-induced hypersensitive cell death [47].

Cryptogein-induced PCD in BY-2 cells accompanies dynamic reorganization of the vacuole prior to the execution of cell death [40]. Cryptogein induces a decrease in the transvacuolar strands (TVS), tubular regions of the cytoplasm connecting the nucleus to the cell periphery, and formation of spherical intravacuolar structures called the "bulb" [39] that has been observed in a wide range of plant tissues [48–50]. The bulb-like structure could be derived from the excess VM comprising the TVS. At the later stage of the PCD, the bulb-like structure disappears and the structure of the large central vacuole gets simpler. Simplification of vacuolar morphology is commonly observed in various PCD processes [51–55], suggesting a general role of VM rearrangement in vacuole-mediated PCD in plants.

The molecular mechanism of the vacuolar shape simplification is still ambiguous. A KEG (KEEP ON GOING) protein that contains functional RING-type E3 ligase domain [56] is involved in vacuolar expansion and cell growth [57]. The KEG proteins are localized at the trans-Golgi network and early endosomes, suggesting its critical roles in membrane trafficking to the vacuole [57, 58]. Interestingly, infection by a powdery mildew fungus *Golovinomyces cichoracearum* causes specific degradation of KEG proteins, suggesting KEG is targeted by fungus effectors to perturb the membrane traffic in the host cells [57]. This plausible story is supported by the putative roles of KEG in recruitment of EDR1 (ENHANCED DISEASE RESISTANCE1) kinase, which is involved in powdery mildew resistance [59], to the trans-Golgi network and early endosomes [58]. Future analysis on its target proteins would clarify the relationship between membrane trafficking and vacuolar morphological changes during defense responses.

4.2.2 Reorganization of Actin Microfilaments and Vacuole-Mediated PCD

MFs are involved in vacuolar morphogenesis including TVS formation in higher plants [60]. Live cell imaging has revealed that cryptogein-induced vacuolar reorganization accompanies MF reorganization in tobacco BY-2 cells [39]. In addition, a MF inhibitor promoted both the simplification of vacuolar structure and the induction of PCD [39]. Based on a series of these observations in the model system, we proposed a hypothetical model for a MF-regulated vacuole-mediated PCD. During the PCD process, MFs running through TVS disappear, but MF bundles appear on large vacuoles. MF rearrangement causes the conversion of intravacuolar

morphology from TVS to bulb-like structures. The MF bundles on the large vacuoles are necessary to maintain the bulb-like structures by covering the large vacuole surface. Disruption of the MF bundles triggers the simplification of the vacuoles, reduction of bulb-like structures, and formation of small spherical vacuoles derived from the bulb-like structures. The simple-shaped vacuoles are easy to rupture by water absorption and cause cell death defined by PM disintegration. In this model, the MF bundles that appear in the process negatively regulate vacuole-mediated PCD [35]. In other words, MF bundling may act as a safety lock against unexpected vacuolar rupture and cell death to keep the bulb-like structures intact.

Vacuolar rupture is observed in virus-induced hypersensitive cell death as well as other types of PCD in plants [46]. Possible involvement of actin MFs in the regulation and execution of the vacuole-mediated PCD in plants is an emerging important research topic. Fusion of the vacuolar and the PMs during bacterial infection-induced hypersensitive PCD has recently been reported by transmission electron microscopy and live imaging of GFP–PIP2a, a PM protein, and mRFP–AtVAM3, a VM protein in *Arabidopsis thaliana* [47]. This heterologous membrane fusion should accomplish quick discharge of vacuolar contents including antibacterial proteins into apoplastic space. A defect in proteasome subunit PBA1 abolishes the membrane fusion and the PCD. An attractive model has been proposed that proteasome-driven degradation of the unidentified heterologous membrane fusion inhibitor(s) triggers the bacterial infection-induced PCD [47, 61].

Functional modification and critical roles of the vacuole during the execution of hypersensitive cell death induced by viruses [45] and eukaryotic pathogens [39] seem to be different from those by bacterial pathogens. In cryptogein-treated tobacco BY-2 cells, the heterologous membrane fusion does not occur at least until VM disintegration because a vacuole-accumulated fluorescent probe leaks into the cytosol, and conversely free GFP-tubulin moves from the cytosol into the vacuolar region [39]. These observations are supported by recent findings on the modulation of a vacuolar protease by cytoplasmic proteins. Activity of a vacuolar papain-like cysteine protease RD21 (RESPONSIVE-TO-DESICCATION-21), which promotes cell death triggered by the necrotrophic fungus *Botrytis cinerea* or *Sclerotinia sclerotiorum*, is suppressed by overexpression of the gene for AtSerpin1, which is a cytoplasm-localized proteinaceous inhibitor of RD21 [62, 63].

In contrast to animals for which viruses and bacteria are major pathogens, the majority of plant pathogens are eukaryotes. These cell biological events in cryptogein-induced PCD in cultured cells may reflect common features of hypersensitive cell death induced by eukaryotic pathogens. Though dynamic functional modification of the VM seems to be a common critical step during execution of various PCD in plants, there are also a range of membrane dynamics and cell biological processes to execute cell death. In the future comparative cell and molecular biological studies of various pathways leading to the execution of the final steps of PCD in plants will be particularly important.

4.3 Early Signaling Events to Trigger Hypersensitive Cell Death

Upon recognition of signal molecules from pathogens, plant cells activate a widespread signal transduction network involving second messengers as early signaling events, which triggers inducible immune responses [64]. Characteristic early signaling events include influxes of Ca^{2+} and H^+; effluxes of K^+ and Cl^-; membrane potential changes, typically transient membrane depolarization [32, 65–68]; ROS production by enzymes such as NADPH oxidase [69, 70]; and activation of a MAPK cascade. These initial responses are followed by biosynthesis of phytoalexins, vacuolar collapse, and PCD.

Treatment of tobacco (*Nicotiana tabacum*) BY-2 cells with cryptogein, a protein from the oomycete *Phytophthora cryptogea*, induces various immune responses such as membrane potential changes, ion fluxes, biphasic ROS production, and MAP kinase activation in a cell cycle-dependent manner [71, 72], followed by cell cycle arrest and PCD [73, 74]. The slow prolonged phase, not the rapid transient phase, of ROS production shows strong correlation with downstream events including expression of defense-related genes and PCD [71, 72].

NADPH oxidase-mediated ROS production has been suggested to play a crucial role in triggering and regulating PCD [75, 76]. Respiratory burst oxidase homolog (Rboh) proteins show ROS-producing activity synergistically activated by binding of Ca^{2+} to their EF-hand motifs and protein phosphorylation [77–79]. Potato StRbohB has been shown to be activated by phosphorylation by calcium-dependent protein kinases StCDPK4 and StCDPK5 [80]. *Arabidopsis* AtRbohF binds CIPK26, a protein kinase activated by binding of calcineurin B-like (CBL) Ca^{2+} sensor proteins, *in planta* [81], and is activated in the presence of Ca^{2+}, CBL1/CBL9, and CIPK26 [82].

Rise in cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ is one of the earliest common responses triggered by various pathogenic signals [64]. Correlation between the temporal pattern of $[Ca^{2+}]_{cyt}$ or Ca^{2+} signature and induction of downstream events including PCD has been discussed. For example, chitin fragments, a typical PAMP/MAMP, triggers a rapid/transient $[Ca^{2+}]_{cyt}$ rise without induction of PCD, while xylanase protein from a fungus *Trichoderma viride* (TvX) triggers a prolonged $[Ca^{2+}]_{cyt}$ rise followed by the induction of PCD in cultured rice cells [83].

Anion effluxes are often accompanied with the induction of immune responses and PCD [32, 84]. $[Ca^{2+}]_{cyt}$ rise is inhibited by several anion channel blockers, indicating the importance of the plasma membrane anion channel for the induction or amplification of $[Ca^{2+}]_{cyt}$ response [32, 84]. *Arabidopsis* SLAC1, an S-type anion channel, functions in cryptogein-induced early signaling events to trigger PCD in tobacco BY-2 cells. Functional characterization of *Arabidopsis SLAC1-overexpressing* lines suggests that SLAC1 mediates cryptogein-induced Cl^- efflux through the plasma membrane to positively modulate the elicitor-triggered activation of extracellular alkalinization, NADPH oxidase-mediated ROS production, and a wide range of defense responses including PCD [85].

4.4 Hypersensitive Cell Death and Cell Cycle

4.4.1 Pathogenic Signal-Induced Cell Cycle Arrest

The cell cycle is a tightly controlled process divided into four (S, M, G1, G2) distinct phases. During the S and M phases, the cell replicates its genome and separates the duplicated genome between the two daughter cells, respectively. Both phases are followed by a gap phase, designated G1 and G2 [86]. In animal cells, the crosstalk between cell cycle progression and apoptosis or immune responses has been well studied [87]. Similar crosstalk has also been suggested to exist in plant cells. Two major PAMPs, flg22 and elf18, induce immune responses including defense-related gene expression along with growth inhibition in *Arabidopsis* plants [88]. A variety of *Arabidopsis* and rice lesion mimic mutants expressing defense-related genes constitutively show dwarf phenotypes [89, 90], suggesting the presence of positive crosstalk between cell cycle progression and immune responses in plants as well as animals.

Synchronous culture of tobacco BY-2 cells using the aphidicolin or the aphidicolin/propyzamide synchronization method [91] has been developed to quantitatively evaluate the interrelationship between cryptogein-induced HR, including PCD, and the cell cycle [73]. The elicitation by cryptogein during S phase causes cell cycle arrest at G2 phase accompanying suppression of cell cycle-related genes *NtCycA1;1* and *NtCycB1;3* in BY-2 cells. In contrast, cells treated with cryptogein in late G2, M, or G1 phases progressed to M/G1 phase and arrested at G1 phase with suppression of *NtCycD3;1* and *PCNA* expression [71, 73]. Cyclins bind and activate cyclin-dependent kinases (CDKs), thus playing a central role in cell cycle regulation. A1- and B1-type cyclins are thought to be involved in the progression from G2 to M phase, and D-type cyclins and PCNA are crucial for the progression from G1 to S phase [92, 93]. Cryptogein suppresses the activity of CDKA and CDKB1 during G2 to M phase along with both suppression of expression of various cell cycle-related genes and degradation of CDKB1 and cyclin [74].

In animal and yeast cells, stress-induced cell cycle arrest is controlled by specific genes, and mutations in these genes often result in increased sensitivity to damaging reagents such as oxygen radicals. Moreover, these genes are commonly mutated in various kinds of cancers, highlighting their importance in maintenance of the cell cycle [94]. One of these genes encoding p53 protein harbors mutations in more than half of all human cancers [95]. p53 takes part in G1 arrest in response to DNA damage. DNA damage-induced cell cycle arrest in the G1 and S phases may partly involve inhibition of the activity of G1 CDKs by the specific CDK inhibitor p21 [96]. Furthermore, the mechanism underlying DNA damage-induced G2 arrest was shown to involve a specific inhibitory phosphorylation of the mitotic kinase CDK1 in human cells [97, 98]. However, no homologs of p53 involved in cell cycle regulation in animals have been found in plants [29]. Cryptogein induced a reduction in the level of NtWEE1, which is thought to be a negative cell cycle regulator [74], suggesting that cryptogein affects multiple targets to inactivate CDKA to induce G2

arrest by mechanisms distinct from known checkpoint regulation. Additional analyses of this system may provide further molecular links between signaling events and cell cycle regulation during stress responses in plants.

4.4.2 Cell Cycle Dependence of Immune Responses and Hypersensitive Cell Death

Plant innate immunity consists of two layers of responses. The first is governed by extracellular transmembrane receptors or pattern recognition receptors. By recognizing conserved PAMP/MAMP/DAMPs, pattern recognition receptors trigger a relatively weak immune response known as pattern-triggered immunity (PTI) that inhibits colonization by invading organisms [99]. The second layer of plant innate immunity is based on highly polymorphic resistance (R) proteins that are activated upon the recognition of highly variable pathogen molecules called avirulence effectors. This effector-triggered immunity (ETI) consists of a rapid and robust response, often associated with HR including PCD to control the spread of biotrophic pathogens [11, 100–103].

Cryptogein induces not only HR and PCD but also growth inhibition and cell cycle arrest in G1 or G2 phase [73]. The pattern of cryptogein-induced HR changes depending on the cell cycle stage. BY-2 cells in the S phase arrest the cell cycle at G2 phase and induce the expression of defense-related genes and PCD immediately [71]. In contrast, BY-2 cells in G2 or M phase arrest the cell cycle at G1 phase and induce these responses at the same time as cells treated with the elicitor in G1 phase, suggesting that HR and PCD, and cell cycle arrest, are induced only at specific phases of the cell cycle in BY-2 cells. In fact, a transient treatment with cryptogein during S or G1 phase induced cell death and growth inhibition in BY-2 cells. By contrast, similar transient elicitor treatment during G2 or M phase did not induce these responses (Fig. 4.2). The suppression of cryptogein-induced PCD during G2 or M phase suggests differences in some of the components involved in defense signaling in each phase of the cell cycle.

4.4.3 Cell Cycle Dependence of Signaling Events to Trigger PCD

4.4.3.1 ROS Production

ROS can function as signaling molecules during cell division, through complex signaling pathways [104], and ROS homeostasis has been suggested to be critical for plant cell division [105]. Oscillations in intracellular ROS level keep pace with respective antioxidant oscillations. Subsequently, ROS acting as signaling molecules contribute to the establishment of Ca^{2+} gradients and participate in the control

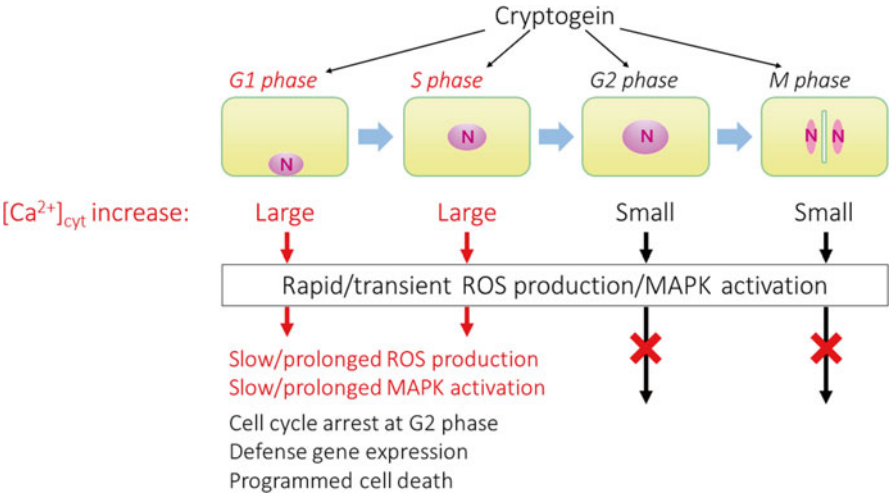

Fig. 4.2 Cell cycle-dependent regulation of cryptogein-induced defense signaling in tobacco BY-2 cells. The partial suppression of the cryptogein-induced $[Ca^{2+}]_{cyt}$ rise and the absence of sustained ROS production and MAP kinase activation at G2 and M phases are well correlated with the absence of induction of defense-related gene expression and PCD. *N* Nucleus. Treatment with cryptogein at G1 or S phase induces changes in $[Ca^{2+}]_{cyt}$, biphasic (rapid/transient and slow/prolonged) ROS production, and biphasic MAP kinase activation, followed by the cell cycle arrest at G1 or G2 phase and induction of defense gene expression and PCD. In contrast, treatment at G2 or M phase only induces smaller $[Ca^{2+}]_{cyt}$ changes, followed by only rapid/transient ROS production and MAP kinase activation (Kadota et al. [71]). The nucleus localizes at the cell periphery at G1 phase, but moves to the center at S/G2 phases (Higaki et al. [39])

of regulatory proteins such as CDKs and possibly aurora kinases. Rbohs have been identified as ROS-producing enzymes in plants, which have recently been shown to play key roles in numerous physiological processes such as tip growth of root hairs [78] and pollen tubes [106], hormonal responses, and abiotic and biotic responses [107]. A positive feedback mechanism involving Ca^{2+}-activated Rboh proteins and ROS-activated Ca^{2+}-permeable channels to regulate tip growth of root hairs has been proposed at the plasma membrane [78]. Rboh-mediated ROS production is implicated in the regulation of cell cycle progression, microtubule organization, nuclear envelope dynamics, and cell plate formation. In animal cells, ROS are involved in cell proliferation, regulating transition through specific cell cycle checkpoints [108].

Nox-/Rboh-mediated ROS production has been suggested to be involved in the regulation of PCD as a HR [109–111]. The rapid and transient phase (phase 1) of Rboh-mediated ROS production, triggered by cryptogein, occurred during any phase of the cell cycle, whereas the slow and prolonged phase (phase 2) was induced only by elicitation during S or G1 phase (Fig. 4.2). However, the relationship between the elicitor-induced oxidative burst and cell cycle regulation remain unclear, and these studies shed light on the novel aspect of the roles of Rboh-mediated ROS production and the immune signaling network in plants.

4.4.3.2 MAPK Activation

Cryptogein also induces biphasic activation of MAPK homologs, salicylic acid-induced protein kinase, and wounding-induced protein kinase [112]. The rapid and transient activation of both MAPKs occurs after elicitation during any phase of the cell cycle, whereas prolonged activation of MAPKs occurs only after elicitation during G1 or S phase [71]. The rapid/transient phase of cryptogein-induced ROS production and MAPK activation is induced at any phase of the cell cycle, suggesting that the elicitor is recognized throughout the cell cycle. In contrast, the slow/prolonged phase of ROS production (phase 2) and MAPK activation shows a strong correlation with the induction of immune responses including PCD (Fig. 4.2).

Suppression of HR during the G2 or M phase is correlated with the absence of prolonged production of ROS and prolonged activation of MAPKs. Other components participating in the induction of HR may be inactivated only during the G2 or M phase [71]. The elicitor-induced expression of *Rboh* genes is suggested to contribute to prolonged ROS production called the oxidative burst [71, 113]. The MEK^{DD} mutant, in which MAPKs are constitutively active, showed enhanced expression of *NbRbohB* and PCD in *Nicotiana benthamiana* [114], suggesting that the MAPK cascade positively regulates *Rboh* expression and ROS production.

Though PTI and ETI share downstream signaling machinery, activated immune responses in ETI are more prolonged and robust than those in PTI, and hypersensitive cell death are only induced by ETI signals. *Arabidopsis* MAP kinases 3 and 6, key regulators of immune responses, are activated rapidly and transiently during PTI, but activated for an extended period during ETI [100, 115]. Prolonged activation of oxidative burst and PCD are correlated with, and presumably require, prolonged activation of MAPKs [71, 116], suggesting that the cell cycle-dependent regulation of MAPK activation may be crucial for the induction of PCD. Suppression of the oxidative burst and PCD in cells treated with cryptogein during G2 or M phase may be attributed to the absence of prolonged activation of MAPKs.

4.4.3.3 Cytosolic Ca^{2+} Rise

Cryptogein induces a biphasic $[Ca^{2+}]_{cyt}$ rise in tobacco BY-2 cells [32]. It is induced at all phases of the cell cycle, but is significantly weaker at G2 and M phases than S and G1 phases in which hypersensitive cell death is induced (Fig. 4.2), suggesting that some signaling components upstream of $[Ca^{2+}]_{cyt}$ rise are regulated in a cell cycle-dependent manner [71]. Although the cryptogein receptor has not yet been identified, the expression of the receptor may be regulated differentially during the cell cycle phases and thus contribute to cell cycle-dependent regulation of immune responses. Transcriptomic analyses of a synchronized culture of *Arabidopsis* revealed that transcripts of two putative disease resistance proteins accumulate during G1 and S phases [117]. A cryptogein receptor may also be expressed more at G1 and S phases than at G2 or M phases. Alternatively, the receptor may be partially inactivated at G2 or M phases. Molecular mechanisms for the cell cycle-dependent

regulation of these signaling events are important issues to be elucidated in future research.

The partial suppression of the cryptogein-induced $[Ca^{2+}]_{cyt}$ rise at G2 and M phases is correlated with the absence of the oxidative burst and the prolonged activation of MAPKs at these phases (Fig. 4.2). Several studies have indicated that Ca^{2+} channel inhibitors and Ca^{2+} chelators inhibit pathogenic signal-induced ROS production and MAPK activation, suggesting that the Ca^{2+} influx is essential for the induction of these responses [32, 118]. The rapid/transient phase of ROS production and MAPK activation was induced even at G2 and M phases, in which $[Ca^{2+}]_{cyt}$ rise were partially suppressed. These results suggest that a relatively small increase in $[Ca^{2+}]_{cyt}$ at G2 and M phases is sufficient to induce the rapid/transient phase of ROS production and MAPK activation, which occurs independently of the slow/prolonged phase. The slow/prolonged phase of ROS production and MAPK activation is only induced at S and G1 phases, where the cryptogein-induced $[Ca^{2+}]_{cyt}$ rise is prominent (Fig. 4.2).

4.5 Conclusions and Future Perspectives

We have described the cell biological events, including intracellular reorganization and cell cycle regulation, during PCD as an immune response. Recent advances in GFP-based fluorescent molecular probes and microscopy have synergistically promoted our understanding on the structural changes in cytoskeletons and vacuoles during immune responses. The intracellular reorganization of organelles including the vacuole is suggested to be governed by cytoskeletons. Future molecular cell physiological studies should reveal the missing link between quantitatively detected early molecular events (e.g., ROS production, MAPK activation, Ca^{2+} influx) and intracellular structural changes (e.g., rearrangement of the cytoskeletons, vacuolar rupture). In this situation, quantitative evaluation of cell biological events must be of growing importance. Acquirement of enormous microscopic image data and its statistical analysis with numeric image features (e.g., skewness of fluorescent intensity distribution in GFP-labeled cytoskeletal images [119]) to evaluate cytoskeletal or vacuolar behaviors will become a growing area of research in the near future.

Besides apoptosis, autophagy is also involved in animal PCD [120, 121] and has also been suggested to play roles in several types of plant PCD including hypersensitive cell death against pathogen infection [122–125]. Autophagic cell death is characterized by the occurrence of double-membrane autophagosomes within the dying cells that remove the cell remnants [126]. In many eukaryotes, autophagy is required for normal development, for example, for dauer development in nematodes and preimplantation in mice [127–129]. Interestingly, autophagy-defective rice mutants show complete male sterility and limited anther dehiscence under normal growth conditions, suggesting that autophagy is crucial in reproductive development in rice [130]. A relatively simple method to quantitatively analyze autophagic fluxes has recently been developed in plants [131]. Such technical advances in combination with genetic analyses may reveal novel aspects of autophagy in PCD in plants.

The strict cell cycle dependence of pathogenic signal-induced immune responses and their suppression suggest that immune responses may be suppressed in dividing cells *in planta*, such as meristems, young leaves, and seedlings (Fig. 4.3). In fact, young leaves are less sensitive to pathogenic signals than mature leaves [132, 133]. In contrast, almost all mesophyll cells of mature leaves are at G0 or G1 phase [134] and induce strong immune responses including HR against pathogens [135], which is consistent with the finding using suspension-cultured cells showing that immune responses and hypersensitive cell death are strongly induced at G1 phase [73]. Molecular characterization of the relationship between the cell cycle and the immune responses in intact plants is a new frontier of research.

Some studies using synchronized suspension cells revealed that different cell cycle phases are associated with slightly different gene expression patterns [136] and several *R* genes exhibit peak expression at S or M phase. This differential expression pattern could have physiological consequences. Cells at different cell cycle phases exhibit different responses to elicitors, and defense gene induction by elicitors is cell cycle dependent [71]. Perturbation of cell cycle regulation triggers plant immune responses via the activation of disease resistance genes [137], suggesting that cell cycle regulation could have an impact on the expression of genes, including *R* genes, in plant immunity. Cell cycle progression is tied to the dynamics of not only DNA but also chromatin [138], which thus could have a profound effect on gene expression. Further investigation should reveal the regulatory mechanisms for gene expression during cell cycle phases and the increase our understanding of the interactions between plants and pathogens.

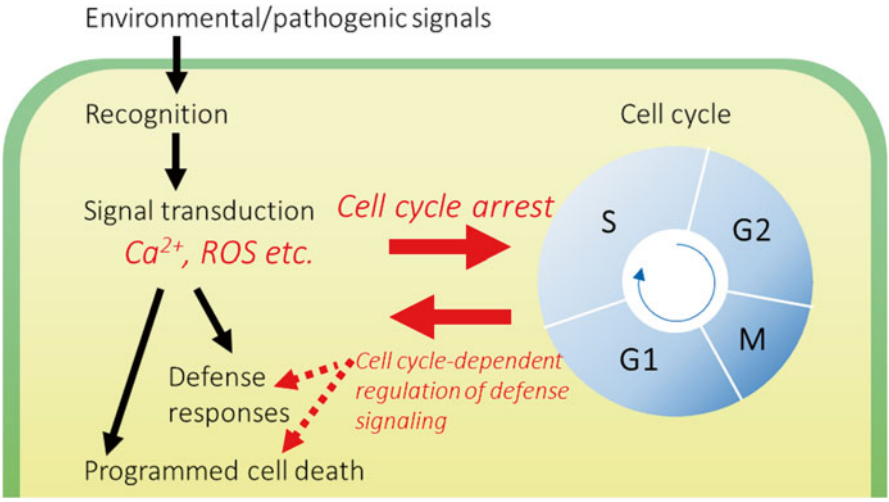

Fig. 4.3 The crosstalk between stress/defense signaling and cell cycle regulation. The strict cell cycle dependence of pathogenic signal-induced immune responses and their suppression in suspension-cultured cells indicate possible relationship between stress/defense signaling and cell cycle regulation in intact plants at various developmental stages

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Chapter 5
Vascular Aerenchyma and PCD

Daniel K. Gladish

5.1 What Is Aerenchyma?

The classic textbook definition of aerenchyma is based on the assumed function of the tissue. Thus, it has been defined as a tissue in a plant that has intercellular air spaces that enhance diffusion of gases, especially oxygen [1–3]. This is an inadequate description because it does not discriminate aerenchyma from typical plant ground tissue, so more recently others have specified that the intercellular spaces must be larger than those found in typical ground tissues [4]. The larger spaces are generally described as being formed by one of two mechanisms: schizogeny or lysigeny [1, 2, 4, 5]. Schizogeny is the physical separation of cells along the middle lamella ("glue" layer between adjacent cell walls) during growth and differentiation [3] and is most commonly found in plants adapted to wetland conditions. Wetland plants would logically benefit from the increased gas-conducting potential of aerenchyma tissue under such chronically hypoxic environmental conditions. This type of aerenchyma is constitutively produced as a normal part of development and will form even when the plants are not submerged [6, 7]. A variant of schizogeny, called expansigeny, that generates a honeycomb pattern of spaces involves cell division following cell separation was described by Seago et al. [7]. Lysigenous aerenchyma, on the other hand, is formed by the organized destruction of cells in order to form extensive gas-filled lacunae. This type of aerenchyma is often induced by acute conditions, such as flooding [1, 2, 4]. Some wetland species produce lysigenous aerenchyma in roots constitutively [4], and the aerenchyma is often more extensive when hypoxia is more severe [8–13]. Schizogeny and lysigeny can occur simultaneously separately in different specific organs or act together in the same organ of the same plant ([9, 14], respectively).

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Consistent with the above functional definition, aerenchyma has primarily been reported as being found in stems and roots of submerged or flooded plants [1, 2, 4, 15–18] or plants otherwise subjected to hypoxia [17]. Studies have shown that the presence of aerenchyma does effectively enhance internal oxygen availability [11, 19–22], and this may well be the primary and most common function of aerenchyma.

On the other hand, a survey of the literature shows that this definition is overly simple. There are numerous examples of evidence that aerenchyma forms for reasons other than avoiding or mitigating hypoxia or anoxia within stems and roots. An early proposal often repeated is that lysigenous aerenchyma that forms in response to hypoxia reduces oxygen demand by removing a significant proportion of the respiring cells in "non-critical" tissues [16, 23, 24]. Perhaps related to this proposal, aerenchyma in roots has been observed forming lysigenously in response to other stressors, such as exposure to elevated osmotic stress [25], nitrogen or phosphorus deficiency [23, 26], sulfur deficiency [27], and mechanical impedance imposed by increased soil density [28]. Induction of lysigenous aerenchyma has even been reported in leaves as a consequence of high soil salinity [29].

The observation by He et al. [28] that mechanical impedance induces lysigenous aerenchyma in roots possibly is a type of mild wound response, given that elevated ethylene production often follows wounding of a plant organ [30–33] and, perhaps coincidentally, ethylene is a signal molecule prominent in the induction pathway of induced lysigenous aerenchyma in several species [3, 18, 28, 34, 35].

An often overlooked example of an aerenchymatous tissue is the spongy parenchyma found in the ground tissue of leaves (mesophyll) of many species [1, 2], the primary functions of which are the influx of carbon dioxide and the efflux of oxygen, the opposite of aerenchyma's role in most reported situations. Spongy parenchyma in mesophyll is also the principle site of water loss from plants, which results in transpiration [1, 2]. Spongy parenchyma in mesophyll is usually produced by schizogeny, extreme examples of which can be found in the floating leaves of aquatic plants, in which case the spongy parenchyma provides the standard function of rapid gas distribution but also the buoyancy that enables the leaves to float [1–3].

One of the most interesting proposals for an alternative role for aerenchyma in roots is the improvement of mineral availability [36, 37]. It has been reported several times that root aerenchyma in some species under flooded conditions allows for radial oxygen loss into the rhizosphere sufficient to enhance growth in adjacent, non-aerenchyma-forming plant species and for microflora in the rhizosphere, and it allows an enhanced path through the plants for the efflux from the soil of carbon dioxide and methane [36, 38, 39]. In an experiment using *Oryza sativa*, Kirk [37] provided evidence that the ability to provide a pathway for oxygen flux into the soil enhances the availability of nitrate by oxidation of ammonium and also reduces the toxicity of reduced metal ions by oxidizing them. Furthermore, there has been some discussion of the value of aerenchyma in enhancing ethylene diffusion to facilitate other ethylene-mediated stress responses [23].

Therefore, aerenchymatous tissues in the various organs of plants while primarily providing aeration also provide enhanced transport of other gases such as carbon dioxide, methane, ethylene, and water vapor fluxes within and out of the plant body and may also provide ecosystem services that are adaptive for the plant species that possesses it and beneficial for other organisms in the plant's surroundings. Of specific interest in the context of this book is the role of programmed cell death (PCD) in the development of induced lysigenous aerenchyma. Evidence that PCD is specifically responsible for lysigenous aerenchyma formation in the cortices of roots has been developed for several species [18, 40, 41] and for an unusual form of aerenchyma found so far only in cool-season herbaceous legumes: vascular cavities [42–44].

5.2 First Reports of Vascular Cavities

In the 1940s and 1950s, issues of how and where along the root axis water and minerals entered were debated. A publication describing the debate and offering to help solve the problem by evaluating the patterns of differentiation of tissues in roots, especially vascular tissues, was published in the *American Journal of Botany* by Richard Popham [45]. He argued that understanding the spatial relationships of differentiation among the several tissues (dermal, ground, and vascular) would be important to understanding the processes of absorption by putting boundaries along the axis where absorption and translocation were possible. He also mentioned, quoting Heimsch [46], that if different environments caused a shift of those relationships, it might allow a mechanism of experimentation to test relevant hypotheses about environmental effects on differentiation.

To establish the developmental patterns and begin testing the environmental modulation premise, Popham [45] imbibed and germinated uniform and surface-sterilized 'Alaska' (semidwarf cultivar) and 'Perfected Wales' (tall cultivar) pea (*Pisum sativum*) seeds in darkness on a screen submerged slightly in a "complete mineral solution." Three days after germination the seedlings were transferred onto opaque 1.9 L "Mason jars" with perforations in their lids that supported the cotyledons with the roots descending into mineral solution inside the jars. Half the jars containing each cultivar were aerated using an aquarium pump and airstone system. The resulting plants were maintained under artificial light with a 15 h light:9 h dark regime and a concomitant 24 °C:16 °C temperature regime. Using light microscopy and freehand and paraffin-embedded sections, he mapped the tissues of the primary roots at two stages of maturity: 5 days and 21 days.

Popham [45] made an observation about the development of the vascular cylinders in those roots that eventually precipitated a controversy that resulted in over two decades of research by the Niki/Gladish group that began in the Rost Lab in 1989. The observation was that all of the primary roots in his study developed extensive lysigenous vascular cavities that began as a stack of lens-shaped lesions near the apical meristem (Fig. 5.1). The level of aeration had no effect on cavity

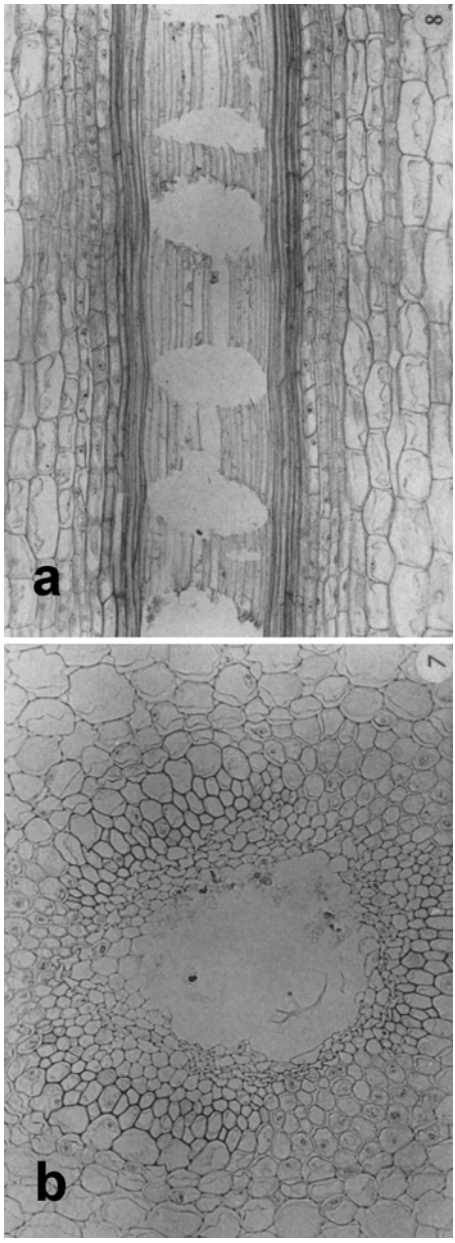

Fig. 5.1 First report of vascular cavities in primary roots of *Pisum sativum* cv. 'Alaska'. (**a–b)** Light micrographs of vascular cylinders about 4 cm from the tip. Scale bars were not provided. A vascular cylinder in that location is probably 200–250 μm in diameter. (From [45], Figs. 7–8; used with permission.) (**a**) Longitudinal section of a vascular cylinder with lens-shaped, lysigenous lesions beginning to become joined into a continuous cavity. (**b**) Transverse section of a vascular cylinder with a cavity

formation, though it did enhance the growth rate. Earlier Popham [47] had also presented two figures showing vascular cavities, though the phenomenon was not mentioned in the text. His observation was noteworthy for several reasons, not the least of which was that, unlike stems, a hollow "pith" in roots had not been previously reported in roots, and it contradicted standard texts (e.g., [48–50]) that dicot root vascular cylinders had either tracheary elements or a parenchymatous pith in their centers. Additionally, it contradicted a specific description of *P. sativum* by Haward [51]. Published cross sections of *P. sativum* primary roots in previous experimental reports did not have central vascular cavities nor were they mentioned [52–54]. Popham offered, "Whether the cavity formation is controlled by genes or whether it is an ultimate product of the environment to which the experimental roots were exposed proposes an open question worthy of investigation" [45].

5.3 A Personal Challenge

In the ensuing years, the validity and ultimate value of Popham's 1955 studies were questioned by several authorities working on root development (M. E. McCully, pers. com., [55]). It was claimed that Popham simply had not known how to grow roots properly; that was why they had "holes" in them. Likewise, after being shown some micrographic data from *P. sativum* roots we had produced showing vascular cavities, T. L. Rost was told by a respected colleague in my presence that he (Rost) and his people were obviously not growing our roots under proper conditions. The production of those data, and the discussion that followed, was the result of criticism that Rost had received following the publication of Rost et al. [56], an article that added fine detail to the work of Popham [45] on *P. sativum* root anatomy and development. Rost et al. [56] had included two micrographs of roots with vascular cavities (Fig. 5.2) and another very respected senior scientist suggested the micrographs were of "mishandled" thin-sections. I urged Dr. Rost, my mentor, to let us accept the challenge of demonstrating that vascular cavity formation in *P. sativum* was a natural process, as Popham had suggested, and to evaluate the conditions under which they form.

5.4 Answering the Challenge

Six different cultivars of *P. sativum* were cultivated in well-watered (field capacity) vermiculite or sand and in aerated nutrient solution (aquaculture) under standard laboratory conditions (in growth chambers with a 12:12 h photoperiod at 25 °C). They were also grown in a greenhouse in commercial potting soil or in sand in terracotta pots and in a Central California garden plot planted at various times from January through May. This study showed that seedlings produced vascular cavities "in almost every primary root" [57]. This study supported Popham's [45]

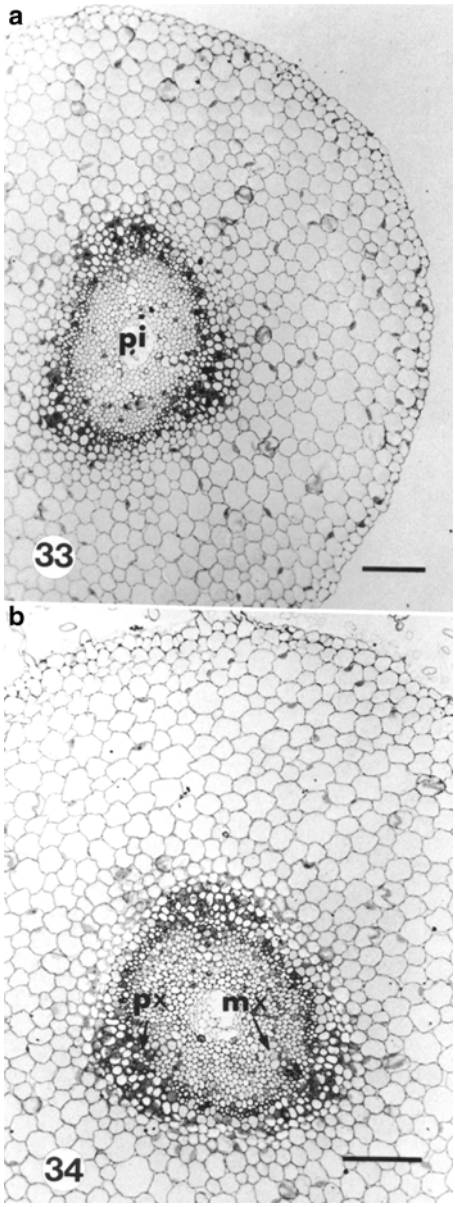

Fig. 5.2 *Pisum sativum* cv. 'Alaska' vascular cylinders with vascular cavities. (**a**–**b**) Light micrographs of transverse sections, scale bars = 100 μm. In the original legend, pi, px, and mx referred to pith, protoxylem, and metaxylem, respectively. (From [56], Figs. 33–34; used with permission.) (**a**) 9.5 mm from the root tip. (**b**) 19 mm from the root tip

description of an initial series of lens-shaped lesions in the center of the vascular cylinder that became joined by further lysigenous cell destruction in the parenchymatous tissue between the lesions (Fig. 5.3a, b). These events followed behind the differentiation of the procambial tissues at a predictable distance in sequence as primary roots grew once they were about 3 cm in length and could result in a lengthy central cavity that extended along the root axis as much as 10 cm. The same study

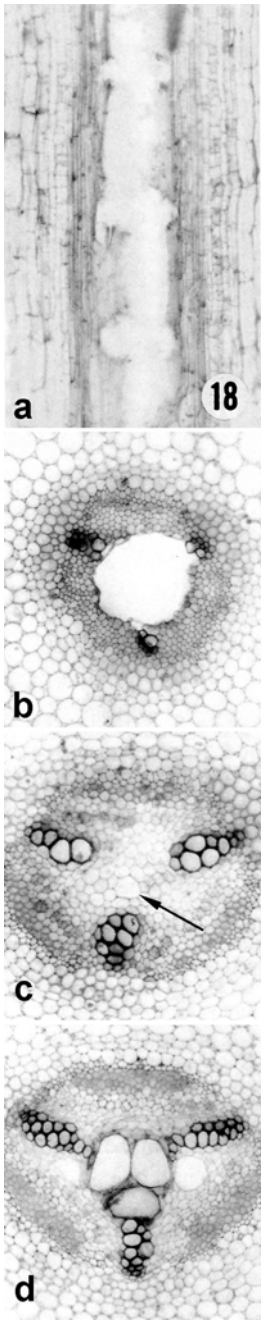

Fig. 5.3 *Pisum sativum* cv. 'Alaska' vascular cylinders. (**a**–**d**) Light micrographs of freehand sections of primary roots grown in medium moistened to field capacity (From [57], Figs. 18, 10, 2, and 6, respectively; used with permission). (**a**) Longitudinal section taken 0.5–1.0 cm from the tip. Scale bar = 150 μm. (**b**). Transverse section taken 6 cm from the tip of a root grown at 25 °C. (**c**) Transverse section taken 20 cm from the tip of a root grown at 10 °C. *Arrow* indicates a nascent late-maturing metaxylem tracheary element. (**d**) Transverse section taken 31 cm from the tip of a root grown at 10 °C showing the replacement of parenchymatous central pith region by late-maturing metaxylem tracheary elements. Nascent secondary xylem tracheary elements can be seen adjacent to them. Scale bar for (**b**–**d**) = 100 μm

revealed that pea primary roots never formed vascular cavities under those conditions at temperatures below 15 °C (Fig. 5.3c, d). It was soon demonstrated that root vascular cavities were mainly limited to cool-season, herbaceous legume species, including *Cicer arietinum*, *Lens culinaris*, *Lupinus latifolius*, *P. sativum*, and *Vicia faba* [42]. Since a wide range of media were used and none of the media used in the study by Lu et al. [57] were thought to restrict gas exchange more than soils normally do under standard conditions, it was concluded that vascular cavity formation was strictly a temperature-dependant phenomenon. Further study at the ultrastructural level revealed many of the details of the lysogenic degradation and cell collapse that actually formed vascular cavities in *P. sativum* [58].

If it was the case that vascular cavities were a "normal" developmental process stimulated by a specific temperature range, the logical next question was whether the response was an evolved adaptation that provided a selective advantage, despite the fact it destroyed tissue that ultimately would have produced very large late-maturing metaxylem tracheary elements. On the other hand, perhaps it was just a pleiotropic result of artificial selection and inbreeding during domestication that served no particular purpose. It was understood that *P. sativum* grew best in cool environments [59, 60] and its root systems generally developed more extensively at soil temperatures ca. 15 °C [61]. If a central cavity was adaptive, what advantage or mitigation did it provide? I asked a fundamental, relevant question: What does a vascular cavity contain, gas or liquid solution? Initially it was thought that vascular cavities were liquid filled [57]; a subsequent study using scanning electron microscopy and X-ray microanalysis revealed that vascular cavities were air filled (Fig. 5.4; [62]). Given the considerable length the cavities were shown to develop (up to 17 cm in length) and the fact that they did not have a negative effect on growth of the aerial parts of the plant [57], it seemed possible that they might enhance aeration within the root. In other words, vascular cavities might be a form of aerenchyma [17].

On the other hand, while in classical literature aerenchyma was described in stems of papilionaceous legumes [49], it was never reported in roots of legumes, much less in their vascular tissue. Furthermore, Gladish and Niki [17] confirmed the observation of Popham [45] that in aqueous culture at 25 °C, though it positively affected growth, the level of aeration in the system made no difference in the frequency of vascular cavity formation in a population of *P. sativum* seedlings. But in that cultivation system, consistent with previous reports cited above, vascular cavities never formed at 10 °C regardless of aeration level, nor was there a significant difference in growth rate. They showed that, when *P. sativum* seedlings were grown at 25 °C in vermiculite wetted with different amounts of water, root growth rate was not significantly affected, but the population frequency of vascular cavity formation (20–100 %) was positively correlated with the relative amount of water in the medium (325–1100 ml water per liter of vermiculite). Confounding the issue further, Gladish and Niki [17] showed, using the low-moisture condition they

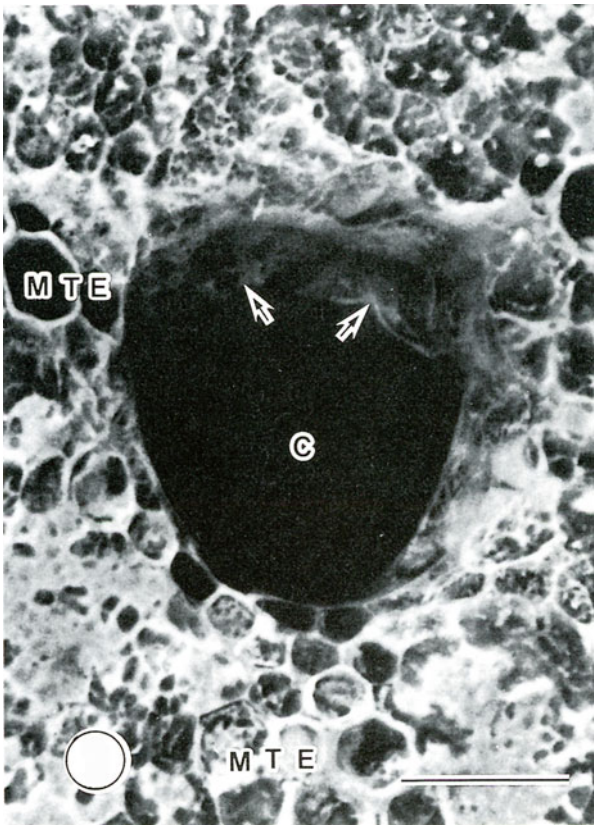

Fig. 5.4 Scanning electron micrograph of the freeze-fractured and freeze-etched surface across a *P. sativum* root showing a cavity empty except for cell debris (*arrows*). *c* vascular cavity, *MTE* metaxylem tracheary elements, scale bar = 75 μm (From [62], Fig. 8; used with permission)

discovered that suppressed cavity formation and infusing the root zone with gas mixtures at a range of oxygen levels (2–21 %), that root growth was positively correlated with oxygen availability, as would be expected, but vascular cavity formation frequency was negatively correlated with it. Questions remained. The aquaculture and wetted vermiculite series experiments suggested that in warm conditions vascular cavity frequency was strictly dependent on the amount of water in the root zone regardless of aeration, but the gas infusion experiment suggested that the correlation was to oxygen availability. Of course, these factors are not entirely unrelated. Was vascular cavity formation a programmed adaptive response (our working hypothesis) or stress-induced necrosis, as was suggested by William Armstrong (pers. com., 1999)? Using a Clark-type oxygen microelectrode, Armstrong et al. [63] had shown that the center of a *Zea mays* primary root has the lowest tissue oxygen level in the organ under good conditions and that at low ambient oxygen levels (10 %) the center of a *Z. mays* root was completely anoxic.

5.5 Are Vascular Cavities Programmed Cell Death-Mediated Functional Aerenchyma?

Programmed cell death (PCD) differs from necrosis (uncontrolled death) due to failure of homeostasis systems, which should be distinguished from necrosis-like PCD [44, 64] in several important and distinctive ways. While there is significant variation among examples in plants, in general and in contrast with true necrosis, PCD is targeted to specific cells or tissues and provides a benefit to the plant (reviewed in [65]). Essentially, necrosis represents failure; PCD represents success. PCD can contribute to development or to relieving the effects of biotic or abiotic stressors [65, 66]. Ultrastructural changes in targeted cells are distinctive but vary among organs and among taxa. Typical events often include condensation of chromatin, lobing and invagination of the nucleus, rupturing of the tonoplast, degradation of other organelles, and alteration or degradation of the cell wall. Some biochemical events have also been observed across different systems, such as increased ethylene synthesis, cytochrome c release from mitochondria, systematic fragmentation of DNA, and activation of caspase-like proteases [17, 18, 40, 41, 44, 66–69].

Although it had long been understood that certain aspects of plant development involved organized cell death (e.g., vessel formation [50, 70]), beginning in the mid-1990s the role of PCD was increasingly and specifically being discussed and explored in plant systems, especially as a factor in cell and tissue development and as a compensatory response to various biotic and abiotic stresses (reviewed in [65]). Some examples included the hypersensitive response to pathogens [71–73], the differentiation of tracheary elements [74], the formation of constitutive cortical aerenchyma in aquatic plants [40], cell death in response to salt stress [75], and flooding-induced cortical aerenchyma in mesophyte roots [18, 41]. Although it was clear that root vascular cavities were gas filled, there was no direct evidence this conferred an adaptive advantage by functioning as aerenchyma, and there was only morphological evidence, such as organelle abnormalities, tonoplast rupture, and rapid cell wall thinning, supporting the idea that the process was managed by PCD (Fig. 5.5; [58]).

Circumstantial evidence of functionality as aerenchyma was obtained when we began experimenting with sudden flooding [43]. The fact that using relatively dry growing medium would suppress the formation of vascular cavities even at warm temperatures [17] provided a technique to explore the sensitivity of vascular tissue at different stages of development and to probe the timing of events associated with vascular cavity formation. The study revealed that primary roots of pea seedlings 4 days old (typically 60–70 mm long) that did not already have a vascular cavity (ca. 80 % of the population) would dependably form a complete cavity from about 10 mm stemward of the tip to within about 20 mm from the cotyledonary node within 24 h. Furthermore, and significantly, such roots would continue to grow directly downward, albeit at a slightly slower rate, and continue to form a cavity acropetally as they did [43]. Seedlings that were 1 day older (and consequently had primary roots ca. 30 mm longer) when suddenly flooded would rapidly slow to a

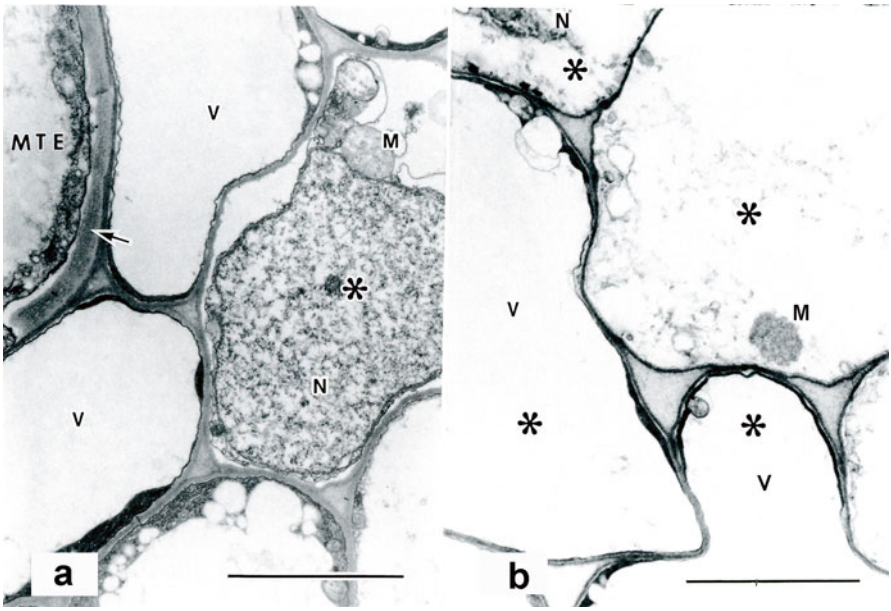

Fig. 5.5 Transmission electron micrographs of cells in a vascular cylinder of *P. sativum* where a cavity was forming. (**a**–**b**) *Asterisks* indicate degenerating cells. *M* mitochondrion, *MTE* metaxylem tracheary element (*arrow* indicates thick secondary wall), *N* nucleus, *V* vacuole, scale bars = 5 μm. (From [58], Fig. 9b, c; used with permission, Copyright Univ. of Chicago Press.) (**a**) Degenerating cells on right adjoin normal cells at *left*. (**b**) Degenerating cells with thinning walls, ruptured tonoplasts, degenerating organelles, and autophagosomes

stop within a few hours and grow no further. Tips of those roots are usually sharply curved, often into a spiral, as they slowed. The roots of 5-day-old seedlings did invariably form vascular cavities extending from near the tip basally within 24 h, but they were about the same length as those formed in 4-day-old seedlings. Consequently, the basal (stemward) ends of the cavities were significantly farther from the cotyledonary node, which was located at the floodline. We interpreted this response to mean two things: (1) cells in the center of the vascular cylinder over time became irreversibly committed to differentiation into late-maturing tracheary elements [55, 57] and were therefore incompetent to contribute to a cavity and (2) vascular cavities contributed internally to aeration of the primary meristem, allowing continued growth, unless their basal ends were too far from the floodline to contribute to enhanced oxygen diffusion. We further hypothesized that vascular cavities were the result of PCD [43].

We discovered that the inhibition of growth was accompanied by, and perhaps was due to, rapid and significant inhibition of mitotic activity and severe internal cell disruptions in the growth zone, which was often asymmetrical and which accounted for the tip curling. Subsequently we showed that the “trauma” had characteristics of PCD and shared some features with vascular cavities, but at a smaller scale. These included condensation of chromatin and morphological distortion of nuclei (Fig. 5.6) that were reactive to the terminal deoxynucleotidyl

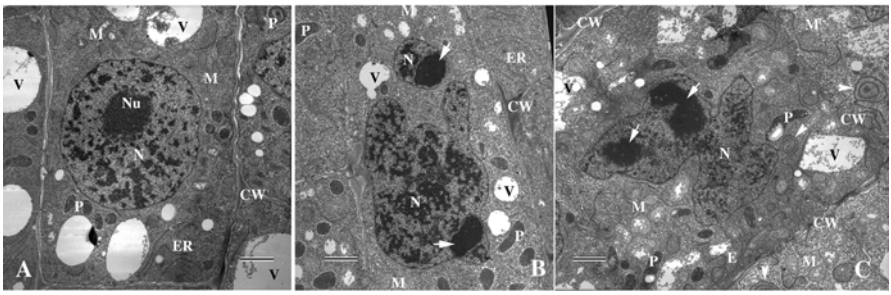

Fig. 5.6 Transmission electron micrographs of nuclei in cells of *P. sativum* root ground meristem cells from roots grown in relatively dry medium at 25 °C. (**a**–**c**) *Arrowhead* indicates possible autophagosome. *Arrow* indicates very condensed chromatin. *CW* cell wall, *ER* endoplasmic reticulum, *M* mitochondria, *N* nucleus, *Nu* nucleolus, *P* plastids, *V* vacuole, scale bar = 2 μm (From [76], Fig. 6A–C; used with permission, Annals of Botany Co.). (**a**) Normal nucleus in a 6-day-old root tip. (**b**) Nucleus in a ground meristem cell flooded for 6 h after 5-day growth showing lobing, possible nuclear fragmentation, and chromatin condensation. (**c**) Nucleus in a ground meristem cell flooded for 24 h after 5-day growth with extreme lobing and chromatin condensation

transferase-mediated dUTP nick end labeling (TUNEL) procedure, disruption of organelles, and eventual rupturing of the tonoplast, sometimes leading to complete collapse of groups of cells to form a series of small, lens-shaped lesions. Some differences included the fact that frequently the nucleus would fragment into small, dense pieces that dispersed throughout the cell and the protoplast would subdivide into a number of "macrovesicles" resembling the apoptotic bodies that form during apoptosis in animal systems (Fig. 5.7). Furthermore, the DNA had undergone systematic, internucleosomal fragmentation in less than 6 h (Fig. 5.8; [43, 76]). From this we understood that root tissue cells in this species were capable of true PCD, and this led us to be more confident that vascular cavities were a PCD-mediated process rather than stress-induced necrosis [4, 77]. It was at that point that we began focusing on demonstrating definitively that vascular cavity formation was caused by PCD.

Ethylene has long been implicated as a regulator of aerenchyma formation in roots [15, 78, 79]. More recently it has been linked to PCD in aerenchyma formation [18, 41, 80] and to other PCD-mediated processes, e.g., endosperm development [81, 82], epidermal cell death in rice [83], and perforation development in the leaves of lace plant [84]. Three lines of evidence indicated that vascular cavities in *P. sativum* primary roots were an ethylene-mediated process: induction by exposure to exogenous ethylene, suppression of cavity formation by inhibitors, and careful measurements of endogenous ethylene after flooding [35]. (1) Using a specialized manifold system developed at the University of California-Davis Pomology Post-Harvest Laboratory for the purpose of managing the exposure of plant organs to various gases (Fig. 5.9), we exposed roots of 4-day-old pea seedlings grown under the low-moisture, cavity-suppressing conditions described above for 24 h to flowing (25 ml/min) ethylene at 5, 10, and 15 μL ethylene per liter of air. Seedlings exposed to ethylene developed cavities at frequencies twofold or greater than controls. (2)

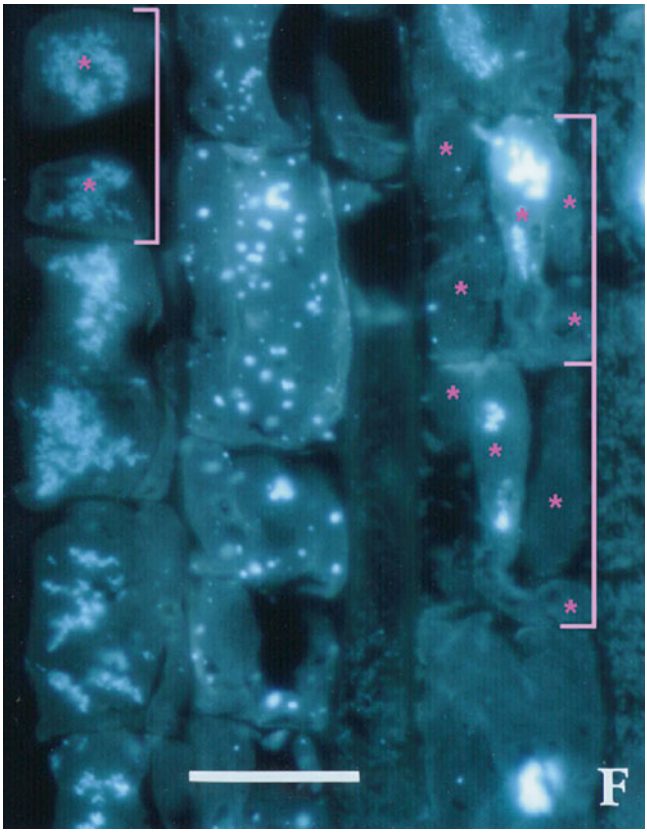

Fig. 5.7 Fluorescent light micrograph of a DAPI-stained longitudinal section of ground meristem cells in the tip of a 5-day-old *P. sativum* root flooded for 24 h. DAPI stains chromatin a very strongly and fluoresces bright pale blue or white when excited with 340–380 nm UV light. Since cell walls do not stain, some individual cells have been indicated with *brackets*. In most of the cells, the nuclei have fragmented and dispersed throughout the cells (brightly stained spots), and protoplasts have subdivided into multiple "macrovesicles" reminiscent of apoptotic bodies (*asterisks*). Scale bar = 25 μm (From [76], Fig. 1 F; used with permission, Annals of Botany Co.)

Roots of 5-day-old seedlings produced cavities at frequencies the same as unflooded seedlings (controls) when suddenly flooded with aminooxyacetic acid (ethylene synthesis inhibitor) at 5 mM. Likewise, similar seedlings flooded with 8 mM silver thiosulfate (ethylene action inhibitor) were suppressed in their cavity formation frequencies to below control levels, and the calcium chelator, ethyleneglycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), which suppresses the activity of 1-aminocyclopropane-1-carboxylic acid oxidase (a key enzyme in the ethylene biosynthesis pathway), reduced cavity frequency by about two-thirds at 85 mM in the flooding solution. (3) Suddenly flooded roots had significantly higher rates of endogenous ethylene production than unflooded controls for at least 21 h after flooding, especially during the first 3 h [35].

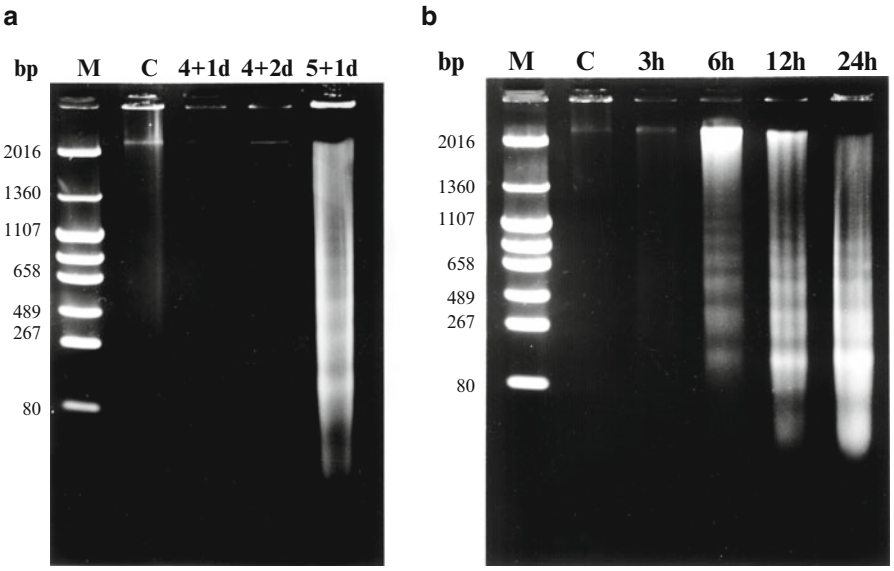

Fig. 5.8 Gel electrophoresis of DNA extracted from tips of *P. sativum* roots showing laddering pattern after 6 h of flooding. *M* size marker DNA. (From [76], Fig. 5A–B; used with permission, Annals of Botany Co.) (**a**) C, 5-day-old unflooded controls; 4 + 1 days, roots grown for 4 days then flooded for 1 day; 4 + 2 days, roots grown for 4 days then flooded for 2 days; 5 + 1 days, roots grown for 5 days then flooded for 1 day. (**b**) C, 6-day-old unflooded controls; DNA extracted from roots grown for 5 days then flooded for 3, 6, 12, and 24 h

One of the most remarkable aspects of PCD is its cell specificity. Cells self-destruct, but surrounding cells are left unaffected [44]. Early on it was observed that vascular cavity formation in *P. sativum* roots was limited to the parenchymatous cells that formed the pith-like center that is present early in the roots' development, cells that under different conditions would likely form late-maturing metaxylem tracheary elements, and that cells of surrounding tissues were rarely affected [57, 58]. Subsequent work reconfirmed this [44, 85]. This includes adjacent primary cell walls that may thin to nonexistence in as little as 3 h or degrade to nothing on only one side of the middle lamella (Fig. 5.10; [85]). We evaluated certain ultrastructural and biochemical hallmarks known to be associated with PCD and established a timeline noteworthy because of its rapidity [44]. The PCD pathway was induced by sudden flooding of roots of seedlings grown for 4 days at 25 °C in relatively dry vermiculite. Using fluorescence microscopy and in situ immunolabeling with anti-cytochrome *c* fluorescein-conjugated monoclonal antibodies and with isolated and segregated mitochondrial and cytosolic cell fractions, we showed that cytochrome *c* was released from the mitochondria into the cytosol within 2 h (Figs. 5.11 and 5.12). Cytochrome *c* release from mitochondria has been implicated in the PCD induction pathway in several systems [86, 87]. Obvious signs of cell wall degradation (Fig. 5.10), rupture of the tonoplast, fragmentation of DNA into 15–30 Kbp pieces in 3 h (Fig. 5.13), and lobing and invagination of nuclei (Fig. 5.6) occur between 2

Fig. 5.9 Manifold system for regulating and distributing dilute ethylene gas mixtures into experimental jars in a walk-in environmental chamber in the Post-Harvest Laboratory of the Department of Pomology at the University of California in Davis, CA, USA

and 3 h. DNA fragments were detectable in situ with the TUNEL procedure until about 6 h after flooding (Fig. 5.14), after which time most affected cells were completely destroyed, and a continuous cavity was present [44].

5.6 Ongoing Biochemical and Molecular Work

Because of its large genome size with a large percentage of repetitive sequences, few genomic tools have been developed for *P. sativum* [88]. While vascular cavities seem to occur only in cool-season, herbaceous legumes, an early study [42] indicated that at least one cultivar of *Glycine max* (unidentified in the report) was capable of producing vascular cavities. Some genomic information and tools were known to be available for *G. max*. So, we decided to screen cultivars of *G. max* that had been developed for cold tolerance to see if they would form vascular cavities under sudden flooding like *P. sativum*. Three cultivars of *G. max* were identified that responded to sudden flooding at 25 °C by forming vascular cavities: 'Traff and 'Fiskeby IV' from Sweden and 'Yukihomare' from Hokkaido, Japan. 'Yukihomare'

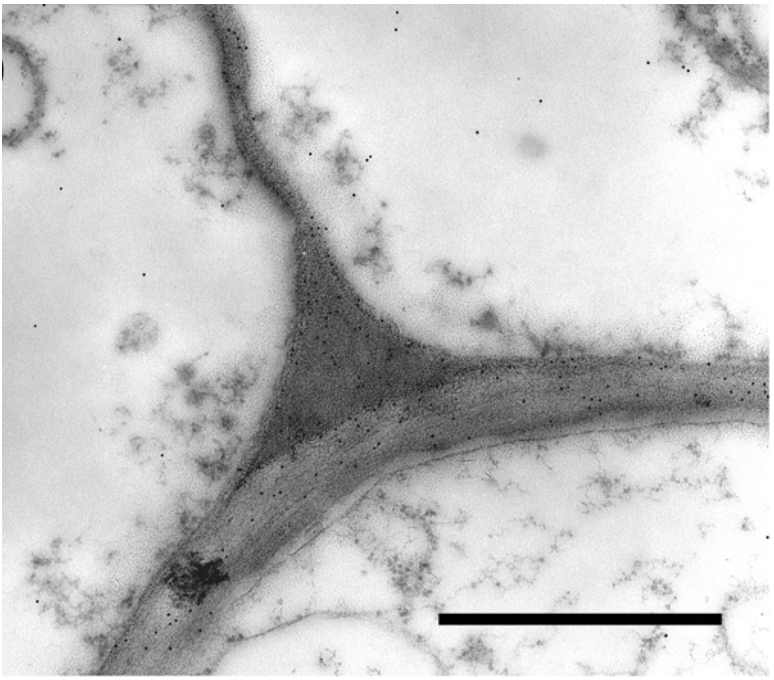

Fig. 5.10 Transmission electron micrograph showing primary cell walls completely hydrolyzed away from the middle lamella adjacent to an apparently unaffected cell wall during vascular cavity formation in *P. sativum* in response to sudden flooding at 25 °C. Scale bar = 1 μm (From [85], Fig. 3D; used with permission)

dependably displayed high frequencies of cavity formation in our system, so we chose it for further study, though the process of cavity formation was slower in those roots [89].

Caspase-like proteases had been implicated in the transduction pathway of plant PCD in several other systems [90, 91]. For example, this was shown to be the case for cell death-mediated selective dominance of axillary branch development following decapitation in *P. sativum* [92] and in the mediation of the hypersensitive response [67, 93]. At the time of our investigations of cavity formation in *G. max*, a link between caspase-like proteases and hypoxia-driven PCD had not been demonstrated [89]. Primary roots of *G. max* cv. 'Yukihomare' exposed to the sudden flooding system developed for *P. sativum* proved to be a good platform for beginning the evaluation of caspase-like protease activity during PCD-mediated cell lysis during flooding. Caspase-specific inhibitor studies using 5 μl of 100 μM Ac-YVAD-CHO (mammalian caspase 1 inhibitor) or Ac-DEVD-CHO (mammalian caspase 3 inhibitor) microinjected 2 cm stemward of the tip every 12 h following flooding suppressed the mean vascular cavity formation frequency by 44 % and 55 %, respectively, compared to flooded controls microinjected with distilled water. A time-course analysis of YVADase and DEVDase activity in tissue collected from

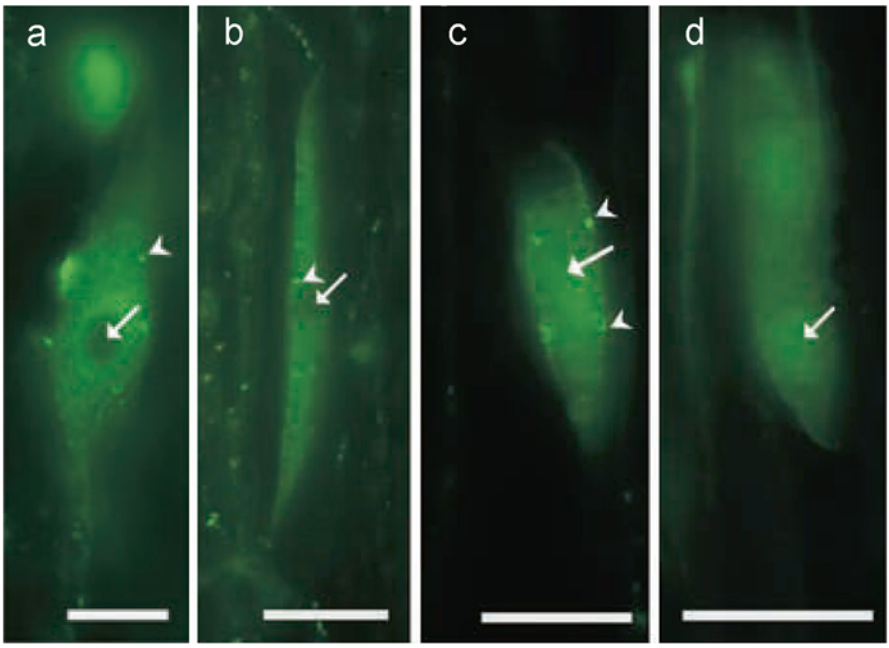

Fig. 5.11 Longitudinal sections of cytochrome *c* immunolocalized in situ in *P. sativum* root central vascular cylinder cells about 2 cm from the tip before and during flooding-induced vascular cavity formation. (**a**–**d**) *Arrows* indicate the nucleus, *arrowheads* indicate strongly labeled mitochondria, scale bars = 10 μm. (From [44], Fig. 4B, D, G, and J, respectively; used with permission. Copyright Physiologia Plantarum.) (**a**) Unflooded root. (**b**) 1 h after flooding some cytochrome *c* had infiltrated the nucleus. (**c**) 3 h after flooding the nucleus was strongly labeled, though mitochondria remained labeled as well. (**d**) 24 h after flooding the nucleus in a cell at the margin of a vascular cavity was strongly labeled, but mitochondria were not labeled

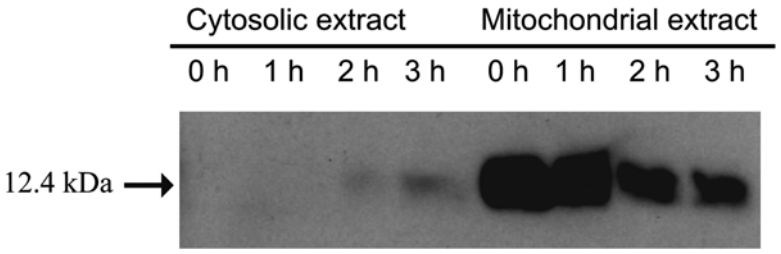

Fig. 5.12 Western blot of cytochrome *c* immunolabeled proteins from isolated cytosolic and mitochondrial fractions of extracts of tissue collected 1–3 cm from the tips of *P. sativum* roots during a flooding time series after growth for 4 days at 25 °C under relatively dry (cavity-suppressing) conditions. Samples were extracted just prior to and 1, 2, and 3 h after flooding, which showed that cytochrome *c* was lost from mitochondria into the cytosol beginning about 2 h after flooding (From [44], Fig. 6; used with permission. Copyright Physiologia Plantarum)

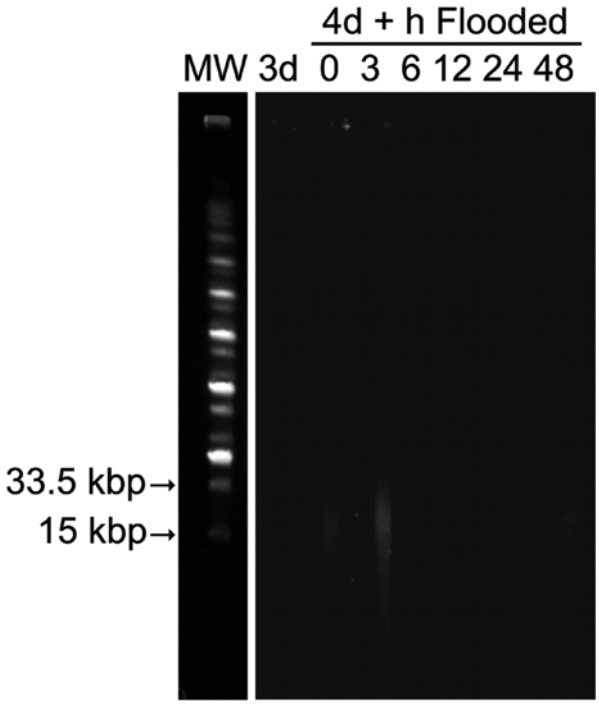

Fig. 5.13 Pulse-field gel electrophoresis of DNA extracted 1–3 cm from tips of *P. sativum* primary roots after growth for 4 days at 25 °C under relatively dry (cavity-suppressing) conditions during a flooding time series (From [44], Fig. 1; used with permission. Copyright Physiologia Plantarum)

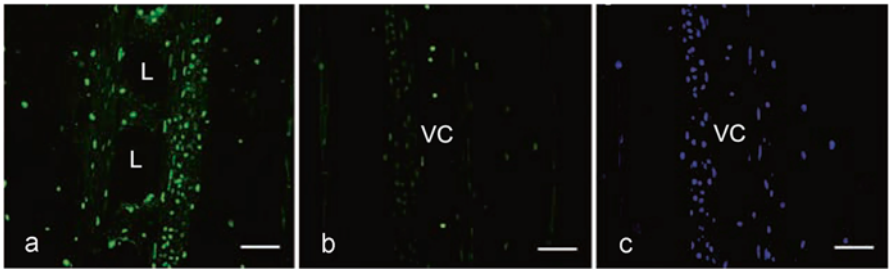

Fig. 5.14 TUNEL-labeled longitudinal sections of vascular cylinder tissues of *P. sativum* roots taken about 2 cm from their tips during flooding-induced vascular cavity formation. (**a**–**c**) *L* Lens-shaped lesion, *VC* vascular cavity, scale bars = 100 μm (From [44], Fig. 2G, I, and J, respectively; used with permission. Copyright Physiologia Plantarum). (**a**) Vascular tissue 5 h after flooding. (**b**) Vascular tissue after a cavity had fully formed (6 h after flooding). Little fragmented DNA remained to react to TUNEL. (**c**) DAPI-stained section from the same root as (**b**) showing intact nuclei around the vascular cavity

the cavity-forming region showed that DEVDase activity began increasing immediately after flooding and peaked at fourfold the activity of controls 5 h after flooding, declined thereafter to about twice the activity of controls, and remained at that level until cavity formation was complete at 24 h. YVADase activity increased gradually to nearly twice control levels in 7 h and declined slightly over the remaining time cavities were forming [89].

Subsequently we began a transcriptome analysis using the 'Yukihomare' cultivar of *G. max* [94]. Microarray technology had previously been used to study transcriptome changes in *Arabidopsis thaliana* due to hypoxia [95, 96]. Agilent Technologies (Santa Clara, CA, USA) had just produced their new 6000 Series II microarray chip for *G. max*. We were told that our lab was the first to experiment with this chip. A time-course experiment was designed for the extraction of total RNA from the region of primary roots most susceptible to vascular cavities (1.5–3.0 cm stemward of the tip). Seedlings were grown so that roots could be sampled at the same time of day (8 p.m.) 0, 3, 6, 12, and 24 h after flooding to avoid circadian effects on gene expression. Extracted RNA was tested for quality using qRT-PCR and electrophoresis. The series was replicated four times, and the samples were processed by the Biomedical Genomics Core at the Nationwide Children's Hospital Research Institute (Columbus, OH, USA). The microarray analysis suggested that expression levels of over 8500 genes were up- or downregulated twofold or more by flooding. Almost 2000 of those changed transcript level fivefold. Logically, the latter included expressed sequence tags (ESTs) representing genes associated with glycolysis, fermentation, and sucrose and nitrogen metabolism. ESTs associated with ethylene synthesis and signaling genes and at least four ESTs representing PCD genes were significantly upregulated. One was homologous to *Arabidopsis* LOL1, which is a positive regulator of PCD. Others were DOX1 and NLA (involved in the hypersensitive response) and PLD, which Drew [5] indicated increases just prior to hypoxia-induced aerenchyma formation in maize. The response appeared to occur in two stages with the first occurring during the first 6 h and the second after 12 h. Many of the genes responded in complex ways [94]. The Agilent 6000 Series II microarray chip for *G. max* includes over 43,000 probes, many of which are for transcripts that have not yet been characterized, so at the current time analysis of these data are underway with very much work left to be done.

Finally, we thought it would be important to confirm by direct measurement that vascular cavities in primary roots of cool-season legumes was functional aerenchyma, i.e., they help mitigate the hypoxia caused by flooding. It was understood that sudden flooding of seedling *P. sativum* roots in medium in our system resulted in a steady decline of available free oxygen in the medium around the roots to about 50 % of the initial 3.8 mg oxygen per liter of suddenly flooded vermiculite medium in less than 24 h [97]. Subsequently, seedlings of *Phaseolus coccineus*, a cool-season bean species whose primary root is very broad and becomes visibly wider where a vascular cavity has developed, were used to obtain data on the oxygen status internally and externally during flooding. An OxyMicro oxygen photomicroprobe (World Precision Instruments, Sarasota, FL, USA) was used to measure the oxygen availability 2 h and 24 h after sudden flooding at the surface of roots at their

tips, at 1 cm intervals along the root axis and opposite swollen regions when they appeared (typically 0.5–1.0 cm stemward of the tip). Surface oxygen level at the tips of most roots tested dropped exponentially from over 6 mg oxygen per liter of medium at the moment of flooding to less than 1 mg/L in less than 2 h but increased thereafter as a cavity began to form. The probe mechanism was installed by the manufacturer in a fine hypodermic syringe that we were able to install onto a computer-controlled micromanipulator. In a manner similar to Armstrong et al. [63], the micromanipulator was used to impale roots 0.5–1.0 cm from their tips and take oxygen measurements at 10 μm increments during penetration. Two hours after flooding, as the probe passed through the root, it revealed an oxygen "profile" very similar to that obtained for *Z. mays* by Armstrong et al. [63]. Such roots were nearly anoxic at the center, whereas after 24 h of flooding the profile had a distinct peak as wide as a typical vascular cavity that corresponded to the center of the root and representing an oxygen level only slightly lower than the value taken at the root's surface (T. Niki, M. Takahashi, and D. K. Gladish, unpublished data), which showed markedly improved oxygen availability had occurred.

At this point we are confident that vascular cavities in the primary roots of cool-season herbaceous legumes are mediated by PCD and that they are functional aerenchyma that is an adaptation to mitigate the effects of flooding during seedling development. Because, in addition to classic hallmarks of PCD such as cytochrome *c* release from mitochondria, nuclear condensation and morphological distortion, and systematic DNA fragmentation, the vacuoles "permeabilize" (rupture) and the cell walls thin until they disappear so that the remaining cell contents eventually are dispersed and absorbed into the surrounding tissue, this phenomenon is probably best described as "mega-autophagic," PCD, as proposed by van Doorn and Woltering [69]. At the present time what remains to be done is to complete the signal transduction pathway and develop a model of it that may be applicable to other examples of PCD in plants.

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Chapter 6
Out for a Walk Along the Secretory Pathway During Programmed Cell Death

Jean-Luc Cacas

Abbreviations

ABA	Abscisic acid
ABI5	Abscisic acid insensitive 5
ABP	Auxin-binding protein
ACD5	Accelerated cell death 5
ACD11	Accelerated cell death 11
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
APX	Ascorbate peroxidase
ATG	Autophagy-related genes
ASK1	Apical apoptosis signal-regulating kinase 1
BCL2	B-cell lymphoma 2
BI1	BAX inhibitor-1
BAX	BCL2-associated X protein
BAK	BCL2-homologous antagonist/killer
BiP	Binding protein
BOS1	Botrytis sensitive 1
CA1	Calcium-ATPase 1
CAT	Catalase
Cer	Ceramide
CHOP	C/EBP homologous protein
CNX	Calnexin
CPA	Cyclopiazonic acid
CRT	Calreticulin
DDR	DNA damage response
EDR1	Enhanced disease resistance 1

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eIF2α	Eukaryotic translation initiation factor 2 alpha
ER	Endoplasmic reticulum
ERAA	ER-activated autophagy
ERAD	ER-associated degradation
ERD2	ER luminal protein receptor 2
ERD15	Early responsive to dehydration 15
ER-QC	ER-quality control
ERR	Endoplasmic retrograde regulation
ETC	Electron transport chain
ETI	Effector-triggered immunity
FA	Fatty acid
FAH	Fatty acid hydroxylase
FB1	Fumonisin B1
GA	Golgi apparatus
GCN2	General control non-depressible 2
GIPC	Glycosyl-inositol-phosphoryl-ceramides
GluCer	Glucosylceramide
GRP78	Glucose-regulated protein 78
HCD	Hypersensitive cell death
HSP	Heat shock protein
HVA22	*Hordeum vulgare* ABA-induced cDNA 22
IPCS	Inositol-phosphoryl-ceramide synthase
IRE1	Inositol-requiring enzyme 1
KEG	keep on going
LAZ5	LAZARUS 5
LCB	Long-chain base
MPK6	Mitogen-activated protein kinase 6
MRR	Mitochondrial retrograde regulation
NAC	Non-apical meristem/*Arabidopsis* transcription activation factor/cup-shaped cotyledon
NAG	Neuroblastoma-amplified gene
NDR1	Non-race disease-specific disease resistance 1
NPR1	Non-expressor of PR1
NRP	Asparagine-rich protein
NTL9	NAC transcription factor-like 9
NTP1,2	NAC targeted by *Phytophthora* 1,2
OST	Oligosacharidyl-transferase
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PDI	Protein disulfide isomerase
PERK	PKR-like ER kinase
PIN	PIN-FORMED
PM	Plasma membrane
PR1	Pathogenesis-related 1

PRR	Pattern recognition receptor
PTI	PAMP-triggered immunity
RCD	Runaway cell death
RIDD	Regulated IRE1-dependent decay
RIP	Regulated-intramembrane proteolysis
ROS	Reactive oxygen species
S1P/S2P	Site-1/site-2 proteases
TM	Tunicamycin
SAR	Systemic acquired resistance
SPT	Serine-palmitoyl-coA transferase
TBF1	TL1-binding factor 1
TEI	TGN/early endosomal interface
TF	Transcription factor
TGN	Trans-Golgi network
TMV	Tobacco mosaic virus
TRAF2	Tumor necrosis factor-receptor-associated factor-2
TSPO	Translocator protein
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VLCFA	Very long-chain fatty acid
VPE	Vacuolar processing enzymes
XBP1	X-box protein 1

6.1 Introduction

Since the first cytological description of apoptosis in the 1970s [1], the fascinating field of programmed cell death (PCD) has been the witness of considerable conceptual progress. With the development and combination of biochemical, functional, and microscopic tools, entire cell death pathways have been deciphered in animal cells. This unveiled intricate signaling networks where the mitochondrion holds a central regulatory role by setting in motion cell death through initiation of irreversible proteolytic cascades that involves caspases. Under specific conditions, the endoplasmic reticulum (ER) has also been shown to cooperate with the mitochondria in sentencing cells to death. Additional cell death pathways, partly dependent on or independent of the mitochondrion, such as the one relying on autophagy, have also been reported ending the exclusivity of apoptosis and opening new perspectives in the cellular control of PCD.

Comparisons with apoptosis have long been a driving force in the plant cell death research field. Although this approach was somehow fruitful as it allowed for the identification of crucial caspase-like enzymes, these enzymes were still found to execute cellular dismantling in a manner unique to plants. In addition, a growing body of findings also lends support to the occurrence of divergent cell suicide

programs in plants and mammals. Nowadays, it has thus become clear that plants have evolved specific cell death modalities sharing possibly mechanistic patterns but only a few common protein regulators and effectors with their animal counterparts. Remarkably, the endomembrane system comprising the ER, Golgi apparatus (GA), vacuole, and autophagic compartment is currently emerging as an essential component of these conserved patterns, and, consequently, it is tempting to speculate that cell secretion activity is intimately linked to cell fate. This chapter provides an in-depth analysis of our current molecular knowledge regarding this new role for the secretory pathway in plant PCD and further tries to puzzle out signaling and regulatory connections between endomembrane organelles occurring throughout the cell's demise process. A special emphasis has been put on the ER because of its strategic and pivotal function in both cell secretion and death.

6.2 Endoplasmic Reticulum in Plants Cells: A Gateway to the Secretory Pathway and Much More!

The ER is a highly dynamic organelle composed of interconnected membrane structures (tubules, cisternae, and vesicles) that organize as an intricate perinuclear and/or cortical network. It coordinates lipid anabolism in photosynthetic cells by feeding the "*prokaryotic pathway*" with lipid precursors to be desaturated in chloroplasts and initiates sphingolipid synthesis [2]. In addition, it is also involved in translation, modification, and maturation of most, if not all, secreted soluble and transmembrane proteins. This illustrates the pivotal role of the ER in basal cell metabolism. However, a growing body of recent findings suggests that this organelle could have a much wider impact on cell physiology. It is currently envisaged in the literature that the ER could sense both abiotic and biotic stresses and participate in signaling cascades leading to appropriate cellular responses in plants, including PCD [3, 4]. How could this be orchestrated at the molecular level? In other words, how can stresses be sensed by the ER? And how, then, can this organelle influence cell fate?

6.2.1 Endoplasmic Reticulum Machinery and Organellar Stress

In eukaryotic cells, except for a specific class of transmembrane proteins which are directly integrated into the ER membrane, all nascent secreted polypeptides are co-translationally translocated across membranes into the lumen through a complex of integral membrane proteins, dubbed the translocon [5]. Aggregation of the entering protein is prevented via interaction with ER-resident chaperones and co-chaperones belonging to the family of heat shock protein 70 (HSP70), namely, glucose-regulated protein 78 (GRP78)/binding protein (BiP). Meanwhile, the

sequence of the nascent polypeptide is also scanned for the presence of glycosylation site recognition motifs (or sequons) by the oligosaccharidyl-transferase (OST), a multimeric protein complex associated with the translocon. When sequons are detected, preformed oligosaccharides linked to the lipid carrier dolichol-pyrophosphate are directly transferred by OST onto asparagine (Asn) residues. Modification of the saccharidic chains then involves glycosidases and the enzymatic duo of lectin-like calcium-dependent chaperones formed by calnexins (CNX) and calreticulins (CRT). Finally, a particular subset of thioredoxins, named protein disulfide isomerases (PDI), catalyzes the formation of appropriate disulfide bonds to complete the maturation process of glycosylated proteins. Together with additional proteins and cofactors aiding in protein folding, the GRP78/BiP proteins, the CNX/CRT cycle, and PDI represent the three components of the so-called endoplasmic reticulum-quality control (ER-QC) system. Besides its active role in acquirement of protein native state, the ER-QC system also monitors protein folding and decides whether a protein is exported to the Golgi apparatus or retained in the lumen for further folding attempts. Upon one more round of CNX/CRT cycle, if proteins are still misfolded, they are targeted for degradation. This implies retro-translocation to the cytoplasm and disposal by ubiquitin-proteasome system (UPS), a pathway known as ER-associated degradation or ERAD [6, 7].

Theoretically, the global folding capacity of the ER is directly dependent on the efficiency of both the ER-QC and ERAD systems and, consequently, relies for the most part on the concentrations of the ER-resident proteins forming this machinery. From this simple statement, one can deduce that this folding capacity is limited but can be adjusted to some extent by augmenting the synthesis of the machinery components when needed. The latter situation can happen when a large amount of proteins has to be secreted. Under physiological conditions, the balance between the load of nascent polypeptides, their folding, and secretion is, however, equilibrated due to tight regulations, and the organelle homeostasis is maintained. Under stressful circumstances, folding demands can exceed ER capacity, leading to unfolded protein accumulation in the lumen. This phenomenon is referred to as ER stress and, if unresolved, can jeopardize cell viability. In animals, excessive or prolonged accumulation of unfolded proteins in the lumen does not result in passive necrotic cell death but instead activates signaling cascades leading to apoptosis (see Sect. 6.2.3.1). In plants, ER stress-induced PCD does exist, as proven by the use of chemicals such as tunicamycin (TM, a fungal antibiotic specifically inhibiting OST activity) and cyclopiazonic acid (CPA, an inhibitor of ER-type IIA calcium-ATPases) [8–11].

6.2.2 Signaling Endoplasmic Reticulum Stress for Protecting Plant Cells

Partially conserved molecular mechanisms have been selected throughout the course of evolution to cope with ER stress in eukaryotic cells. The onset of these retrograde ER-to-nucleus signaling pathways, the so-called unfolded protein

response (UPR), warns cells about harmful situations originating from biotic or abiotic constraints and further allows the prioritizing of cellular responses. UPR can be subdivided into two branches acting sequentially: the adaptative one, destined to mitigate ER stress, and the pro-death one, devoted to eliminate malfunctioning cells via PCD when attempts to restore ER homeostasis have failed.

Mammalian cells employ distinct strategies for alleviating ER stress. The amount of incoming polypeptides into the lumen is restricted by diminishing or even blocking general translation. ER-associated mRNA is also specifically degraded. The global folding capacity of the ER is increased through quality control (ER-QC) transcriptional activation. ERAD is stimulated, which accentuates unfolded protein disposal. At the molecular level, these events rely on three ER membrane-embedded protein receptors defining the corresponding arms of the adaptive UPR (Fig. 6.1a): the PKR-like ER kinase (PERK), the inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). All these proteins encompass, besides their single transmembrane region, cytoplasmic and luminal portions [12]. They use their luminal domain to assess the protein folding environment and their cytosolic part to signal ER stress. When unfolded protein accumulates, PERK and IRE1 self-associate to form active homodimers. PERK, which harbors a cytoplasmic kinase domain, contributes to ER stress alleviation by attenuating general protein synthesis through phosphorylation of the eukaryotic translation initiation factor 2α, or eIF2α [13]. Upon homodimerization, IRE1 autophosphorylates, activating its own cytosolic RNAse domains responsible for mRNA splicing and subsequent translation of the bZIP transcription factor (TF) X-box protein 1 (XBP1). Then, active XBP1 relocates to the nucleus to upregulate the expression of genes encoding ER-resident chaperones and ERAD components [14, 15]. Additionally, a phenomenon known as regulated IRE1-dependent decay (RIDD), involving the RNAse domain of the protein, reinforces translation inhibition through degradation of ER membrane-associated mRNA coding for secreted proteins [16]. Finally, the activation of the bZIP TF ATF6 requires its translocation to the GA where it is sequentially cleaved by site-1-resident and site-2-resident proteases (S1P and S2P). Following this regulated-intramembrane proteolysis (RIP), free ATF6 cytoplasmic domain relocalizes to the nucleus to exert its cytoprotective function via transcriptional stimulation of ER-QC genes [17, 18].

Despite our considerable progress in the understanding of plant UPR over the last few years, whether and how protein synthesis is regulated during plant UPR remains an open question. Searching for sequence homologues of PERKthrough genomic databases has resulted in a dead end [19]. Possibly, divergent kinase proteins, like the general control non-depressible 2 (GCN2) and $P58^{IPK}$, may control translation in ER-stressed plant cells, as assumed by Duwi-Fanata et al. [20]. Apart from PERK orthologs, other UPR sensors/transducers have been identified in plants (Fig. 6.1b). IRE1 sequence homologues have been found in many plant genomes. For instance, the two *Arabidopsis* proteins IRE1a and IRE1b share similar structural properties with their animal counterparts and were demonstrated to be involved in ER stress response [21–23]. Although there is no doubt that AtbZIP60-encoding mRNA is targeted for splicing by IRE1 upon TM application, to date, the respective

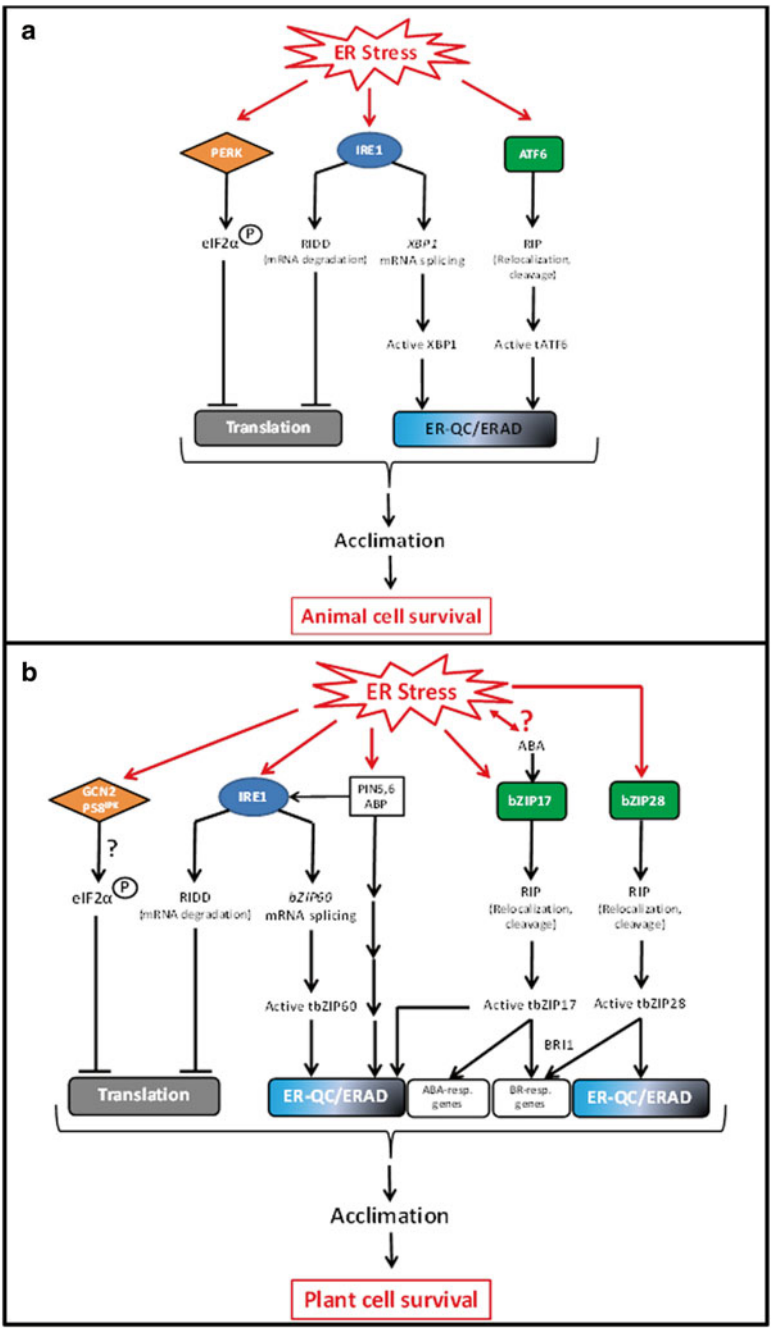

Fig. 6.1 Adaptive unfolded protein response in animal (**a**) and plant (**b**) cells. *ABA-* or *BR-resp. genes* Abscisic acid- or brassinosteroid-responsive genes, *BRI1*, brassinosteroid insensitive 1; *tATF6* or *tbZIP* truncated ATF6 or truncated bZIP TF

contribution of IRE1a and IRE1b to this step is still controversial. AtbZIP60, unlike its animal ortholog XBP1, is an integral protein displaying a luminal part and a cytoplasmic TF domain. Processing of *At-bZIP60* mRNA occasions an ORF frame-shift, resulting in loss of the C-terminal transmembrane anchor and remobilization of the soluble TF domain to the nucleus [21, 22]. Recent data indicates that the TF NAC103 is a downstream effector of the bZIP60 arm [24]. Also, RIDD of ER-tethered mRNA has been seen in *Arabidopsis* [25].

The subfamily D of *Arabidopsis*bZIP TF comprises four proteins tethered to the ER membrane: bZIP17, bZIP28, bZIP49, and bZIP60. While the role of bZIP49 in UPR remains elusive, bZIP28 and bZIP17 were reported to initiate two adaptive UPR arms. Under ER stress conditions, bZIP28, like ATF6, undergoes proteolytic release of its TF domain at the Golgi apparatus followed by remobilization to the nucleus [26, 27]. More recently, Che and coworkers [28] described a similar S1P-/S2P-dependent RIP activation mechanism for bZIP17. Using a yeast two-hybrid system, Liu and Howell [29] further showed that bZIP28 has an intrinsic propensity for homodimerizing as well as forming heterodimers with all bZIP TF of the class D. Thus, plant UPR is finely regulated, combining double transcriptional and post-translational control levels. Moreover, adding to this complexity, recent works uncovered novel cross-talk between UPR and hormonal signaling. Reverse genetic studies established that (1) ER-localized auxin receptor (auxin-binding protein, ABP) and auxin transporters (PIN-FORMED 5, PIN5, and PIN-FORMED 6, PIN6) are necessary for full UPR activation in *Arabidopsis*, and (2) this induction is only partly dependent on IRE1 [30]. In addition, Yang et al. [31] reported on a putative role for abscisic acid (ABA) in controlling bZIP17 arm in maize. Remarkably, exogenous ABA application was able to upregulate *bZIP17* and ER-QC genes. Overexpressing an active truncated form of bZIP17 also stimulated the expression of both ER stress- and ABA-responsive genes. Finally, RIP of bZIP17 and bZIP28 was proposed to relieve abiotic stress-contingent growth impairment in *Arabidopsis* seedling via brassinosteroid signaling enhancement [28]. Altogether, these data shed light on unexpected ER-orchestrated coordination between plant growth, ER stress, and abiotic stress acclimation response.

Pioneering work pointed out the beneficial role of the three adaptive UPR arms in plant acclimation to abiotic stress. *bzip28* null mutants have decreased tolerance to heat shock [32], and *ire1a*/*ire1b* knockout lines display exacerbated sensitivity to elevated temperatures [21]. Furthermore, gain of function of either of the two bZIP17- and bZIP60-controlled arms renders plants less sensitive to salt stress [33]. In accordance with these results, mimicking adaptive UPR activation through ER-QC component overexpression prevents abiotic constraint-triggered damages, including PCD in some cases [34–40]. Conversely, loss of function of ER-QC components confers hypersensitivity to heat, cold, salt, water, or osmotic stress [41–45] with the notable exception of the *leaf wilting 1* mutant defective in dolichol biosynthesis. But, the latter transgenics show enhanced constitutive *bZIP60* expression potentially accounting for their tolerant phenotype toward osmotic stress [46]. Thus, given that most, if not all, aforementioned stresses are able to induce PCD and likely provoke ER stress, one can assume that UPR cytoprotective function is conserved across

kingdoms and prevents or delays plant PCD by alleviating unfolded protein accumulation in the ER. Above a certain threshold of ER stress that could be cumulative in terms of persistence and intensity, plant cells may engage a PCD program.

6.2.3 *Endoplasmic Reticulum Stress, Unfolded Protein Response, and Programmed Cell Death*

6.2.3.1 Apoptosis Initiation at the Endoplasmic Reticulum in Animal Cells

In animal systems, UPR activation can have antagonistic outcomes. It can either rescue cells from mild stress or kill them in case of acute/chronic stress. The corresponding regulatory nodes that sentence cells to death have been identified. All three UPR sensors/transducers, i.e., ATF6, PERK, and IRE1, were found to participate in the regulation of ER stress-induced apoptosis (Fig. 6.2). On the one

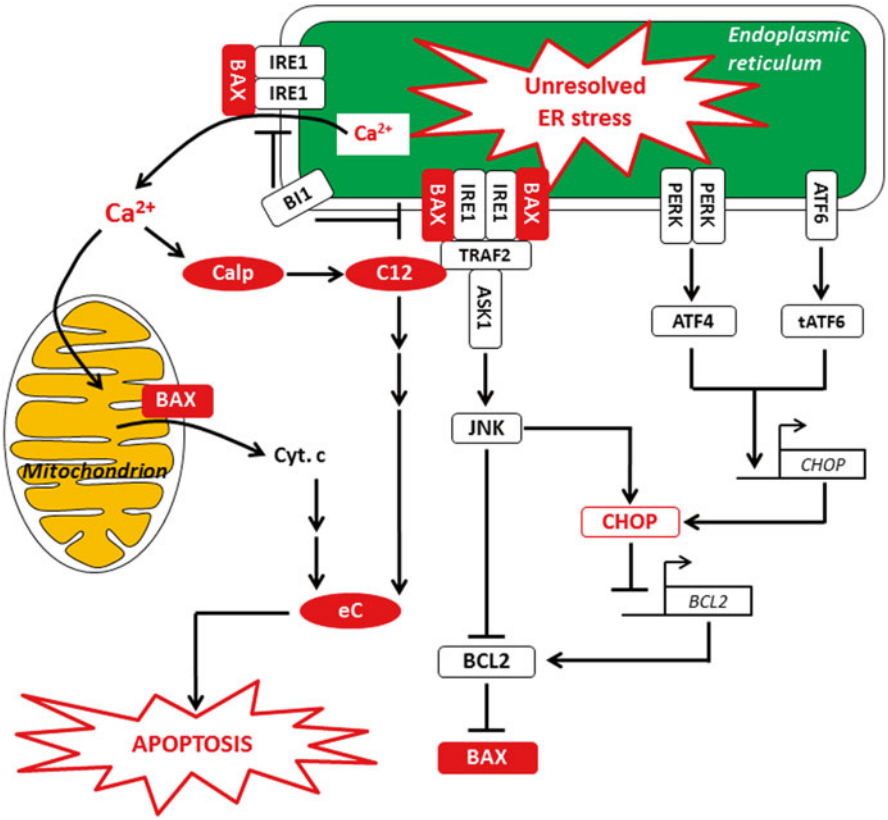

Fig. 6.2 Simplified view of the pro-apoptotic cell death pathway initiated by unfolded protein response sensor/transducers at the endoplasmic reticulum in mammalian cells

hand, IRE1 can set in motion cell death by directly or indirectly recruiting and, consequently, activating pro-apoptotic factors such as caspase-12 and members of the BCL2 family, like BAX and BAK [47, 48]. The latter proteins promote calcium release from the ER, causing rapid cation uptake by mitochondria, cytochrome c release, and activation of downstream caspase effectors. Such calcium signaling events can be relayed by calpain-activated caspase-12 or prevented by BAX inhibitor-1 (BI1) [49]. In addition, IRE1 is also able to interact with the adaptator tumor necrosis factor-receptor-associated factor-2 (TRAF2), which in turn can recruit apical apoptosis signal-regulating kinase 1 (ASK1), triggering a phosphorylation cascade that ends by the inactivation of the anti-apoptotic molecule BCL-2 [50]. Once phosphorylated, the TF CHOP (C/EBP homologous protein) represses *BCL-2* expression, altering indirectly the self-inhibitory equilibrium between BCL-2 and BAX/BAK that normally keeps cells alive. JNK-dependent phosphorylation of BCL-2 further contributes to free the pro-cell death proteins BAX/BAK, reinforcing their targeting to the ER and mitochondria. On the other hand, PERK and ATF6 drive signaling pathways that converge on *CHOP* upregulation. PERK-generated signals are transduced by the activating transcription factor 4 (ATF4), whereas truncated ATF6 directly binds to the *CHOP* promoter to mediate its transcriptional activation [51, 52].

6.2.3.2 Abiotic Stress-Induced Plant Programmed Cell Death

In plants, except for BI1, there is no conclusive evidence thus far for the conservation of any ER-regulated apoptotic machinery cogs. Moreover, the role of plant UPR sensor/transducers in PCD remains poorly understood due to little investigation. Single mutants *bzip17*, *bzip28*, *bzip60*, and *ire1* have no obvious developmental phenotypes, probably because of functional redundancy. By contrast, they exhibit drastic germination inhibition and growth retardation upon treatment with ER stressor agents [25, 28, 31, 53, 54]. Double mutants where *bZIP28* and *IRE1* functions are disrupted are lethal, and *bZIP60* mRNA is constitutively spliced in reproductive organs [54, 55], suggesting that UPR is a key actor of plant development at specific stages. Consistently, rice hybrid sterility which restricts gene flow between populations relies on a killer/protector system involving ER stress-dependent PCD [56]. It is also worth noting that deficiency for RIDD pathway in *ire1a*/*ire1b* knockouts leads to enhanced TM-induced cell death [25].

Recently, putative overlapping functions for IRE1 and the β-subunit of the G-protein heterotrimeric complex were proposed [23]. The Gβ protein was originally described as a positive regulator of TM-induced cell death in *A. thaliana* [57]. Subcellular fractionation and Western blotting methods showed that the protein is predominantly localized to the ER. Unlike *G*α-null mutants, those impaired for the *G*β-encoding gene (*AGB1*) exhibited markedly less cell death than the WT plants and an attenuated UPR response when infiltrated with TM [57]. Intriguingly, reinvestigation of AGB1 involvement in UPR pointed to contradictory results. Triple mutants *ire1a*/*ire1b*/*agb1*, indeed, presented exacerbated

TM-induced developmental phenotype compared to *ire1a*/*ire1b* and *agb1* lines [23], indicating that AGB1 may act as part of the adaptive UPR branch. In the light of our current knowledge, it seems difficult to reconcile these two works, though exact experimental conditions and allelic mutant combinations tested might represent usual suspects to be checked for explaining such an apparent discrepancy. Nonetheless, Chen and Brandizzi [23] and Wang et al. [57] provided consistent evidence for *AGB1* transcript downregulation and degradation of the protein during UPR, respectively. Taking into account these transient regulation profile and conflicting data, one may assume that the *G*β protein may participate in switching from pro-survival to pro-death UPR mode.

In animal cells, IRE1 interacts with more than 15 different proteins (see [58]), among which is the small transmembrane protein BI1 that negatively regulates apoptosis. BI1 proteins are present in numerous organisms including several plant species [59, 60] and appear to mainly reside in the ER [61, 62]. Animal and plant orthologs are functionally exchangeable [63], and the latter can modulate cell death intensity in response to many abiotic cues, as well as chemically induced ER stress [64–67]. Although potential physical association between IRE1 and BI1 has not been challenged in plants, it is possible based on microarray data that *Arabidopsis BI1* transcription could be directly upregulated by IRE1-bZIP60 arm when ER homeostasis is disturbed [55]. Noteworthy, attenuation of ER stress-triggered PCD by BI1 seems unrelated to ER-QC activation [66]. Overexpression of At-*BI1* significantly decreases the cytosolic calcium peak triggered by CPA application and confers enhanced tolerance to this drug [68]. In the same work, At-BI1 was shown to interact with calmodulin 7 *in planta*. Moreover, CPA treatment is known to sequentially elicit ER stress, cytochrome *c* release from mitochondria, and cell death in soybean cell suspensions [10]. Therefore, by analogy with mammalian models [62], it is conceivable that the cytoprotective effect of plant BI1 is intimately linked to its capability of lessening the available luminal calcium resource. Nevertheless, whether mitochondria do buffer cytoplasmic calcium released from the ER following stress and trigger cell death signaling pathways upon reaching a certain ion threshold remains to be clarified in plants. Genuine mitochondrial downstream effectors of plant PCD have yet to be identified.

Studying nuclear DNA protective mechanisms, Vanderauwera et al. [69] brought to light novel cross-talk between BI1 and oxidative stress. The authors demonstrated that safeguarding genome integrity not only relies on reactive oxygen species (ROS)-scavenging pathways but also involves ER signaling events. In previous work [70], they found that tobacco transgenics deficient for both peroxisomal catalase (CAT) and cytosolic ascorbate peroxidase (APX) exhibit higher tolerance to oxidative stress and high light (HL) than WT plants. Upon confirmation of this unexpected phenotypic trait in *Arabidopsis* seedlings, Vanderauwera et al. [69] then undertook a genome-wide transcriptome analysis of double *cat*/*apx* mutants that revealed constitutive DNA damage response (DDR) characterized by specific gene markers, even in the absence of DNA lesions. Such a constitutive DDR was accompanied by *At-BI1* gene upregulation and knockout *bi1* mutants were hypersensitive to HL. Chemical chaperones known for relieving ER stress were also able to rescue catalase-deficient lines from HL-induced cell death lesions [69]. These findings

point out that the beneficial impact of DDR is dependent, at least in part, on adaptive UPR. In line with cellular protection from oxidative stress by the ER, recent works identified three TF integrating both mitochondrial and ER retrograde regulation (MRR and ERR, respectively). Among them, *Arabidopsis* ER-resident TF NAC013 and NAC017 (for no apical meristem/*Arabidopsis* transcription activation factor/cup-shaped cotyledon 13 and 17) undergo RIP when the mitochondrial electron transport chain (ETC) is overwhelmed and ROS accumulate. As a consequence of RIP, freed activating TF domains of the two proteins remobilize to the nucleus to upregulate genes coding for mitochondrial antioxidative proteins (like alternative oxidase), thereby mediating oxidative stress tolerance [71, 72]. Although far from being clear, the interdependency between NAC017 and IRE1/bZIP60 paths has been established. While *bZIP60* expression is positively regulated by NAC017, IRE1 can dampen *NAC017* transcript steady-state levels [72], perhaps through RIDD. A third TF responsive to hydrogen peroxide, WRKY15, may also act as a master switch of the regulatory node MRR/ERR, as overexpressor lines display constitutively activated UPR associated with impaired MRR. It was hypothesized that WRKY15-coordinated UPR may perturb cellular calcium homeostasis and, consequently, desensitize mitochondria to the cation, abolishing MRR and leading to salt stress hypersensitivity [73]. With regard to the use of CPA in the latter study, it is tempting to speculate that calcium fluxes that launch MRR originate from the ER and are under the control of BI1. Therefore, since stress tolerance and PCD likely represent "two sides of the same coin," it is plausible that antagonism between MRR and UPR decides cell fate in relation to ROS balance in response to abiotic stress (Fig. 6.3a).

Apart from the MRR/ERR cross-talk described above, a new ER-coordinated PCD signaling pathway is currently being deciphered in soybean plant by the group of Elizabeth PB Fontes (Federal University of Viçosa, Brazil). Asparagine-rich protein (NRP)-encoding genes were initially isolated among a gene set that requires both ER and osmotic constraints for full activation [74]. In *BiP*-overexpressing lines that fail to initiate UPR in response to TM, the normal increase in *NRP* expression was unaffected [75], indicating that the NRP-contingent path is independent of classical UPR. In addition, transient expression of *NRP* was proven to trigger caspase-3-like activity, DNA fragmentation, and PCD [75]. Further works allowed for the identification of NRP downstream effectors as well as an upstream TF. Using promoter transactivation approach, it was demonstrated that the nuclear TF early responsive to dehydration 15 (ERD15) directly interacts with the *NRP-B* promoter to positively control its expression [76]. Yeast two-hybrid experiments also showed that the TF NAC81, which is transcriptionally upregulated by NRP [77], can associate with another TF, NAC30 [78]. The heterodimer NAC81-NAC30 then cooperates for targeting the promoter of genes coding for the well-characterized vacuolar processing enzymes (VPE) that serve as PCD executioners [78]. Interestingly, VPE orchestrate cell death through vacuole dismantling using their caspase-1-like activity, whereas NRP induces caspase-3-like activity. Only one specific subunit of the proteasome (PBA1), which is responsible for plasma membrane fusion with the tonoplast during PCD, was found to bear a caspase-3-like activity in plants (see

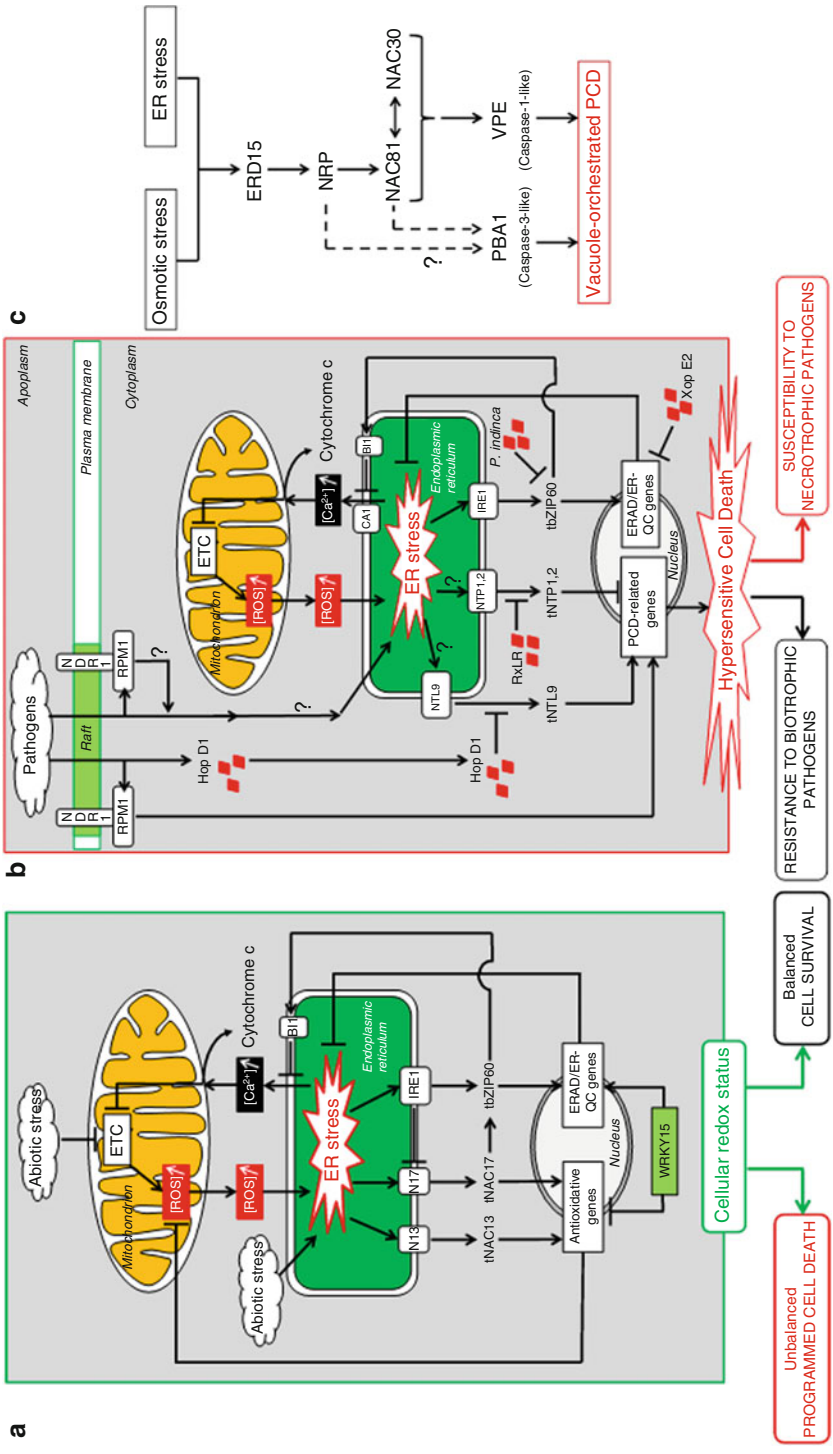

Fig. 6.3 Models explaining how the endoplasmic reticulum could orchestrate programmed cell death in plants. (**a**) Abiotic-induced cell death, (**b**) Hypersensitive cell death triggered by pathogens, and (**c**) soybean pro-death pathway controlled by the endoplasmic reticulum

Sect. 6.4.1). Thus, these data raise the question as to whether NRP could direct cells to one or the other death execution modality (Fig. 6.3c)

6.2.3.3 Endoplasmic Reticulum-Orchestrated Plant Responses to Pathogens

To cope with pathogen attacks, plants have evolved sophisticated defense mechanisms at the local and systemic levels. At the infection site, innate immunity mainly relies on two defense layers governed by specific signaling cascades initiated at the plasma membrane (PM) and any strategic subcellular compartments whose high-jacking would benefit pathogens. Briefly, the first line of defense involved PM-localized receptors, dubbed pattern recognition receptor (PRR), that detect pathogen-associated molecular pattern (PAMP) molecules commonly found in pathogen microorganisms, like sugars and proteins. The second line of defense utilizes intracellular resistance protein receptors highly specialized in direct or indirect recognition of specific invader effectors. In order to manipulate host cell metabolism, pathogens first need to overcome PAMP-triggered immunity (PTI). To do so, they inject effectors into plant cell cytoplasm. If the presence of effector proteins is not perceived by the resistance proteins (or "guards"), then PTI is successfully inhibited, microorganisms multiply and propagate, and plants develop disease. Alternatively, if effectors or any cellular alterations they promote are sensed by "guards," effector-triggered immunity (ETI) signaling pathways are activated leading to plant defense onset and ultimately disease resistance [79, 80]. The former and latter situations are referred to as compatible and incompatible interactions, respectively. It is noteworthy that incompatible interaction often culminates in hypersensitive cell death (HCD), a PCD process that can adopt multiple morphotypes depending on pathogen species and lifestyle.

Upon an infection attempt, a third defense barrier, which is systemic, is also established to prevent any further invasion attempt. The onset of this reaction, or systemic acquired resistance (SAR), is under the control of the two TF non-expressor of pathogenesis-related protein 1 (NPR1) and TL1-binding factor 1 (TBF1). Accumulation of the phytohormone salicylic acid (SA) in systemic tissues activates NPR1 and provokes its translocation from the cytoplasm to nucleus where it stimulates the binding of TGA TF to the promoters of pathogenesis-related genes and other genes involved in protein folding and secretion [81–83]. Although the activation mechanism of TBF1 is less understood than that of NPR1, it has been demonstrated that it can also induce ER-QC gene expression [84]. This transcriptional reprogramming is thought to augment ER folding capacity and facilitate the externalization of the pathogenesis-related (PR) proteins (like PR1), setting up apoplastic defense associated with SAR. Interestingly, bZIP60-encoding mRNA is spliced in response to SA treatment [85]. The UPR mutants *bzip60* and *ire1a*/*ire1b* phenocopy *npr1* and *tbf1* knockout lines, exhibiting SAR deficiency accompanied by diminished PR1 secretion [83–85]. Altogether, these data suggest that IRE1/bZIP60 arm could control the cell's secretory activity to permit extensive

export of antimicrobial proteins. This is reminiscent of what has been reported in animals, when B cells activate IRE1/XBP1 path to anticipate and accommodate massive secretion of immunoglobulins [86]. In plants, it is also possible that UPR is employed to sustain high levels of secretion during ETI, as supported by indirect evidence [55, 85].

Of particular interest is the transcriptional and translational upregulation of the ER-QC system that also occurs in the context of PTI and ETI [87]. What is the biological relevance of this cellular response? Actually, most PRR are glycosylated proteins that enter the ER where they are properly folded and matured, before being sorted via the regular secretory route. It is believed that the ER-QC system impacts PTI by quantitatively and qualitatively adjusting PRR delivery at the PM following infection. Classic and reverse genetic approaches carried out by independent research groups agree with this assumption [88–91]. On the contrary, understanding how ER-QC components could influence ETI outputs is currently much more challenging. Loss- and gain-of-function strategies performed thus far clearly underscored that affecting components of the ER-QC and ERAD systems, the UPR, the secretory pathway or some ER-localized proteins can either lead to enhanced susceptibility or reinforce resistance to pathogens with respect to the targeted gene (Table 6.1). Distinguishing among the respective contribution of HCD and apoplastic defense to these phenotypes is thus not obvious, especially when considering that biotrophic pathogen-induced cell death can be decoupled from resistance [95, 110]. Nevertheless, ER-QC function is undoubtedly related to HCD) regulation. Recent works argue in favor of this conclusion, even though they reported on contradictory phenotypes (see Table 6.1, [83, 95–97, 100, 111]). It seems that genetically manipulating the folding capacity of the ER in the context of biotic stress may not be as straightforward as expected and may result in specific side effects that can account for the observed discrepancies. Firstly, it can be envisaged that both silencing and overexpressing QC genes compromise the export and trafficking of a specific client immune receptor/PCD mediator and prevent HCD from being launched, as exemplified in Ondzighi et al. [112], Park et al. [113], and Caplan et al. [99]. Secondly, it can be hypothesized that high SA levels recorded in *BiP* and *CRT* overexpressor plants [96, 98] dominate over potentiation of the protein folding capacity, masking the primary effect of the gain of function. Thirdly, unusual increases in BiP concentrations may curtail UPR sensor/transducer relocalization [114], refraining adaptive response and exacerbating HCD. Fourthly, changing the luminal folding environment is not without perturbing ER calcium homeostasis, which has been shown to be important for HCD development [105]. Therefore, although not unequivocal evidence for a "rheostat model" where ER stress levels would decide cell fate, these data raise the crucial question as to whether UPR signaling could control pathogen-induced cell death. Seeking for transcriptional hints in microarray databases revealed that *At-bZIP60* is the only bZIP TF-encoding gene among the subfamily D which expression is specifically stimulated in multiple incompatible interactions (Cacas JL, unpublished data). Combined with the fact that both the ER-localized calcium-ATPase 1 (CA1) and BI1 are needed for coordinating HCD [105, 106], this strongly suggests that part of the model proposed for abiotic

Table 6.1 Overview of selected published works on the relationship between endoplasmic reticulum and defense mechanisms in plants

	Plants	Genotypes	Pathogens/treatments	Phenotypes	References
ERAD	*Medicago truncatula*	*Makibishi1*^{KD} (ERAD-type E3-ubiquitin ligase^{KD})	–	Enhanced saponin production and secretion	Pollier et al. [92]
	Nicotiana benthamiana	*CDC48B*^{OE}	ORMV	Restricted local virus propagation	Niehl et al. [93]
er-qc and secretory pathway	*Arabidopsis thaliana*	*sec61α*^{KO}, *bip2*^{KO}, *dad1*^{KO}, *sec61α*/*bip2*^{DKO}, *dad1*/*bip2*^{DKO}	*Psm* (ES4326)	SAR deficiency Reduced PR1 secretion	Wang et al. [83]
		bip2^{KO}	SA analogs	Hypersensitivity to SA analogs Cell death	
	Hordeum vulgare	*sec61β*^{KD}	*Bgh*	Enhanced disease susceptibility	Zhang et al. [94]
	N. benthamiana	*bip*^{KD}	*Pst* (DC3000)	Comparable to WT	Xu et al. [95]
			Xoo (Pxo99)	Decreased HCD Unaffected bacterial growth	
		crt3^{KD}	*Pst* (DC3000)	Decreased HCD Unaffected bacterial growth	
			Xoo (Pxo99)	Comparable to WT	
		uggt^{KD}	*Pst* (DC3000)	Decreased HCD Unaffected bacterial growth	
			Xoo (Pxo99)	Comparable to WT	
		erd2a/*erd2b*^{DKD}	*Pst* (DC3000)	Enhanced HCD Unaffected bacterial growth	
			Xoo (Pxo99)	Enhanced HCD Unaffected bacterial growth	
			p50/N	Enhanced HCD	
			Cf9/Avr9	Enhanced HCD	
			Pto/AvrPto	Enhanced HCD	

	Glycine max and *Nicotiana tabacum*	*bip*^{KD}	*Pst*	Decreased HCD	Carvalho et al. [96]
		BIP^{OE}	*Pst*	Enhanced HCD Increased SA levels	
	N. benthamiana	*bip5*^{KD}	*BAX* or *INF1* expression	Cell death suppression	Lee et al. [97]
	A. thaliana	*CRT2*^{OE}	–	Increased SA levels	Qiu et al. [98]
		CRT2^{OE}	*Pst* (DC3000)	Increased bacterial growth	
		crt2^{KO}	*Pst* (DC3000)	Decreased bacterial growth	
	N. benthamiana	*CRT2*^{KD}, *CRT3*^{KD}, *ERp57*^{KD}, *P5*^{KD}	TMV	Partial loss of resistance	Caplan et al. [99]
UPR	*N. benthamiana*	*BiP4*^{OE}	TGBP3p	Decreased cell death	Ye et al. [100]
		bZIP60^{KD} *skp1*^{KD}	PVX	Restricted infection and systemic propagation	
	A. thaliana	*bzip60*^{KO}, *ire1a*/*ire1b*^{DKO}	*Psm* (ES4326)	Enhanced local bacterial growth	Moreno et al. [85]
		ire1a/ire1b DKO	*Psm* (ES4326)	SAR deficiency Reduced PR1 secretion	
	N. benthamiana	*bZIP60*^{KD}	*Pc*	Enhanced bacterial growth	Tateda et al. [101]

(continued)

Table 6.1 (continued)

	Plants	Genotypes	Pathogens/treatments	Phenotypes	References
ER-localized or ER-related	*Capsicum annuum*	WT	*XopB* + *Xcv*	Inhibition of both HCD and cell secretion activity Abolition of ETI	Schulze et al. [102]
	A. thaliana	*ntl9*^{KO}	*Ps* (AvrRpt2)	Decreased resistance	Block et al. [103]
			Ps (AvrRpm1)		
		NTL9^{OE}	*Ps* (AvrRpt2)	Increased resistance	
			Ps (AvrRpm1)		
	N. benthamiana	*ntp1*^{KD}, *ntp2*^{KD}	*P. infestans*	Enhanced susceptibility	McLellan et al. [104]
	N. benthamiana	*CA1*^{KD} (Ca^{2+}-ATPase^{KD})	Cf9/Avr9	Enhanced HCD	Zhu et al. [105]
			Pto/AvrPto	Enhanced HCD	
			Cryptogein	Enhanced HCD	
			Pst (DC3000)	Enhanced HCD	
			TMV	Enhanced HCD	
	A. thaliana	*bi1*	*Pst* (AvrRpt2)	Enhanced HCD	Kawai-Yamada et al. [106]
	H. vulgare	*BI1*^{OE}	*Bgh*	Increased disease susceptibility	Hückelhoven et al. [107]
	A. thaliana	*rpn2*	*Ec*	Decreased disease resistance	Weis et al. [108]
		cyp83a1	*Ec*	Increased disease resistance	
	A. thaliana	*tga1*/*tga4*^{DKO}	TM	Hypersensitivity to TM	Wang and Fobert [109]
			Pst (DC3000 $hrcC^{-}$)	Increased bacterial growth Decreased PR1 secretion	
			Psp (*1448a*)	Increased bacterial growth Delayed PR1 secretion	
	A. thaliana	*tbf1*^{KO}	TM	Hypersensitivity to TM	Pajerowska-Mukhtar et al. [84]
			SA	Decreased *BiP2*/*CRT3* expression Reduced PR1 secretion	
			SA + *Psm* (ES4326)	SAR deficiency	
			Psm (ES4326)	Increased local bacterial growth	

Abbreviations for genotypes and phenotypes: *OE*, overexpressor; *KO*, knockout; *KD*, knockdown; *DKO*, double knockouts; *DKD*, knockdowns; *ETI*, effector-triggered immunity; *HCD*, *hypersensitive cell death*; *SAR*, systemic acquired resistance. Abbreviation for pathogens and treatments: *Bgh*, *Blumeria graminis* f.sp. *hordei*; *Ec*, *Erysiphe cruciferarum*; *ORMV*, oilseed rape mosaic virus; *Ps*, *Pseudomonas syringae*; *Pc*, *Pseudomonas cichorii*; *P. infestans*, *Phytophthora infestans*; *Psm*, *Pseudomonas syringae* pv. *maculicola*; *Psp*, *Pseudomonas syringae* pv. *phaseolicola*; *Pst*, *Pseudomonas syringae* pv. *tomato*; *PVX*, potato virus X; *TGBP3*, Triple Gene Block Protein3; *TM*, tunicamycin; *TMV*, tobacco mosaic virus; *SA*, salicylic acid; *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*. Abbreviations for genes and proteins: *BI1* Bax inhibitor 1, *BiP* binding protein, *CDC48* cell division cycle protein 48, *CRT* calreticulin, *DAD1* defender against apoptotic cell death 1, *NTL9* NAC transcription factor-like 9, *NTP1-2* NAC targeted by *Phytophthora* 1-2, *PR1* pathogenesis-related protein 1, *RPN2* ribophorin 2, *TBF* TL1-binding transcription factor. *CYP83a1* encode a cytochrome P450 enzyme involved in glucosinolate synthesis. ERp53 and P5 belong to protein disulfide isomerase family of proteins. *SKP1* code for a E3-ubiquitin ligase-type of enzyme. Xop B is a bacterial pathogen effector secreted by the bacteria *Xanthomonas campestris*

stress-induced cell death (Fig. 6.3b) could be applied in the context of plant immunity. Indirect insights also point to a regulatory function for UPR in the orchestration of HCD. Bosis et al. [115] demonstrated that the bacterial effector Xop E2, which represses HCD *in planta*, is able to dampen the transcription of a reporter gene controlled by an UPR-responsive element-containing promoter in heterologous system. In addition, the altruistic fungus *Piriformospora indica*, which needs PCD to take place for successfully colonizing *Arabidopsis* roots, switches off *bZIP28* and *bZIP60* expression by means of an unknown mechanism [116], probably for abrogating the cytoprotective UPR branch. Thus, future investigation will certainly provide exciting clues about this emerging PCD pathway in plants.

6.2.4 Novel Programmed Cell Death Signaling Pathways Initiated at the Endoplasmic Reticulum

Besides the aforementioned PCD regulators, three TF of the NAC family are embedded in ER membranes by a single transmembrane domain and released to orchestrate ERR-mediated resistance to pathogens. The *Arabidopsis* NAC transcription factor-like 9 (NTL9) is a key player in the ETI response to the biotrophic bacterial strain *P. syringae* (DC3000::AvrRpm1). Upon indirect recognition of the effector AvrRpm1 by the "immunity guard" RPM1, signaling cascades are initiated that leads to HCD. To overcome this defensive barrier, another bacterial effector Hop D1 is injected by bacteria into host plant cells, specifically targeting and preventing NTL9 relocalization to the nucleus and abolishing AvrRpm1-initiated HCD. Accordingly, plants expressing a constitutively active, truncated version of NTL9 insensitive to Hop D1 are more resistant to virulent and avirulent bacteria [103]. Similarly, the nuclear remobilization of the two potato NAC targeted by *Phytophthora* (NTP) 1 and NTP2 is inhibited through direct interaction with a RxLR effector from the necrotrophic oomycete *Phytophthora infestans*. However, the outcome of this effector/TF association is different as it leads to enhanced plant susceptibility due to increased cell death that profits the pathogen [104]. Since the potential interplay between these new PCD regulators and ER stress has not yet been studied, it is difficult to affirm if these proteins act in an UPR-contingent manner or if they are part of independently regulated cell death pathway(s) (Fig. 6.3b). Once again, these two studies highlight the pivotal role for the ER in the generation of context-specific signals that contribute to organize plant cell responses—like PCD—to environmental constraints.

6.3 Endoplasmic Reticulum-Golgi Apparatus Interface in Programmed Cell Death Regulation

The GA is a motile compartment composed of multiple stacked cisternae. Besides its function in subcellular trafficking in eukaryotic cells, the GA can act as a sensor of cellular homeostasis perturbations in mammals [117, 118] and initiate

caspase-2-dependent signaling pathways that organize its own demise, pushing cells in the direction of apoptosis under irreparable stress conditions [119–122]. In plant cells, many PCD regulators concentrate in the GA, as previously described [123]. However, again, underpinning molecular mechanisms of GA-regulated PCD seems quite divergent from what has been reported in animals.

6.3.1 Anterograde and Retrograde Protein Transport

The GA can be subdivided in a proximal *cis*-network, which exchanges proteins with the ER via vesicular transport, a medium part most likely involved in lipid and protein maturation, and a distal portion associated with the *trans*-Golgi network (TGN) specialized in molecule sorting to PM, vacuole and other compartments. As mentioned earlier, PCD could be negatively regulated via the processing of UPR sensors/transducers in a RIP-dependent manner. Although still uncharacterized, the activation mechanism of all ER-localized NAC TF (NAC013, NAC017, NTP1, NTP2, and NTL9) may be similar and require anterograde trafficking. Some works also reported on GA morphological modifications in several developmental PCD models [124–126]. Early pharmacological study using brefeldin A, a fungal antibiotic inducing apoptosis by disrupting retrograde protein transport, further linked GA to plant PCD [8]. Yet, it is unclear if the GA, by itself, can sense cellular perturbations and induce PCD or if it depends on ER signaling events to do so. The fact that TM-treated sycamore cell suspensions present a similar set of physiological events as those reported upon application of brefeldin A [9] could argue in favor of the second hypothesis. Accordingly, RNA interference experiments where components of the retrograde GA to ER transport machinery were targeted provided evidence for ER stress-triggered cell death. Indeed, deficiency for the tobacco *neuroblastoma-amplified gene* (*NAG*) coding for a protein of the syntaxin 18 complex results in DNA degradation, mitochondrial membrane potential modification, upregulation of *BI1*, and ER chaperone-encoding genes as well as bZIP28 nuclear relocation [127]. Likewise, abrogating the retrieval pathway that brings back luminal proteins to the ER by knocking-down both tobacco ER luminal protein receptors (ERD) 2a and 2b causes BiP to escape in cytoplasm, hypersensitivity to ER stressor agents, and drastic exacerbation of hypersensitive symptoms in response to non-host pathogens [95]. Altogether, these data suggest that protein trafficking disturbance in the ER/GA interface can activate cell suicide programs, irrespective of the possible ability of GA to signal PCD. The work of Qiao et al. [128] on rice *spotted leaf 28* lesion-mimic mutants altered for the clathrin-associated adaptor protein 1 further extends this scenario to the TGN. In plant immunity, this makes full sense as pathogens try to repress cell secretion activity that is required for successful mounting of apoplastic defense. For instance, the bacterial effector Xop B that targets GA was recently shown to inhibit both HCD and vesicle trafficking [102]. Thus, it is not surprising that some "guard" proteins, like At-RPP1 which confers resistance to specific fungi, are found in the early secretory pathway [129], where they could be

in charge of monitoring protein fluxes and launching the hypersensitive response when effector-induced perturbations are encountered.

6.3.2 *Does the Golgi Apparatus Control Abscisic Acid Signaling During Cell Death?*

In addition to bZIP17 (Fig. 6.1b), two *Arabidopsis* proteins designated MAIGO2 (MAG2) and translocator protein (TSPO) connect the early secretory pathway to ABA signaling. The first one is embedded in ER membranes by a single transmembrane domain and is part of the NAG-containing complex that participates in protein retrograde trafficking. *mag2* null mutants exhibit oversensitivity to ABA and ER stress, potentially due to a defect in bZIP17 signaling [130]. As for the second protein, its human counterpart is a polytopic mitochondrial protein that controls steroid and porphyrin import as well as binding to benzodiazepine-like molecules and has many physiological implications including cell death by apoptosis [131]. Although not prone to depression, *Arabidopsis* plants possess a single, conserved orthologous TSPO protein that resides in the ER/GA and can relocate to the chloroplast upon perception of abiotic stress [132]. Even if the ins and outs of the TSPO function still have to be clarified in plants, it has been demonstrated that (1) the *Arabidopsis* protein is induced by exogenous ABA application, (2) it has an intrinsic propensity for binding hemes, and (3) its heme-bound form is not degraded by the ERAD system but involves a specific autophagic-dependent process [133]. Vanhee and Batoko [134, 135] further proposed a model where At-TSPO would not only modulate the increasing amounts of toxic free hemes following stress but also indirectly regulate the ER-localized cytochrome P450, CYP707A, that requires heme as a prosthetic group for initiating ABA catabolism through hydroxylation. Reinforcing the idea of a regulatory function for the endomembrane system in ABA signaling, ABA glucosyl esters were recently reported to be sequestered in the vacuole and presumably the ER [136].

Importantly, the enzymatic duo composed of the kinase enhanced disease resistance 1 (EDR1) and the multidomain protein keep on going (KEG) functions in both ABA and PCD signaling pathways. Loss-of-function mutation in the *Arabidopsis EDR1* gene confers enhanced resistance to powdery mildew accompanied by larger hypersensitive lesions at the infection sites and causes spontaneous cell death in response to drought stress as well as faster ethylene-induced senescence when compared to WT plants [137]. Microarray data revealed specific gene sets that are constitutively downregulated by EDR1 in uninfected wild-type plants and unrepressed upon fungal attack. These enriched gene ontology annotations include a bench of defense TF and endomembrane system-related genes [138]. Consistently, KEG recruits EDR1 at the TGN/early endosomal interface (TEI) and controls vesicular trafficking, contributing to apoplastic defense [139]. A missense mutation (*keg-4*) in the C-terminal part of KEG results in mislocalization of the protein and suppresses all *edr1*-mediated phenotypes [140], indicating that location in the TEI is crucial for

KEG function during pathogen-induced cell death. Moreover, KEG harbors a cytoplasmic RING-E3 ubiquitin ligase domain that is responsible for targeting the TF abscisic acid-insensitive 5 (ABI5) to proteasomal degradation [141]. To date, whether the functional EDR1/KEG node requires ABI5 for restricting powdery mildew-triggered HCD is, however, uncertain. Alternatives could be that (1) EDR1/KEG interaction facilitates the formation of PCD signaling complex at the TEI; (2) these two proteins tightly regulate PCD mediator trafficking; or (3) other TF than ABI5, which are negative PCD regulators, could be substrates of KEG. In line with the latter speculation, the TF identified by Christiansen et al. [138] are likely candidates to be phosphorylated by EDR1 and further ubiquitinated by KEG. More recently, knocking-out the nuclear TF *Botrytis* sensitive 1 (BOS1)/MYB108-encoding gene was found to provoke an ABA-contingent runaway cell death (RCD) in wounded *Arabidopsis* plants [142], unveiling a new regulatory mechanism whereby BOS1/MYB108 would limit cell death from spreading by diminishing ABA accumulation. Based on this study and the above analysis, it seems that the ABA signaling aspect that has long been neglected in the context of PCD may be of utmost relevance and therefore deserves further investigation in relation to the secretory pathway.

6.3.3 Sphingolipids Connect the Early Secretory Pathway to Programmed Cell Death

The enigmatic family of plant sphingolipids is synthesized in the early secretory pathway, starting in the ER for long-chain bases (LCB), ceramides (Cer), and glucosylceramides (GluCer) and being completed in the GA for more complex glycosylated sphingolipids or glycosyl-inositol-phosphoryl-ceramides (GIPC) (Fig. 6.4). Sphingolipids play a conserved dual role across kingdoms as structural membrane elements and signaling molecules in response to biotic and abiotic stress. GluCer, and probably GIPC, are also involved in maintaining homeostasis by regulating protein sorting [143, 144]. When chemically perturbed or genetically disrupted, most steps of the sphingolipid biosynthesis pathway can lead to conditional cell death phenotypes or spontaneous hypersensitive-like foliar lesions. The tightly regulated equilibrium between unfettered LCB/LCB-phosphate (LCB-P) seems to represent one conserved master switch of plant cell fate, keeping cells alive when in favor of LCB-P or initiating cell's demise otherwise. Compelling evidence for this notion was provided by exogenous LCB application, the use of the mycotoxin fumonisin B1 (FB1) that inhibits ceramide synthase (CerS), and mutation (*fumonisin-resistant 11*, *fbr11*) in a subunit of the LCB-forming enzyme, or serine-palmitoyl transferase (SPT) [145, 146]. Another crucial regulatory node may rely on the Cer/Cer-phosphate (Cer-P) balance, as substantiated by genetic data regarding the ceramide kinase accelerated cell death 5 [147] and inositol-phosphoryl-ceramide synthase (IPCS) [148]. In plants, it is assumed that, like in animals, accumulation of free Cer would kill cells, whereas that of Cer-P would have

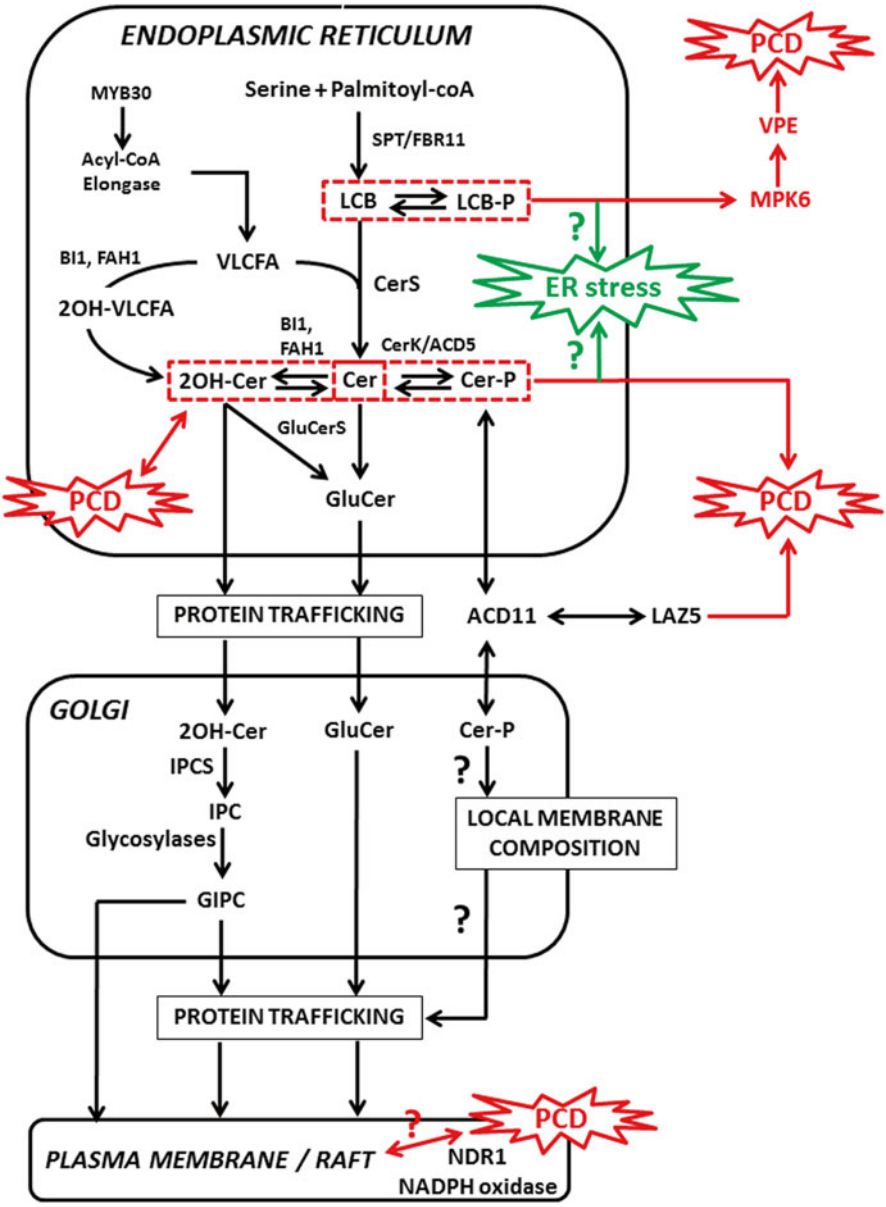

Fig. 6.4 Regulation of plant programmed cell death by sphingolipids. Sphingolipid synthesis is initiated in the ER. It starts by the condensation of a serine and a palmitoyl-coA catalyzed by the serine-palmitoyl-coA transferase (SPT) and producing nine distinct long-chain base (LCB) species. VLCFA are then amidified to LCB by the ceramide synthase (CerS). A glucose moiety can be added to ceramides by the glucosylceramide synthase (GluCerS) in the ER. Alternatively, ceramides can be exported to the Golgi apparatus where the inositol-phosphoryl (IPC)-ceramide synthase forms IPC that can be further glycosylated before being sorted to plasma membrane rafts. Main regulatory nodes and mediators of sphingolipid-induced cell death are represented in *red*

survival-promoting effects. Despite accumulating data in the literature, downstream effectors of sphingolipid-induced cell death remain elusive in plants. Recently, Sacedo-Garcia et al. [149] identified the *Arabidopsis* mitogen-activated protein kinase 6 (MPK6) as the first effector of one such signaling pathway. The authors demonstrated that the kinase is rapidly activated upon FB1 and LCB exogenous supply and *mpk6* mutants are compromised in both FB1- and LCB-triggered PCD, though they endogenously accumulate sphingoid bases. Taking into account that FB1-induced cell death implies vacuole-orchestrated events [150], it is conceivable that MPK6-dependent phosphorylation cascade ends up in VPE activation, highlighting possible links between sphingolipid signaling, ER stress, and NRP (Fig. 6.3c). In animals, luminal increases in Cer and sphingosine-1-phosphate concentrations promote ER stress and activate a protective autophagic response. Although ER stress-activated autophagy has been evidenced in plants (see Sect 6.4.2), the ability of sphingolipids to alter ER homeostasis has yet to be investigated.

Again, the *accelerated cell death 11* (*acd11*) mutant hints the importance of sphingolipids as mediators of HCD. *ACD11* encodes a soluble Cer-1-phosphate transferase [151] that most likely shuttles between the ER and GA by means of physical association with multiple protein interactors implicated in vesicle fusion at the GA, facilitation of ceramide transport, and translocation of soluble proteins to the ER [152]. It has been suggested that ACD11 could act in concert with ACD5 to control the Cer/Cer-P balance [151]. Alternatively, ACD11 may also modulate local membrane composition through its lipid-transfer activity, indirectly influencing protein trafficking. Whatsoever, this protein must exert a key function judging by the strong RCD phenotype of *acd11* under restrictive conditions. ACD11 was proposed to be the "guardee" of unknown resistance protein(s); its deficiency in mutant lines can result in inappropriate activation of the "guard(s)" and subsequent ectopic HCD development [153]. The recently identified immune receptor LAZARUS 5 (LAZ5) is a good candidate for such a "guard," in spite of its apparent lack of interaction with ACD11 *in planta* [154].

The *Arabidopsis* MYB30 TF is a positive regulator of hypersensitive defense and cell death. Genes coding for the acyl-coA elongase subunits represent the main targets of MYB30 and are specifically upregulated during incompatible interactions with pathogen bacteria. Upon infection, this transcriptional reprogramming significantly augments the synthesis of very long-chain fatty acids (VLCFA) that are precursors of cuticle components and sphingolipids [155]. Although changes in wax composition can account, at least in part, for MYB30-driven resistance, the authors also highlighted the possibility that VLCFA may serve as cell death signals. How can pro-death information be relayed by VLCFA? Nagano et al. [156, 157] provided a possible answer to this question. They showed that At-BI1 interacts with the electron donor, cytochrome b5, the latter of which can in turn associate with fatty acid hydroxylase 1 (FAH1) catalyzing the hydroxylation of VLCFA on carbon position 2. Overexpression of At-BI1 was also correlated with higher hydroxylated VLCA (hVLCFA) contents and decreased cell death. Conversely, knockdown *FAH1* plants displayed decreased hVLCFA amounts and enhanced sensitivity to hydrogen peroxide, suggesting that At-BI1 protects cells by activating FAH1 in these specific

conditions. Now, given that hCer exhibit pro-survival properties in animal cells [158], one can postulate that the hCer/Cer balance under the control of BI1/FAH1 dictates the progression rate of HCD in foliar tissues, partly counteracting MYB30 function. This attractive theory cannot, however, explain by itself the massive increase in VLCFA concentrations reported in response to pathogen attack, even though such a burst is all the more sufficient for signaling purposes. Another hypothesis to be considered could be related to the structuring properties of GIPC that are highly enriched in hVLCFA and drives the majority of this cellular FA pool. In addition to their proposed role in coordinating immune receptor sorting at the TGN [146], GIPC are believed to be the main lipids of plant PM microdomains or rafts [144]. These islands have been documented as signaling platforms during apoptosis in mammalian cells [159, 160], and it is likely that protein lateral segregation into rafts relies on PM lipid composition. Therefore, one can assume that pathogen-triggered modifications in (h)VLCFA contents could affect GIPC composition and/or concentration and, consequently, impact raft signaling events. NADPH oxidase was isolated from detergent-insoluble fractions corresponding to rafts [161], and another plant PCD-related protein, non-race-specific disease resistance 1 (NDR1), is suspected to segregate into rafts. Unfortunately, this provides only small clues about how PM microdomains may be involved in cell death regulation.

6.4 Cross-Talk Between the Early Secretory Pathway, Vacuole, and Autophagy

Beyond the theoretical point of no return, cells engage in the execution phase of PCD, during which they organize their self-dismantling. Vacuoles and autophagic structures can participate in this ultimate step in cell's life, whereas the early secretory pathway plays a regulatory role in PCD as aforementioned. What could be the interplays between all these subcellular compartments during plant cell death?

6.4.1 Vacuole and Cell Death Execution

Outstanding works from the Hara-Nishimura's laboratory (Kyoto University, Japan) put forward the case for vacuole-resident hydrolytic enzymes or VPE as executioners of HCD [162]. Since their identification, VPE were also found to be part of developmental cell death programs [163]. They share with plant metacaspases and genuine animal caspases the typical caspase-hemoglobinase fold [164]. Tobacco VPE harbor caspase-1-like activities and are rapidly upregulated in response to tobacco mosaic virus (TMV). Silencing VPE results in abolition of virus-induced hypersensitive lesions associated with the absence of tonoplast disruption, normally observed and followed by vacuolar collapse in WT plants [162]. Another cell dismantling process under the control of the proteasome subunit PBA1

requires vacuole-dependent events for successful execution [165]. Using a RNAi strategy targeting PBA1 in *Arabidopsis*, the authors demonstrated that proteasome-associated caspase-3-like activity is necessary for mediating fusion between tonoplast and PM during an incompatible interaction with *P. syringae* bacteria. Noteworthy, the vacuolar content of cells undergoing HCD was shown to display both antibacterial and death-inducing activities. Combined, these results illustrate two adaptive means whereby plant cells can respond to and dispose of invaders with respect to their mode of infection. VPE-controlled vacuolar collapse ending by self-digest of host cells seems to be employed to cope with intracellular infection by viruses, whereas PBA1-regulated discharge of vacuolar content into apoplasm would be restricted to bacterial pathogens that proliferate extracellularly.

Substrates of VPE and PBA1 are still unknown, hampering our full understanding of the molecular mechanisms that govern tonoplast breakdown or fusion with PM, respectively. The trigger of VPE cell death pathway, nonetheless, may be hypothesized to be controlled by cell secretion activity. VPE belong to the legumain family of endoproteinases that travels in the endomembrane system as inactive proenzymes to reach the vacuole. Originally identified as proteolytic actors in the maturation of storage proprotein precursors in seeds, VPE probably self-processed in acidic conditions as they were found active in the vacuole lumen [166]. This finding implies that direct cell death-promoting substrates of VPE are not present in vacuolar content of healthy cells; otherwise, PCD would ensue. Upon infection, however, protein substrates may be synthesized in the ER and sorted to vacuoles via the secretory pathway in order to seal cell fate. Transcriptional upregulation of VPE observed during defense response would thus either contribute to accelerate tonoplast destruction or aid processing vacuolar antimicrobial proteins.

6.4.2 Dual Role of Autophagy in Programmed Cell Death

Macroautophagy (before and hereafter referred to as autophagy) is a recycling process highly conserved across kingdoms, the function of which is to maintain cellular homeostasis throughout organism life under physiological and stressful conditions, such as starvation and pathogen attack. Autophagy involves pre-autophagic structures (PAS) that encircle large region of the cytoplasm and coalesce to seal and finally create double-membrane vesicles or autophagosomes. The fusion of autophagosomal external membrane with the tonoplast allows the release of the internal vesicle as an autophagic body in the vacuolar lumen, where hydrolysis takes place. During the last decade, around 40 *Arabidopsis AUTOPHAGY* (*ATG*)-related genes participating in the above cited steps have been identified [167].

Using a VIGS-based high-throughput screen, Liu et al. [168] identified ATG6/BECLIN1 as a suppressor of TMV-induced cell death. ATG6/BECLIN1 encodes a protein involved in the nucleation of autophagosome vesicles. *ATG6*-deficient lines display a typical RCD that eventually engulfed the whole plant, while viruses were not found outside of the inoculated area. Silencing other *ATG* genes also led to a

comparable phenotype following viral infection, shedding light on autophagy as part of the machinery dedicated to the restriction of HCD development in response to TMV. Testing for additional pathosystems in tobacco (bacteria, oomycete elicitors, fungal, and bacterial effectors) and *Arabidopsis* plants [168, 169] suggested that the cytoprotective function of autophagy during HCD may be a conserved feature. This work, supported by others [110, 142, 170], underlines the possibility that yet-uncharacterized pro-death signals could be generated in infected dying zones and move into healthy systemic organs. Thus, one can speculate that pro-death signals are literally consumed by autophagosomes in WT plants in order to neutralize them and prevent the onset of undesired systemic HCD.

Hofius et al. [171] further analyzed hypersensitive symptom intensity in relation to autophagic response in *Arabidopsis* upon inoculation of distinct avirulent *P. syringae* strains (DC3000::AvrRpt2; DC3000::AvrRps4; and DC3000::AvrRpm1). The first bacterial strain did not activate any autophagic vesiculation in cytoplasm of WT cells, indicating that execution of RPS2-coordinated cell death is independent of autophagy. By contrast, the two other strains did trigger an autophagosome-contingent process, and autophagy-deficient mutants showed unexpected behaviors. On the one hand, knocking-out *ATG* genes was responsible for a significant delay in AvrRps4-initiated HCD, strongly suggesting a pro-death role for autophagy under these specific conditions. On the other hand, a marked decrease in hypersensitive lesion rate was only observed when *atg* mutants were coinfiltrated with both DC3000::AvrRpm1 and cathepsin B inhibitors. In addition to a death-promoting function for autophagy, the latter data lend support to the idea that multiple execution death pathways could cooperate simultaneously in the same plant cell.

The antagonistic outcomes of autophagy in plant immunity might not be surprising since this process can either protect metazoan cells from apoptosis or be used as a tool that undertakes cellular dismantling [172, 173]. Likewise, ER-activated autophagy (ERAA) seems conserved across kingdoms. Chemically induced ER stress triggers IRE1-contingent autophagic vesiculation degrading the ER, and IRE1 downstream splicing targets seem dispensable for this phenomenon in both animal and plant systems [174, 175]. In *Arabidopsis*, neither bZIP60 nor bZIP28 were necessary for ERAA induction, indicating that, as in animals, IRE1 kinase activity may be responsible for initiating a yet-to-be identified signaling pathway leading to the onset of cytoprotective autophagy. Furthermore, death-regulating cross-talks between the secretory pathway and autophagy are not restricted to ERAA. The small *Arabidopsis* Rab GTPase RabG3b shares a high degree of homology with the animal Rab7 and yeast Ypt7p that has been implicated in vesicle docking and fusion with distinct compartments such as autophagosomes, vacuoles, and endosomes. Overexpressing a constitutively activated mutant form of RabG3b (RabG3bCA) results in enhanced autophagy correlated with an increased number of tracheary elements [176], whose ultimate differentiation steps consist of PCD. Such transgenics also display markedly exacerbated HCD in response to the bacterial

strain DC3000::AvrRpm1 compared to WT plants, but this phenotype is only partially suppressed by *ATG5* loss of function [177]. With reference to the work of Hofius et al. [171], one can assume that the cathepsin-mediated cell death path takes over the autophagic-dependent one in *atg5*/*RabG3bCA* lines, still allowing for part of the hypersensitive program to be executed. Another component of the secretory pathway, the *Hordeum vulgare* ABA-induced cDNA 22 (HVA22), that has many sequence homologues in yeast, plants, and animals and colocalizes with ER/GA fluorescent markers, regulates autophagy [178]. Its yeast ortholog, Yop1p, functions in ER-to-GA vesicular trafficking [179, 180]. HVA22, and a related *Arabidopsis* protein HVA22D, can repress gibberellin-mediated vacuolation and PCD when overexpressed in barley aleurone cells [181]. Perhaps, ABA-induced HVA22 accumulation in aleurone cells inhibits secretion of hydrolytic enzymes that digests the juxtaposed starchy endosperm for nutrient mobilization during cereal seed germination. It is also conceivable that HVA22 family of proteins reinforces its control on germination by blocking autophagy-dependent nutrient recycling that is necessary for de novo synthesis of the hydrolytic enzymes.

6.5 Conclusions

The keen interest of the plant scientific community in both biotic and abiotic stress tolerance has uncovered novel and exciting signaling pathways related to PCD (Fig. 6.5). In this regard, the early secretory pathway holds a privileged position in this intricate web; the ER playing a double role as sensor of intrinsic and extrinsic stimuli and coordinator of downstream cellular responses. Of utmost importance in this context are the newly identified retrograde signaling pathways involving many ER-localized NAC TF that not only orchestrate HCD but are also likely to cooperate with mitochondria for fine-tuning abiotic-induced cell self-destruction. In the future, investigating such networks in relation with UPR would certainly lead to key discoveries. Furthermore, although not fully understood, the involvement of the GA in PCD becomes more and more manifest as data accumulate, pointing to the fact that this organelle, by itself or in combination with the ER, could control sphingolipid- and ABA-dependent cell death. Last, but not least, beyond the physical links between the vacuole, autophagic compartment, and the early secretory pathway, there are functional connections that would deserve deeper investigation to answer simple but crucial questions. What are the underlying molecular mechanisms of vacuole dismantling and fusion with the PM? How does autophagy switch from a pro-survival mode to a pro-death one? And, is autophagy a genuine executioner of PCD or does it degrade negative regulator(s) of PCD to promote cell suicide?

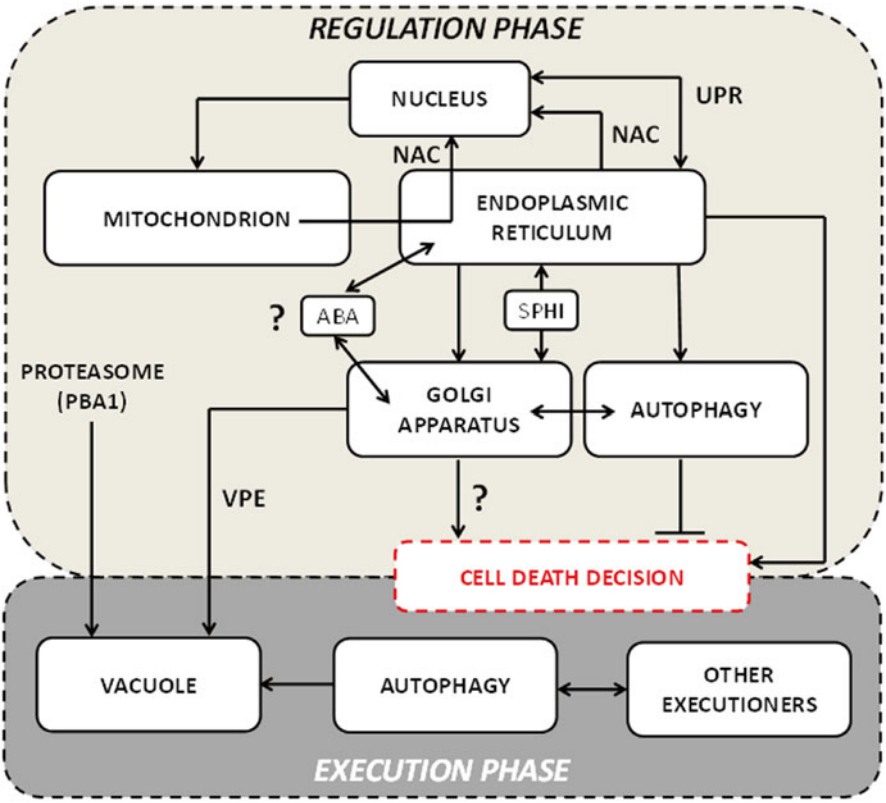

Fig. 6.5 Relationships between endomembrane organelles during plant cell death regulation and execution. Upon stress perception, retrograde signaling pathways relying on classical UPR and NAC TF are initiated from the ER. Mitochondrial-to-nucleus pathways can also involve ER-localized NAC TF. This regulation phase may imply molecular communications between compartments by means of protein trafficking, sphingolipid (SPHI), and ABA signaling. Following abiotic and biotic constraints, it is likely that cells launch the onset of multiple cytoprotective mechanisms, like UPR, MRR, or even autophagy, in an attempt to restore cellular homeostasis. When it fails, the self-destruction decision is taken and death executioners are engaged

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Chapter 7
Nuclear Dismantling Events: Crucial Steps During the Execution of Plant Programmed Cell Death

Fernando Domínguez and Francisco Javier Cejudo

Abbreviations

DAPI	4′,6-Diamidino-2-phenylindole
ER	Endoplasmic reticulum
INM	Inner nuclear membrane
KASH	Klarsicht/ANC-1/Syne-1 homology
MAPK	Mitogen-activated protein kinase
NPC	Nuclear pore complex
Nup	Nucleoporin
ONM	Outer nuclear membrane
PCD	Programmed cell death
ROS	Reactive oxygen species
SIPK	Salicylic acid-induced protein kinase
SUN protein	Sad1/UNC84 protein
TE	Tracheid element

7.1 Introduction

The elimination of unwanted cells by programmed cell death (PCD) is a key feature for the development of multicellular organisms. PCD is a relevant process for the formation of organs with defined morphology and the elimination of unneeded or damaged cells [1], being thus essential for normal development and correct control

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of cellular homeostasis [2]. In animal systems, two main types of PCD have been identified, apoptosis and autophagy [3], which occur with clear differences at the morphological and biochemical levels. Apoptosis is characterized by ultrastructural alterations such as cell shrinkage, cytoplasm contraction, chromatin condensation, and final engulfment by phagocytic cells [4]. At the molecular level, apoptotic cell death is characterized by the participation of cysteine- and aspartate-specific proteases (caspases) [5]. In contrast, autophagic cell death is a self-degradation process characterized at the morphological level by the presence of double-membrane autophagic vesicles, the autophagosomes, which sequester portions of cytoplasm and organelles and fuse with lysosomes or vacuoles for cleavage by resident hydrolases [6]. Genetic studies have revealed that apoptosis and autophagy share common genes [3].

In plants, PCD takes place with peculiar features and there have been different attempts to classify plant PCD taking into account these peculiarities. There is general agreement that plant PCD is not an apoptotic process, though the finding of some apoptotic hallmarks led different authors to propose an apoptotic-like PCD class [7]. Different morphological criteria have been proposed to distinguish among the different types of PCD in plants, based on which plant PCD may be classified into autolytic and non-autolytic. Autolytic PCD keeps some relationship with autophagic PCD in animal cells, whereas non-autolytic PCD can be associated to necrotic PCD [8]. Autophagic processes have been classified in macroautophagy and microautophagy according to the size of the cytoplasmic portion taken up for destruction and the type of vesicle involved [9]. Macroautophagy occurs when large portions of cytoplasm are sequestered into the double-membrane vesicles called autophagosomes. In contrast, microautophagy is characterized by direct invagination of the vacuole membrane to form simple-membrane vesicles surrounding a small portion of cytoplasm releasing it to the vacuolar lumen for degradation [9].

PCD is a genetically controlled program, which in plants, as in other multicellular organisms, has important functions for growth, development, and interaction with the environment [10]. Thus, PCD serves plant development and differentiation, but it is also an important component of plant interaction with biotrophic or necrotrophic pathogens and in response to unfavorable abiotic conditions [11]. Concerning developmental processes, PCD acts as a final step in the formation of tissues such as tracheary elements and has also an important participation in abscission [11–13]. Concerning the interaction with the environment, plants show different types of PCD in response to pathogens. In the hypersensitive response (HR), a rapid cell death at the site of infection takes place, thus limiting the propagation of the pathogen and having a potential systemic acquired response in adjacent cells [14]. Biotrophic pathogens have developed mechanisms to suppress PCD in plant tissues, thus facilitating their spread; in contrast, necrotrophic pathogens trigger PCD of their hosts with the aim of using dead tissues as a source of food [13]. Finally, unfavorable abiotic factors such as hypoxia, temperature, drought, and UV light may also induce PCD in plants [13].

The nucleus of eukaryotic cells may be considered as the central hub for the determination of cell fate. In response to environmental stimuli, the activity of the nucleus changes, thus modifying the pattern of gene expression and determining whether the cell will differentiate, proliferate, and adopt a specific metabolic state, or go into senescence or apoptosis [15]. Moreover, the nucleus is one of the main targets of the cell degradation machinery during PCD. Among the changes undergone by the nucleus in dying cells are chromatin condensation, DNA fragmentation, and nuclear envelope disassembly (reviewed in [16]). The fate of the nucleus in cells undergoing PCD is variable and depends on the system being analyzed. Animal cells undergoing apoptosis show the formation of apoptotic bodies and the engulfment by phagocytes for the lysosomal digestion of apoptotic corpses (reviewed in [17, 18]). A selective process of autophagy targeted to the nucleus, called nucleophagy, has been described in some organisms.

Nucleophagy was first described as a novel way in which unwanted, nonessential, or damaged components of yeast nuclei are degraded. By forming nucleus–vacuole junctions, yeast cells begin a process of microautophagy by which nuclear blebs are released into the vacuole lumen and are degraded by soluble hydrolases [19]. Nucleophagy also occurs in mammalian cells [20] and fungi [21]. Ciliated protozoa have developed a peculiar process for nuclear dismantling throughout conjugation, called programmed nuclear death or nuclear apoptosis, which selectively eliminates the parental macronucleus, whereas coexisting nuclei remain unaffected [22]. When programmed nuclear death begins, the envelope of the parental macronucleus suffers alterations by exposing certain sugars and phosphatidylserine that act as “eat me” signals. Small autophagic vesicles containing mitochondria and lysosomes fuse with the nuclear envelope and release into the nucleus the following components: (1) mitochondrial apoptosis-induced factor (AIF) and endonuclease G-like DNase, which promote chromatin condensation and DNA fragmentation at early stages, and (2) acidic lysosomal enzymes, which are responsible for the final reabsorption of the macronucleus [23]. A similar process mixing macroautophagy and apoptotic-like DNA fragmentation appears in yeast gametogenesis to remove uncellularized nuclei which do not develop as spores [24].

In some plant interspecific hybrids, a curious phenomenon has been developed to selectively eliminate uniparental chromosomes. During the development of crosses between wheat (*Triticum aestivum*) and pearl millet (*Pennisetum glaucum*) hybrid embryos, all the pearl millet chromosomes are eliminated in a process that involves spatial segregation of parental genomes, release of pearl millet chromatin–nuclear extrusions, formation of micronucleus, the subsequent heterochromatinization, DNA fragmentation, and disintegration of micronucleus [25]. No other evidence has been reported of a specific nucleophagy program in plants, but the nucleus, together with portions of the cytoplasm, may undergo either a process of macroautophagy or microautophagy [9]. Another possible scenario is the elimination of nuclear contents after tonoplast disruption and release of vacuolar hydrolases, the so-called mega-autophagy [26], which degrade whatever is left in the cell at the moment of tonoplast disruption.

7.2 Nuclear Molecular Architecture

Most of the events that take place during nucleus dismantling keep a tight relationship with its molecular architecture. The nucleus is delimited by a complex membranous bilayer, the nuclear envelope, and the inner (INM) and outer nuclear membrane (ONM) defining the perinuclear space. The nuclear envelope is penetrated by nuclear pores, structures connecting nucleoplasm and cytoplasm. At the internal side of the nuclear envelope, there is a fibrous network underlying the INM, which acts as a skeleton defining nuclear size and shape [27, 28]. In animal cells, the protein network of the nuclear envelope is formed by four classes of protein components: lamins, INM proteins, the nuclear pore complex proteins, and chromatin proteins [27]. A fifth group localized at the nucleus–cytoplasm interphase, ONM proteins, has been recently identified as a key nuclear component [29, 30] (Fig. 7.1). A considerable effort is now underway to identify the corresponding counterparts in plant nuclei (reviewed in [31–33]).

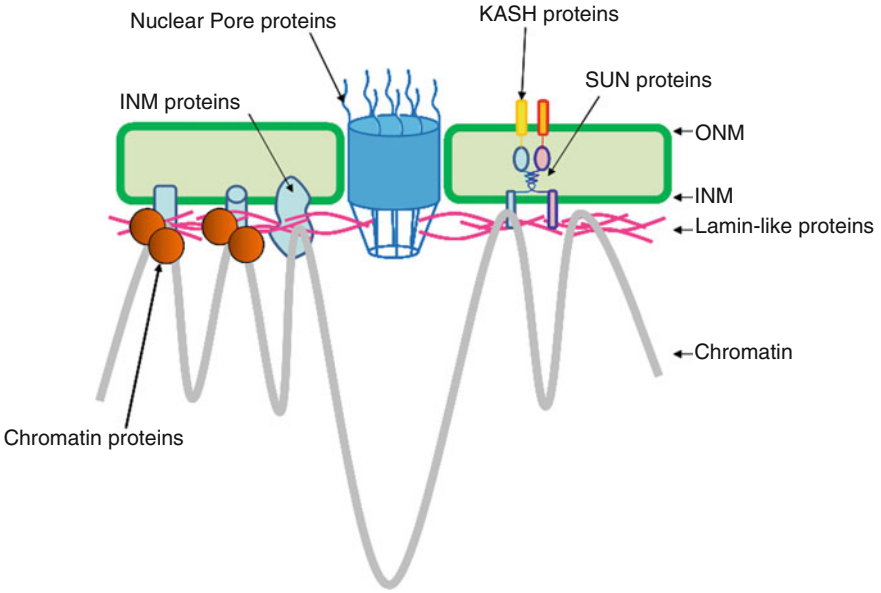

Fig. 7.1 Molecular architecture of the plant nuclear periphery. The nuclear envelope is defined by a double membrane, the outer nuclear membrane (ONM) and the inner nuclear membrane (INM), separating the perinuclear space. The envelope is perforated by nuclear pore complexes (NPCs), which communicate the cytoplasm with the nucleoplasm. By analogy to the nucleus of animal cells, five classes of proteins may be distinguished: lamin-, INM-, ONM-, NPC-, and chromatin-like proteins. Nuclear dynamic is determined by interactions of these proteins with the chromatin and the cytoskeleton

7.2.1 Nuclear Lamina

The nuclear lamina is formed by lamin proteins, which belong to the V intermediate-type filament proteins and can be subdivided into three groups called lamins A, B, and C, which function in nuclear organization and nucleic acid metabolism and processing [27]. Recently, in-lens field emission scanning electron microscopy analyses have allowed the identification of a lamina-like structure in plant nuclei, which resembles the nuclear lamina of animal cell nuclei. This structure forms a network of filaments underlying the INM, which is connected to the nucleoplasmic rings of the nuclear pore complex [34]. These filaments are formed by coiled-coil proteins including the nuclear matrix constituent protein 1 (NMCP1) identified in *Daucus carota* [35] or LINC1 and LINC2 in *Arabidopsis* [36]. Based on the phenotype of mutants deficient in LITTLE NUCLEI 1 and 2, which show reduced nuclear size and changes in nuclear morphology, it has been suggested that plant lamin-like proteins exert scaffold and nuclear support functions [36].

7.2.2 INM and ONM Proteins

INM proteins are integral membrane proteins, which have a nucleoplasmic domain specialized in linking the INM to the lamina and/or chromatin. INM proteins in animal cells include lamin B receptor (LBR), lamin-associated polypeptide 2α (LAP2α), emerin, Man1, and SUN domain proteins (from Sad1/UNC84 protein). In plants, the best characterized INM proteins belong to the SUN domain protein group [37–39], of which five members were identified in maize and two in *Arabidopsis*. Plant SUN proteins form dimers and have been proposed to have an important function in determining nuclear morphology. INM-localized SUN proteins form protein bridges with ONM-localized KASH (Klarsicht/ANC-1/Syne-1 homology) proteins at the perinuclear space, thus connecting the nucleoplasm with the cytoplasmic cytoskeleton [40]. Plant SUN-KASH bridges have also been proposed to play a role in RanGAP anchoring and nuclear shape determination [40].

7.2.3 Nuclear Pore Complex Proteins

Nuclear pore complexes (NPCs) are multiprotein structures, comprising about 30 transmembrane and soluble proteins, the nucleoporins, which form an aqueous channel that allows nucleocytoplasmic transport and supports structural functions [27]. Eight fibrils protrude from the core of the NPC to the cytoplasmic side of the nuclear envelope, whereas a fish trap-like structure protrudes to the inner side, defining the so-called cytoplasmic filaments and nuclear basket, respectively [27]. Plant NPCs show octagonal symmetry and resemble those of yeast and animal cells [34]. The components of the nuclear pore complex have been recently identified in

Arabidopsis thaliana. Although plant NPCs lack seven components, which are present in animal cells, the rest of nucleoporins are present and show high sequence identity with the corresponding animal counterparts displaying a similar domain organization [41].

7.2.4 Chromatin Proteins

This group of proteins, which interact with lamins and INM proteins, is formed by histones, heterochromatin protein 1, and several peripheral chromatin proteins.

In summary, it may be considered that the nuclear envelope is important for three overall functions: (1) constitutes a physical barrier separating cytoplasm from the inner content of the nucleus, (2) controls the movement of macromolecules through the nuclear pores, and (3) serves as anchorage surface for cytoskeletal components and chromatin [42]. Although the present knowledge of the nuclear envelope in plant cells is much scarcer, several components may serve as potential markers for studying nuclear envelope dynamics in plants and their implication in nuclear dismantling during PCD [32]. These include the lamin-like proteins NMCP1/2 or LINC1/2 [35, 36], SUN1/2 INM proteins [37, 39], NPC proteins [41], and WIP1/2/3 and WIT1/2 ONM proteins [40, 43].

7.3 Crucial Steps in Nuclear Dismantling

The comparison of nuclear disintegration in apoptotic animal cells with the present knowledge of plant systems has allowed the identification of several crucial steps in this process.

7.3.1 Processing/Maturation of Key Proteins

Some apoptotic pathways in animal cells progress through the concerted action of caspases, which may act as initiators or executors. Proteolysis is a crucial regulatory process of apoptosis responsible for the activation or inactivation of effector proteins, the translocation of proteins between cell compartments and protein degradation. The analyses of caspase substrates based on proteomic approaches have revealed that most of the targets are proteolyzed for the generation of active effectors and, thus, act in a cascade based on proteolytic activation [44]. In plants, no real caspases have been reported, though enzymes with similar catalytic domains, called metacaspases, have been identified [45]. These proteases, which cleave peptide substrates after Arg and Lys but not Asp residues, have attracted increasing attention in plant PCD studies. However, whether metacaspase activity is part of a

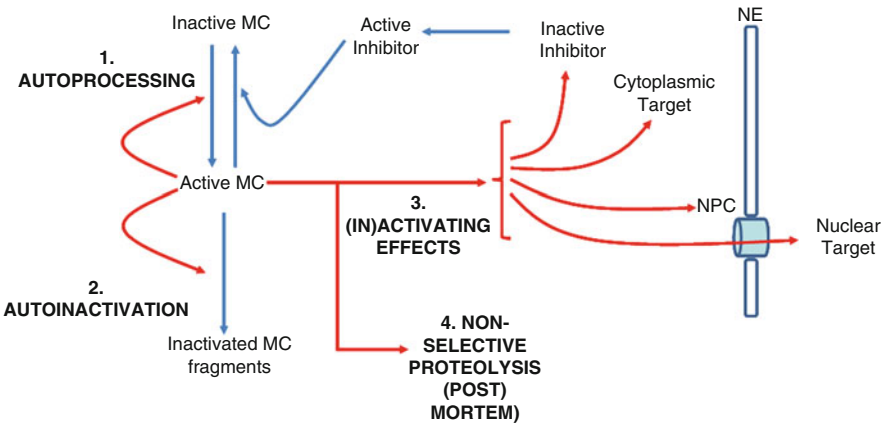

Fig. 7.2 Roles of metacaspases in processing key proteins during PCD include self-processing (1), auto-inactivation (2), and (in)activating effects (3). The latter effects may be subdivided in those affecting cytoplasmic or nuclear targets. Effects involved in cleavage of NPC proteins are especially relevant by facilitating nucleocytoplasmic disruption and translocation to the nucleus. A postmortem nonselective proteolytic activity carried out by metacaspases has been recently described (4). *MC* metacaspase, *NE* nuclear envelope, *NPC* nuclear pore complex

proteolytic cascade is not yet known. It should be noted that plant genomes code for a large number of proteases, though much remains unknown about their natural substrates [46].

Many of the proteolytic events during plant PCD play a regulatory role. In this regard, metacaspases seem to be involved in up to three regulatory functions (Fig. 7.2). First, these enzymes undergo auto-processing, as a mechanism to control the conversion of inactive into active proteases. At least one cleavage site for auto-activation has been identified in type II metacaspases in *Arabidopsis* [47, 48] and *Picea abies* [49]. Probably, these enzymes have additional processing sites that allow a fine-tuning regulation of their activity [50]. Although type I metacaspases have been hypothesized to contain autolytic sites, the autolysis of these proteins has not been yet demonstrated [50]. Second, type II metacaspases remain in active form during a rather small period of time because these enzymes suffer a rapid and irreversible auto-inactivation by cleavage in additional sites. This mechanism of auto-inactivation has the function of reducing the amount of active metacaspases, thus avoiding non-desired effects of a possible overaccumulation [48]. Finally, there is a third regulatory mechanism, identified by yeast two-hybrid assays, which consists in the binding of metacaspases with suicide substrate inhibitors. This is the case of AtMC9, which cleaves AtSerpin1, a serin-protease inhibitor, which in turn, when processed, inhibits the metacaspase [51].

Natural targets acting downstream of plant caspases in the proteolytic cascades are poorly known. The processing of the nonnuclear protein Tudor staphylococcal nuclease (TSN) by mcII-Pa has been described, an enzyme localized at the perinuclear region. This metacaspase is involved in stress tolerance and stabilization of

mRNAs encoding numerous protease inhibitors and other secreted proteins [52, 53]. However, the role of the cleavage of this enzyme in PCD induction remains to be demonstrated. Multiple transcription and translation elongation factors, ribosomal proteins, and structural proteins were identified when the AtMC9 degradome was analyzed [54], suggesting that the nucleocytoplasmic localization of MC9 is essential for its function.

Nuclear proteins also seem to be affected by caspase-like activities during plant PCD. As in apoptotic animal cells [55], poly (ADP-ribose) polymerase (PARP), a nuclear protein involved in DNA repair, replication, transcription, and cell death, is cleaved by caspase-like enzymes in plant cells undergoing PCD. This proteolysis, which has been described in the process of destruction of incompatible pollen to avoid self-fertilization, is Ca^{2+} dependent, inhibited by DEVD peptide, and occurs prior to the detection of DNA fragmentation [56].

7.3.2 Disruption of the Nucleocytoplasmic Barrier

The processes of DNA replication and transcription, which take place in the nucleus, and translation, which takes place in the cytoplasm, are spatially delimited by the nuclear envelope. This physical separation implies the transport of macromolecules between the two compartments, so that soluble transporters must specifically recognize their cargoes to allow their pass through the NPC in the correct direction, as has been established in animal cells [57]. Overall, the characteristics of the nucleocytoplasmic transport are conserved in plants and other eukaryotic organisms [58].

During apoptosis, the massive reorganization of the nucleus, which includes chromatin condensation and DNA fragmentation, as well as global changes in gene expression leading to the activation of the executioner cascade, suggests that alterations in the nucleocytoplasmic transport are needed. There are several signaling and execution factors that migrate in and out of the nucleus specifically during apoptosis [59, 60]. Although there are some differences between apoptotic pathways, two phases may be identified in nucleocytoplasmic transport, protease independent and protease dependent (Fig. 7.3a). Among the protease-independent nuclear transport alterations, it was suggested that signals, such as changes in nuclear calcium levels, might increase the exclusion size through the NPC from ca. 30 kDa to more than 70 kDa, allowing the transport of different factors to the nucleus (Fig. 7.3a,1) [61]. At this stage, which is prior to caspase activation, soluble transport factors, such as Ran, importin-α, and importin-β, suffer an abnormal redistribution across the nuclear envelope, while mRNA is accumulated inside the nucleus probably by blockage of its export mechanism. Furthermore, it has been described that in actinomycin D-treated cells, importin-α is sequestered in dense nuclear bodies [62]. The second phase depends on the limited cleavage of nuclear pore complex and nuclear transport machinery components by caspases, calpains, and other proteases, thus allowing the accessibility of pro-death factors into the nucleus (Fig. 7.3a,2) [61–63]. Despite the cleavage of different nucleoporins, the nuclear envelope

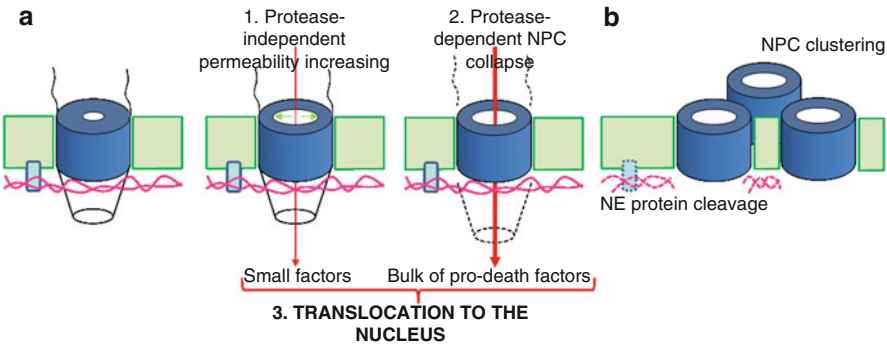

Fig. 7.3 (**a**) Disruption of the nucleocytoplasmic barrier. This process may be divided into a protease-independent phase, which promotes an increase of the diameter of the NPC channel (1, *green arrows*), and a protease-dependent phase, which provokes the degradation of peripheral nucleoporins (2, *dashed lines*). This disruptive process triggers the translocation of pro-death factors to the nucleus (*red arrow*). (**b**) Cleavage of INM proteins and lamin-like proteins (*dashed lines*) provokes NPC clustering

appears intact at the ultrastructural level until the end of the apoptotic process. These two phases are well established in animal apoptosis; however, little is known in plants due to the lack of molecular markers.

7.3.3 Translocation to the Nucleus

Nuclei from dying cells suffer an important modification of their protein content, with the incorporation of new proteins including kinases, transcription factors, proteases, and DNases, among others. Most of these proteins are translocated from the cytoplasm to the nucleus to participate in signaling, execution, and the nuclear dismantling phases. Translocation of proteins to the nucleus is facilitated by the disruption of the nuclear envelope [63] (Fig. 7.3a). In plant cells, proteins that are translocated to the nucleus have been shown to be implicated in different signaling pathways. This is the case of the HR-like cell death in tobacco, which depends on the activation of the transcription factor WRKY1 through direct phosphorylation by the salicylic acid-induced protein kinase, SIPK, which is a mitogen-activated protein kinase (MAPK). Whereas WRKY1 is localized predominantly in the nucleus, SIPK shows cytosolic and nuclear localization [64]. Although SIPK might exert its activating effect at the cytosol, HR-like cell death seems to require the nuclear localization of SIPK. Whether SIPK is phosphorylated and activated in the cytosol and then translocated to the nucleus or is first translocated to the nucleus and then activated is still a matter of debate [65]. In any case, dynamic changes in the nucleocytoplasmic re-localization of MAPK cascade appear as key events for the activation of innate immune responses in plants, as shown in pathogenesis-related gene

expression in parsley [66]. There are also examples of transcription factors, which are translocated to the nucleus during plant PCD. The rice transcription factor OsNAC4 is translocated into the nucleus in a phosphorylation-dependent manner in pathogen-triggered hypersensitive response, activating a Ca^{2+}-dependent nuclease which carries out nuclear DNA fragmentation [67].

During embryogenesis, the metacaspase mcII-Pa is translocated from the cytoplasm to the nucleus in cells that undergo PCD [49]. In the suspensor, the pattern of localization of this protease correlated with the level of DNA degradation. Cells with degraded DNA but still with an intact nuclear envelope accumulated mcII-Pa at the perinuclear cytoplasm, while in cells with fragmented nuclear envelope, mcII-Pa localized inside the nucleus. Moreover, the immunogold labeling revealed that mcII-Pa was associated to nuclear patches of electron-dense chromatin and areas of NPC disassembly.

In cultured tobacco cells treated with staurosporine, it was observed that PCD was accompanied by nuclear-localized caspase-like activities. In early stages of PCD, proteins labeled with fluoroisothiocyanate conjugated to a VAD-fmk inhibitor were localized to the cytoplasm, whereas at late stages of PCD, labeled proteins showed nuclear localization [68]. TATDase, a caspase-like activity involved in TMV-induced hypersensitive response, has also been suggested to be nuclear localized [69]. Caspase-like activities were also detected in the process of self-incompatibility, the elimination of incompatible pollen to avoid self-pollination. Thus, DEVDase, a caspase 3-like activity, was localized in cytoplasm as well as in vegetative and generative cell nuclei from the *Papaver* pollen tubes, suggesting its translocation from the cytoplasm in both cases [70]. The nucleocytoplasmic localization of metacaspase 9, as well as its targets inside and outside the nucleus, suggests that this enzyme carries out an activating role [54]. However, during xylem differentiation, metacaspase 9 showed a change of the subcellular localization from the nucleus and cytoplasm to patches of cell remnants after vacuolar disruption, suggesting a postmortem executor role [71].

Regarding the translocation of nucleases participating in plant cell death to the nucleus, the information is still scarce. Nuclear-localized endonucleases have been identified in nuclear extracts from cells of cereal seeds undergoing PCD [16, 72, 73], and based on these results, it was proposed that these nucleolytic activities are involved in the internucleosomal DNA fragmentation occurring in this process.

7.3.4 The Cytoplasmic Connection: Cytoplasmic Factors Needed for Nuclear Dismantling

Several factors, localized in different cytoplasmic organelles, show a direct or indirect effect on nuclear disintegration in plants (reviewed in [16]). In some cases, these factors are specifically translocated to the nucleus, while in others, translocation to the nucleus depends on the disruption of the vacuolar tonoplast with the consequent degradation of the organelles.

Many of the proteins involved in developmental and/or stress-induced cell death are localized at the endomembrane system of the cell [74]. In this way, proteins that mediate PCD are stored, and somehow sequestered, until their activity is required. This is a mechanism to prevent nuclear protein and DNA degradation when it is not required. Several ER-derived compartments have been reported to provide pro-death factors; among them, it is worth mentioning ER bodies and ricinosomes, which accumulate PCD-related cysteine proteases to be released after rupture [75–78]. Similar ER-derived compartments have been described to contain ribonuclease LX, which remains inactive until the moment of promoting selective cell death processes such as endosperm mobilization, xylem differentiation, and leaf and flower senescence [79]. Besides ER bodies, ER filaments, which contain cell death- and senescence-associated BFN1 nuclease, also participate in cell death. As PCD progresses, BFN1 nuclease-containing filaments are clustered around the nucleus; at later stages, the filaments fuse with fragmented nuclei, thus forming vesicles in which the nuclease is in contact with its substrate, the nuclear DNA [80]; hence, a secondary compartment is formed for the degradation of the nucleic acids. Protein disulfide isomerase-5 exerts an inhibitory effect on cysteine proteases sequestered at the endomembrane system, thus avoiding precocious activity of these enzymes during the trafficking from the ER, Golgi, and trans-Golgi network to protein storage and lytic vacuoles [81]. In this way, protein disulfide isomerase is involved in the regulation of the timing of PCD. The vacuole also plays important roles in plant PCD, allowing maturation of precursors of PR proteins and hydrolases by vacuolar processing enzyme (VPE) and serving for their accumulation. Different hydrolytic enzymes including proteases and nucleases have been shown to be vacuolar localized in mature form, and ready to act upon vacuole rupture [82].

Additional organelles providing pro-death factors are mitochondria and chloroplasts. An Mg^{2+}-dependent nuclease released from the mitochondrial intermembrane space promotes high-molecular-weight DNA fragmentation during PCD, whereas there are some other mitochondrial factors which participate in the induction of DNA laddering [83]. Moreover, ROS produced in mitochondria and chloroplasts appear to be key factors in PCD [83–85], not only by their action promoting oxidative damage to DNA and nuclear membranes but also by their important activity as second messengers [11, 86, 87].

7.3.5 Nuclear Envelope Protein Cleavage and Nuclear Pore Complex Clustering

The composition of the nuclear matrix shows a substantial modification during apoptosis in animal cells. The content of different proteins including lamins and lamin-associated proteins, DNA-binding proteins, hnRNPs, transcriptional regulators, and nuclear trafficking-related proteins is decreased [88]. The decrease of nuclear matrix proteins that takes place during apoptosis is due to caspase cleavage [89, 90]; however, the release of these proteins to the cytoplasm is also possible [88].

One of the key events of apoptotic nuclear dismantling is the proteolysis of matrix attachment region-binding proteins. These proteins are involved in the maintenance of the integrity and proper conformation of the chromatin loops in the nucleus through their interactions with the nucleoskeleton [91, 92]. Important components of these interactions are DNA topoisomerases I and II, which participate in the modification of DNA topology and the linkage of the chromatin to the nuclear matrix; PARP, which is involved in DNA repair and anchorage of chromatin to nuclear scaffold; small nuclear RNP U, implicated in pre-mRNA splicing; and RNA polymerase 1 upstream binding factor, which interacts with the nuclear matrix. All of them are typical caspase substrates [89, 90]. The site-specific proteolysis of the matrix attachment region-binding proteins, which is usually localized at the DNA-binding domain, may promote the release of the proteins from the nuclear matrix, thus opening sites of nuclease hypersensitivity and allowing DNA fragmentation [92].

Lamina, INM, and NPC proteins are also caspase substrates during the apoptotic process. Most of these proteins are localized at the inner side of nuclear envelope and interact with chromatin. Therefore, proteolysis of lamins, lamin-associated proteins such as LBR and LAP2α, and nucleoporins, such as Nup 153, promotes the detachment of chromatin from the nuclear envelope. Moreover, limited proteolysis of lamin-like structure and nucleoporins provokes a more free movement of NPCs in the nuclear envelope, so that clustering of NPCs is an additional consequence of this proteolysis [93, 94] (Fig. 7.3b). Recently, the functional association of SUN1 proteins with NPCs and lamin A has been described. This association promotes the uniform distribution of NPC [95], which argues in favor of the NPC clustering as consequence of the nuclear envelope protein cleavage.

In contrast with the present knowledge of the proteolytic events taking place at the nuclear envelope in apoptotic animal cells, very little is known in plant cells. Though scarce, available ultrastructural and biochemical data suggest that proteolysis of nuclear envelope components also occurs in plant PCD. In developmental PCD processes, such as the elimination of embryonic cells during *Picea abies* embryogenesis [96], or in pathogen-induced PCD, such as the death of cells around the infection site during the hypersensitive response [97, 98], the cleavage of high-molecular-weight DNA to produce 50-kb fragments is the result of the detachment of chromatin from the nuclear envelope, thus increasing sensitivity to nucleases. Bozhkov et al. [99] suggested that NPCs form clusters in areas of segmentation close to apoptotic nuclear lobes, previous to the disassembly of NPC, that release nuclear segments and to the subsequent leakage of chromatin into the cytoplasm.

7.3.6 Posttranslational Protein Modifications During PCD

During the last years, increasing evidence is showing that posttranslational modifications of nuclear proteins constitute critical events regulating nuclear dismantling. In this regard, histones, the main components of chromatin, are the proteins that have received more attention. It is well known that these proteins undergo extensive

posttranslational modifications, which are determinants of the overall chromatin structure and exert a great influence in the regulation of nuclear functions. Different histone modifications occur during apoptosis and suggest a characteristic apoptotic histone code [100]. Other nuclear proteins also suffer covalent modifications influencing cell death; this is the case of lamins and HMGA1a proteins, which become hyperphosphorylated just before DNA fragmentation [92]. Covalent modifications have also been suggested to be related with PCD in plants; these include histone (de)methylation and (de)acetylation in uniparental chromosome elimination [25] or histone (de)acetylation in hypersensitive response-related lesions [101]. Deacetylation also appears as a negative regulator of elicitor-induced cell death in tobacco HR, but in this case, the posttranslational modification occurs in nonhistone proteins [102]. Other posttranslational modifications of nuclear proteins have been suggested to play a role in plant PCD in response to stress [103]. Some examples are the activation by phosphorylation of the transcription factor WRKY1, which mediates HR-like cell death [64], the caspase 3-like activation mediated by nitric oxide, and the mitogen-activated protein kinase MPK6 in response to Cd [104].

An additional protein posttranslational modification that has received high attention is S-nitrosylation, which is the NO-mediated oxidation of reduced sulfhydryl to nitrosothiol groups [103]. Examples of protein S-nitrosylation in plants are the transcription coactivator NPR1 (non-expressor of pathogenesis-related 1) [105, 106], the modification of which provokes oligomerization and the subsequent blockage of the nuclear translocation. *Arabidopsis thaliana* metacaspase 9 zymogens are also S-nitrosylated at their active sites, which cause the suppression of the autoprocessing of the enzyme [107].

7.4 Morphological Hallmarks of Nuclear Dismantling During Plant PCD

While the morphological features associated with nuclear dismantling in animal apoptotic cells are homogenous and have been well defined, nuclear dismantling in plant systems occurs with diverse morphologies. This is probably the consequence of the high diversity of tissues undergoing PCD in plants, either as a developmental process or in response to biotic or abiotic stresses. Here, we summarize morphological features of nuclear dismantling in different plant PCD systems.

The formation of reproductive organs such as anthers implies the death of different tissues [108]. Morphological characteristics of nuclear dismantling in these tissues include shrinkage, chromatin condensation, irregular shape, and invaginations of the nuclear envelope [78]. Nuclei at the abscission zone of tomato flowers and leaves show amoeboid shape, chromatin disorganization and condensation, as well as DNA fragmentation [109]. Gymnosperms, as other higher plants, have evolved the monozygotic polyembryony as a reproductive strategy in which multiple embryos develop from a single zygote. After the selection of the dominant embryo, the subordinate embryos are eliminated by PCD. The autolytic self-destruction

program of these embryos includes nuclear changes, such as lobbing and accumulation of clumps of heterochromatin at early stages and complete nuclear lysis after tonoplast rupture [110].

PCD is also an important process of seed development and germination. Nuclei of perisperm, suspensor, and endosperm cells of quinoa seeds undergoing PCD show lobe-shaped morphology, size increase, and chromatin condensation in the lobes until the nucleolus and the nuclear membrane disappear [111, 112]. The nucellus is a maternal tissue, which degenerates during seed development. Characteristic nuclear events of dying nucellar cells are the appearance of vacuolar dilations in the space between the inner and the outer nuclear envelope and heterochromatinization [113]. After germination, the aleurone layer undergoes PCD [114]. As PCD progresses, aleurone nuclei adopt irregular shapes with discontinuities of the nuclear envelope, which is finally completely degraded [73]. Similarly, the scutellum, which is localized at the interphase between the starchy endosperm and the embryo axis, undergoes PCD upon germination [115]. Nuclei from scutellum dying cells show highly condensed chromatin and deep invaginations, the presence of rest of heterochromatin inside provacuoles leaking to the central vacuole, which is a clear symptom of nuclear degradation. In *Vicia faba* seeds, the endosperm is a single multinucleate cell, and these nuclei show vacuolar inclusions in the nucleoplasm and discontinuities in the nuclear envelope during death [116].

Root aerenchyma is produced after the collapse of root cortex cells by PCD. Nuclei of these cells show disruptions of the nuclear envelope (but conserving nuclear pores), chromatin condensation, fragmentation, and apparent engulfment by the vacuole [117]. In maize, nuclei of cortex cells during aerenchyma formation show nuclear membrane shrinkage at the initial stages, and chromatin condensation at the nuclear periphery towards the end of the process [118].

In lace plants, nuclei from epidermal and mesophyll cells suffer a complete transformation. At stage 1 (pre-perforation), nuclei appear large and round, with condensed chromatin distributed throughout the nuclei; at stage 2 (window perforation), nuclei stay intact with unalterable nuclear envelopes, but show increasing patches of condensed chromatin and eventually lobes in the periphery; finally, at stage 3, which is defined by the appearance of a hollow from a side of the leaf to the other, nuclei appear as discrete bodies with electron translucent nucleoplasm and highly condensed chromatin [119].

PCD in the tuber apical bud meristem is one of the mechanisms that regulate potato apical dominance. At early stages of PCD, nuclei appear surrounded by numerous provacuoles and plastolysome-like structures, or plastids that transformed into autophagic vacuoles. Then, nuclei become lobed showing clusters of nuclear pore complexes, and nuclear fragments seem to release from a budding-like segmentation process. Finally, at late stages of PCD, inclusions of condensed chromatin and remnants of nuclear pore complexes can be observed within the provacuoles and/or the central vacuole [120].

Concerning PCD in response to biotic stress, nuclei of tobacco cells undergoing TMV-induced PCD do not show apoptotic characteristics, such as chromatin condensation and marginalization to the nuclear periphery. However, these cells

show symptoms of chromatin reorganization and high-molecular-weight DNA fragmentation [97, 121].

7.5 Biochemical Events of Nuclear Dismantling During Plant PCD

It is speculated that plant PCD may show characteristics of apoptosis or autophagy depending on the tissue and the time of cell death execution [122]. Apoptosis-like PCD seems to be adopted when cells are committed to die rapidly, such as in response to pathogen attack. In contrast, an autophagic-like PCD appears when the decision of cell death is taken more slowly, which is the case of senescence or tracheary element differentiation. Thus, timing appears as a key factor to distinguish between two types of nuclear dismantling in plant PCD: (1) premortem nuclear dismantling, mainly observed in apoptotic-like processes, which implies the ordered, step by step, decomposition of the complex nuclear macromolecular structure in minor units and (2) postmortem nuclear disintegration, of autophagic-like processes where the nucleus remains apparently intact until the final stage of the death process, in which the collapse of the vacuole promotes the complete destruction of the nucleus [16].

7.5.1 Premortem Nuclear Dismantling

In a previous review, we have summarized the sequence of events of nuclear dismantling during plant PCD emphasizing the similarities with those of apoptotic animal cells [16]. These events may be subdivided according to their influence on chromatin structure, DNA integrity, or nuclear envelope permeability. One of the hallmarks of PCD is chromatin condensation, which progresses with a redistribution around the inner side of the nuclear envelope, to form discrete patches of heterochromatin until the collapse of the nucleus. During animal apoptosis, three phases of chromatin condensation are distinguished: stage I or ring condensation, stage II or necklace condensation, and stage III or nuclear collapse/disassembly [123, 124]. Nuclei from plant cells undergoing PCD also show a process of heterochromatinization [73, 113, 115], but the three stages are less clearly defined, with the exception of those which are used as targets in cell-free assays [72, 125]. Nuclear apoptosis-inducing factors from wheat nucellar cells promote chromatin condensation in animal and plant nuclei (Fig. 7.4a), suggesting that degradation of the nucleus is morphologically and biochemically similar in animal and plant cells. Two types of morphological characteristics of chromatin condensation have been observed during petal senescence cell death: (1) fragments of condensed chromatin inside the same nuclear envelope with chromatin fragmentation and (2) a large number of

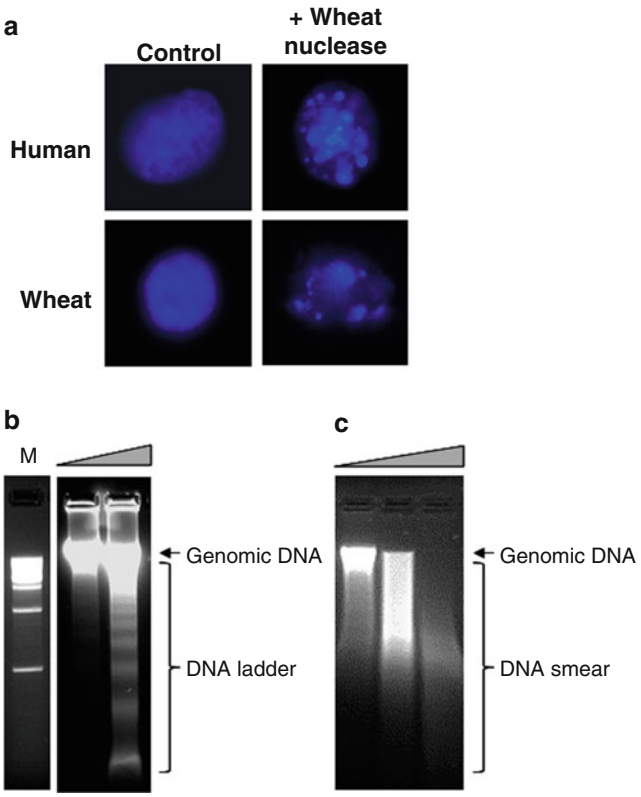

Fig. 7.4 Biochemical events associated with nuclear dismantling during PCD. (**a**) Clumps of condensed chromatin can be observed in human and wheat cell nuclei after incubation with plant apoptosis-inducing factors from wheat grain nucellar cells. (**b-c**) Different levels of PCD-related DNA degradation: low-molecular-weight DNA fragmentation promoted by nuclear-localized nucleases able to act on chromatinic DNA (**b**), while unspecific cleavage of naked DNA may be due to the nonselective action of cytoplasmic nucleases, which need complementary proteases to disrupt chromatin structure (**c**)

DNA spherical bodies, each enveloped by a membrane, which seems to be due to nuclear fragmentation [126, 127].

In parallel to chromatin condensation, DNA fragmentation proceeds in a precise order. The first stage of DNA fragmentation corresponds to the cleavage of chromatin loops producing high-molecular-weight DNA fragments of 50–300 kb. The second stage is the internucleosomal, low-molecular-weight, DNA fragmentation, which provokes the characteristic DNA ladder of multiples of ca. 180–200 bp. The last stage is the cleavage of naked, double- or single-stranded DNA fragments, finishing the DNA degradation process. In apoptotic animal cells, different nucleases have been identified in the three stages [128]. PCD-related nucleolytic activities associated with each stage have also been identified in different plant systems.

Chromatin loop endonucleolytic activities were observed in developmental processes, such as embryogenesis [96] and pathogen-induced HR cell death processes [97, 98]. Endonucleases participating in internucleosomal DNA fragmentation have been detected in different tissues of cereal seeds such as the nucellus [72, 113], the starchy endosperm [129–131], the aleurone layer [73], and the scutellum [115]. Moreover, endonucleases involved in DNA ladder formation were also found when PCD occurs in response to abiotic stress [132]. The final digestion of the DNA requires the action of nucleases able to cleave naked, double- or single-stranded DNA fragments. In animal apoptosis, this type of nucleases is called waste-management nucleases, because their action is carried out in the engulfment-mediated DNA degradation within macrophages or even in the extracellular space where these enzymes degrade DNA released from necrotic cells [128]. In plants, naked DNA cleavage appears in almost any PCD system, including xylem vessel differentiation [133] or senescence [80]. In some plant PCD-related developmental processes, endonucleases promoting high- and low-molecular-weight DNA degradation were localized to the nucleus (Fig. 7.4b), whereas endonucleases involved in naked DNA cleavage seem to be cytoplasm localized (Fig. 7.4c) [72, 73, 115].

Nuclear dismantling events affecting nuclear envelope permeability and disruption are tightly regulated in animal cell apoptosis, where morphological observations are corroborated by biochemical analyses. During apoptosis, the disassembly of the nuclear pore complex may be divided in three stages, which are associated with the corresponding progress of chromatin condensation and DNA degradation (reviewed in [16]). Stage I is defined by the selective proteolysis of specific nucleoporins of the central core complex framework and peripheral nucleoporins comprising the cytoplasmic fibrils of the outer side of the nuclear envelope [134]. This limited proteolysis affects nucleocytoplasmic transport by altering the interaction with other nucleoporins, but essentially the morphology of the nuclear pore complex remains intact until late phases of apoptosis. Stage II is characterized by the cleavage of peripheral nucleoporins and the breakdown of lamins and lamin-associated proteins. In this second stage, detachment of condensed chromatin from the nuclear envelope and clustering of nuclear pore complexes contribute to the anchorage of the nuclear pore complex to the nuclear envelope [135]. Stage III, at the end of apoptosis, is characterized by the loss of nuclear turgor, whereas the nuclear envelope softens, enabling the formation of nuclear fragments [136]. In plants, there is no biochemical knowledge of nuclear envelope disassembly during PCD. Taking advantage of the structural similarities of the plant nuclear pore complex and lamina-like framework with those in yeast and animal cells [34, 41], it has been speculated that plant nucleoporins such as Nup 93, Nup 96, Tpr/NUA, Nup 136, Nup 50, and Nup 214 and proteins from the plant lamina-like structure might be potential substrates for PCD-related proteases [16].

The study of a nuclear dismantling program not related with PCD in animal cells, such as the breakdown of the nuclear envelope during open mitosis, allowed the identification of several processes as key steps of the reorganization of the nuclear membranes, which are caspase independent. The breakdown of the nuclear envelope comprises several steps such as NPC disassembly, nuclear lamina depolymer-

ization, and dissociation of INM proteins [28]. These processes are regulated by mitotic kinases, which upon activation promote the phosphorylation of nuclear envelope proteins, which disrupts their interaction with chromatin. For example, Nup98, a peripheral nucleoporin localized at both sides of the nuclear envelope, which has 13 phosphorylation sites, suffers successive phosphorylation events by multiple kinases, including CDK1 and Neks [137]. The removal of Nup98 after phosphorylation triggers the subsequent wave of disassembly of the central NPC subcomplexes and nuclear envelope permeabilization during mitosis. Spreading of nuclear membranes is also mediated by a mechanism of microtubule-dependent pulling and a process of vesicle formation [138]. Thus, microtubules linked to the cytoplasmic side of the nuclear envelope exert mechanical forces promoting nuclear invaginations and discontinuities [28]. Moreover, apoptotic nuclear disintegration has been associated with hyperphosphorylation of nuclear proteins [139], actin–myosin contraction, and microtubule contractile forces [140, 141]. Morphological studies suggest that events of nuclear envelope disassembly take place also in plant cells undergoing PCD, the most characteristic hallmarks being disruptions and discontinuities of the nuclear envelope [73, 111–113, 117] and clustering of nuclear pore complexes [96, 120].

7.5.2 Postmortem Nuclear Dismantling

Senescence and tracheary element differentiation are among the processes showing postmortem nuclear disintegration in plant PCD systems. Organelles from cells of senescent leaves are degraded in a precise order, chloroplasts being the first organelles to be degraded, while mitochondria and nuclei appear unaltered until the end of the process [142]. Senescence is characterized by the so-called senescence-associated genes (SAGs), a large set of genes encoding hydrolytic enzymes and other factors, which participate in the regulation of this catabolic phase [143, 144]. At the end of senescence, vacuolar collapse promotes the release of hydrolytic enzymes and the consequent degeneration of the nucleus. In a similar way, tracheary element differentiation culminates in vacuolar disruption, which provokes postmortem DNA degradation, the nucleus remaining as the last organelle to be removed [145]. After vacuolar collapse, nucleus degradation is a rapid process culminated within 10–20 min [146]. At the morphological level, tracheary element PCD can be divided into three stages: vacuolar disruption, autolysis, and clearance of the cell corpse. Before tonoplast rupture, no evidence of nuclear degradation is observed; the cytoplasm appears intact but showing dilated ER membranes and vesicle-like structures. During early stages of autolysis, organelles remain intact and vacuolar contents begin to enter in contact with the cellular material. At late stages, cell degradation has progressed sufficiently so that the cell lumen appears as a granular mass with no detectable subcellular structures and the cell lumen is completely cleared [71].

At the molecular level, several proteases and nucleases have been identified as potential enzymes that participate in nuclear destruction. Among them, xylem cys-

teine peptidases 1 and 2 [147, 148], VPEs and metacaspase 9 [149], *Zinnia* endonuclease 1 [133], bifunctional nuclease 1 [150, 151], and LX ribonuclease [79] have been identified in xylem differentiation and may be responsible of nucleus dismantling of TE cells. Unlike TE formation, xylary fibers, the secondary xylem cells, undergo a progressive DNA degradation, nuclei being dismantled prior to cell death [149]. Chromatin condensation and DNA laddering, which are well-established hallmarks of nuclear dismantling, not always are detected in tissues in which the vacuolar collapse determines the rapid DNA degradation and cell death. This is the case of differentiating xylem cells [152] or tissues in which PCD is mainly asynchronous, such as senescing petals [153].

7.6 Identifying Critical Targets of Nuclear Dismantling in Plant PCD

Despite the relevance of PCD in plants, the in-depth knowledge of the process of cell death has progressed mainly in animal cells. Nevertheless, PCD in different plant cell types has been well characterized and is providing valuable information at the morphological and biochemical level [11].

Chromatin has been identified as a critical target of nuclear dismantling both in apoptotic animal cells and plant PCD [16]. Chromatin condensation in apoptotic animal cells is carried out by at least two types of factors, ring condensation factors and ATP-dependent condensation factors [124]. Acinus, a caspase 3-activated protein, is also involved in chromatin condensation [154]. In contrast, the molecular players promoting chromatin condensation in plant cells are still unknown. As in animal cells, DNA with different levels of compaction may also act as target for different endonucleolytic activities in plants, most of them involved in PCD-related processes (reviewed in [16, 155]), but their molecular characterization is very limited.

The increasing knowledge of the proteases involved in plant PCD is not correlated with the scarce knowledge of their substrates [46]. Vacuolar processing enzyme [156], phytaspase [157], saspase [158], and metacaspases [45] have been identified as important enzymes promoting cell death, but no true proteolytic cascade nor initiator or effector proteases have been identified. However, the characterization of the *Arabidopsis thaliana* metacaspase 9 degradome is a promising step forward for the identification of natural substrates involved in PCD [54]. Similar approaches for the characterization of new PCD-related protease degradomes will allow the identification of key targets in plant nuclear dismantling. As in studies concerning animal cell death, a major objective in plant systems is to decipher the hierarchical cascade of proteolytic events involved in nuclear envelope disassembly during PCD. INM-, NPC-, and lamin-like proteins are potential markers to study the timing of dismantling. Recently, novel SUN-KASH bridges were identified in plant systems, forming linkers of nucleoplasm to cytoplasm involved in nuclear shape determination [40]. Some KASH–SUN combinations connect microtubules, actin

filaments, or intermediate filaments to the outer side of the nucleus and were proposed to participate in cell cycle control, nuclear import, and apoptosis, in such a way that defects in SUN and KASH proteins have been associated with human diseases [29, 30]. Therefore, SUN-KASH bridges may be putative targets of nuclear dismantling by proteolysis.

The availability of plant mutants with impaired components of the nuclear lamin-like structure [36, 159], NPC basket [41, 160], or SUN–KASH bridges [39] may improve our knowledge of the involvement of these structures in nucleus dismantling, since the disruption or overexpression of these components provokes nuclear morphology alterations. Apart from identifying nuclear targets for proteolytic enzymes, new findings will be required for understanding the role of posttranslational modifications in nuclear dismantling. Hyperphosphorylation or S-nitrosylation of nuclear regulatory or structural proteins may be key events for nuclear dismantling to be deciphered in the future.

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Chapter 8
Programmed Cell Death Regulation by Plant Proteases with Caspase-Like Activity

Yao-min Cai and Patrick Gallois

8.1 Introduction

In plants the molecular mechanisms involved in the execution of programmed cell death (PCD) are important from a basic cell biology perspective and from an applied perspective. Many processes linked with crop yield stability and crop storage have a PCD component, and manipulating PCD using specific genetic backgrounds or chemicals may open the door to crop improvements. Although we know many regulators of plant PCD and have begun to connect components of the core mechanism [1, 2], we still only have a patchy picture of the network(s) activated. Because the proteases called caspases are central components of the apoptotic pathway in animal cells, similar enzymatic activities detected in plant extracts have attracted a lot of attention for more than a decade. The term caspase-like activity is used in the plant literature because there is no caspase orthologue in plant genomes and the plant proteases closest to animal caspases, the metacaspases, are unable to cleave synthetic caspase substrates. The plant caspase activities therefore originate from proteases clearly distinct from animal caspases. In addition, referring to a specific caspase-like activity, e.g. caspase-3-like, is convenient but can be misleading in the absence of true plant caspases; caspase-like activities are therefore often referred to by the amino acid sequence, in single letter code, of the synthetic substrate used. For example, an enzymatic activity against the caspase-1 substrate Tyr(Y)-Val(V)-Ala(A)-Asp(D) might be referred to as a YVADase activity. Using various experimental systems, several research groups were able to identify plant proteases that possessed caspase-like enzymatic activities and regulated PCD. This chapter focuses on these proteases because caspase-like activities are commonly used in published plant PCD studies as markers of PCD activation.

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8.2 Background on Caspase-Like Activities in Plants

Caspases is short for *c*ysteine-dependent *a*spartate *s*pecific *p*rote*ases*. As the name suggests, animal caspases cleave their substrates after an aspartate residue (in the P1 position of the cleavage site; the P1 amino acid (aa) becomes the C-terminal aa after cleavage). This led to the development of synthetic tetrapeptides conjugated with fluorogenic reporters to measure caspase enzymatic activities. The preferred synthetic substrate has often ended up symbolising a specific caspase even though they are not 'caspase specific', and various caspases have overlapping specificities [3]. For example, YVAD is used to measure caspase-1 activity in animal cells because it is the four-amino acid pattern preferentially cleaved by caspase-1. From this, YVADase activity has tended to become synonymous to caspase-1 in the plant literature although it can be cleaved in vitro by other animal caspases. In addition, it should be noted that YVAD is cleaved by proteases unrelated to caspases, such as legumains [4, 5].

Sequencing the *Arabidopsis* genome in 2000 and in the following years sequencing the genomes of several plant species revealed the absence of orthologous caspase sequences in plants [6]. Plant metacaspases, being distantly related to animal caspases [6], were for a short time prime candidates for being the proteases behind the plant caspase-like activities until it was clearly established using recombinant forms that these proteases did not have caspase-like activity (reviewed in [7]). Despite being unable to cleave caspase synthetic substrates, metacaspases have been found to participate in the control of PCD in plants, among other functions (reviewed in [7]). Whether metacaspases are functionally equivalent to caspases and whether a common origin with caspase explains a metacaspase control of PCD is still open to debate. Some authors argue that metacaspases act as caspases in plants [8], while others argue that there is no justification to link metacaspase and caspase beyond the commonality in names [9].

Nevertheless it is a fact that caspase-like activities have been detected in plant extracts and in vivo using mostly synthetic tetrapeptide substrates designed as the preferred cleavage site consensus of members of the mammalian caspase family. These activities have been detected multiple times in various plant species and in various tissues or cell types (reviewed in [10]). Among them, YVADase and DEVDase have been the most studied, and both activities have been detected in the majority of PCD situations, illustrating their ubiquity. Additional activities such as VEIDase, IETDase, VKMDase, LEHDase and LEVDase have been reported as the commercial availability of these substrates increased (reviewed in [10]); of course some of these substrates may be cleaved by more than one protease in a given extract and vice versa.

In itself, the detection of caspase-like activities that correlates with PCD induction would not demonstrate a regulatory role in the process. Proof however that some of these caspase-like activities are required for completion of PCD has been obtained using caspase inhibitors. The pan-caspase inhibitor p35, a baculovirus protein, was shown to be effective at blocking cell death in several experimental

systems: *Agrobacterium tumefaciens*-induced PCD using embryonic callus in maize [11], *Alternaria alternata* f. sp. *lycopersici* AAL toxin-induced cell death [12], hypersensitive-response (HR) cell death in tobacco plants infected with *Pseudomonas syringae* pv. *phaseolicola* or tobacco mosaic virus (TMV) [13], UV-induced PCD in *Arabidopsis* [14] and kiss-of-death (KOD)-induced PCD [15]. In addition, synthetic inhibitors designed to block caspase activities have blocked or reduced plant PCD (reviewed in [10]). Generally these inhibitors are remarkably efficient at suppressing plant PCD, except in a few experimental systems where specific inhibitors have been reported to have no effect. For example, acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) had no effect on the formation of necrotic lesions in tobacco induced by TMV, while AC-YVAD-CHO had an effect [16, 17]. On the contrary, AC-YVAD-CHO did not suppress PCD of pollen tubes in *Papaver*, while AC-DEVD-CHO did [18]. Ac-YVAD-CHO did not reduce PCD in the *Zinnia* xylem differentiation system [19]. And finally, it has been reported that HR induced by *P. syringae* pv. *maculicola* in tobacco was not blocked by either Ac-DEVD-CHO or Ac-YVAD-CHO [20], suggesting that different pathovars may trigger different pathways.

Overall, it can be said that proteases with caspase-like activities are involved in most PCD responses, suggesting that these proteases are part of the core mechanism of plant PCD. Because of overlapping specificities for the synthetic substrates used, it is difficult to interpret caspase-like activity profiles or inhibition profiles in whole plant extracts. Identifying the proteases responsible behind the activity detected is therefore an important step that opens new experimental avenues that are more direct than relying on inhibitors. To date, there are three proteases that have been shown to cleave caspase synthetic substrates and to be involved in PCD (see Table 8.1).

8.3 The Protease Vacuolar Processing Enzyme Has YVADase Activity

The plant vacuolar processing enzyme (VPE) is a legumain and as such a member of the CD clan of cysteine proteases which also includes caspases, gingipains and clostripains [21]. In early papers, VPE was described as enzymatically active against the synthetic substrates ESEN and AAN and as being involved in various protein maturation processes in seeds or leaves [22]. In addition to this, virus-induced gene silencing (VIGS) of VPE in *Nicotiana benthamiana* linked VPE enzymatic activity, PCD, and the cleavage of the caspase-1 substrate YVAD. VPE downregulation in VIGS lines suppressed the vacuole collapse that leads to TMV-induced hypersensitive cell death. Activity labelling combined with a pull-down approach confirmed that VPE in plant extracts had affinity for a caspase-1 inhibitor Ac-YVAD-CHO [16]. *Arabidopsis* has four VPE genes, α-VPE, β-VPE, γ-VPE, and δ-VPE, and a quadruple-KO mutant had no VPE or YVADase activity detected, suggesting that

Table 8.1 Plant proteases with caspase-like activities and PCD

	Vacuolar processing enzyme (VPE)	Proteasome subunit β1 (PBA1)	Phytaspase
Species studied	*Arabidopsis*, soybean, *N. benthamiana*	*Arabidopsis*, poplar	*N. tabacum*, rice
Preferred synthetic caspase substrate	YVAD, caspase-1-like	DEVD, caspase-3-like	VEID, caspase-6-like
Other synthetic substrates cleaved	ESEN, AAN	APnLD, GPLD	YVAD, IETD, LEHD
Known protein substrates	Storage protein, e.g. phaseolin (not PCD)	Ubiquitinated proteins	ViRD2
Optimum pH	pH 5	pH 7	pH 5.5
PCD regulation	Controls tonoplast rupture during PCD. Positively regulate ER stress-induced PCD	Mediates PCD during HR. Activity detected during ER stress-induced PCD	Mediates HR and ROS-induced PCD. Not reported in ER stress-induced PCD
Subcellular localisation	Vacuole	Cytoplasm and nucleus	Cell wall, then internalised
Catalytic type	Cysteine	Threonine	Serine
Key references	Hatsugai et al. [16], Qiang et al. [27], Mendes et al. [34]	Hatsugai et al. [42], Han et al. [44], Gu et al. [43] Kisselev et al. [57].	Chichkova et al. [38]

there was no other protease with a YVADase activity in the experimental conditions used [23].

The YVADase activity of VPE was formally confirmed in enzymatic assays using a recombinant γ-VPE produced in insect cells. Recombinant γ-VPE was shown to be active against both ESEN (VPE substrate) and YVAD (caspase-1 substrate) and inactive against DEVD (caspase-3 substrate) [23]. Other legumains, such as pig legumains, are also able to cleave the caspase-1 substrate Ac-YVAD-AMC with a pH optimum at pH 5 [5], so an activity of VPEs against YVAD is not plant specific. The Km of recombinant γ-VPE for Ac-YVAD-AMC was found to be 40 μM and was comparable to the Km for Ac-ESEN-AMC, 30 μM [23]. Because the Km of purified human caspase-1 for Ac-YVAD-AMC is 14 μM [24] and VPE belongs to the same protease clan as caspases, it is often considered that VPE plays a caspase analogue role in plant cells. However, at this stage, it cannot be excluded that the VPE activity against YVAD has no relevance in vivo, and this cannot be settled until the identification of an in vivo VPE protein substrate with a YVAD cleavage site. A VPE was purified from *Papaver rhoeas* pollen, and a detailed characterisation of the recombinant PrVPE1 showed that it had YVADase activity. Unlike in *Arabidopsis*, however, the protease exhibited DEVDase and IETDase

activities in addition to YVADase [25]. This illustrates the difficulty in interpreting substrate and inhibitor data in plant extracts.

The enzymatic activity of VPE has been clearly linked to the regulation of PCD in biotic stress, abiotic stress and development. VPE was first shown to be required for TMV-induced PCD, consistent with the inhibition of this process by the inhibitor Ac-YVAD-CHO [16]. Subsequent experiments with single and multiple *Arabidopsis* KO lines have shown VPE to be required for PCD induced by the mycotoxin fumonisin [23] or an incompatible *P. syringae* (*avrRpm1*) [26]. VPE was also shown to be a positive regulator of ER stress-induced PCD, during the beneficial colonisation of *Arabidopsis* roots by *Piriformospora indica* [27]. These mutualistic fungi induce a localised ER stress and PCD in order to successfully colonise *Arabidopsis* roots. A hallmark of PCD during the colonisation of *P. indica* was vacuole collapse, and a VPE-null mutant line displayed evidence of reduced vacuolar membrane collapse, in keeping with VPE suspected mode of action during PCD [27]. Concerning abiotic stresses, VPE overexpression lines and expression data suggested that VPE is involved in promoting radish floral bud abortion under heat stress [28]. VPE is also possibly involved in aluminium-induced PCD in cells of the *N. tabacum* BY-2 line, as 150 μM aluminium causes increased VPE expression, PCD, and a vacuolar collapse that can be blocked by applying the inhibitor Ac-YVAD-CHO [29]. Finally, a ∂VPE KO line highlighted a role for VPE in a form of developmental PCD specifically in two cell layers that are part of the seed integuments in *Arabidopsis* [30]. It is likely that VPE is required only for PCD that uses the vacuolar pathway, and the PCD induced by pollen self-incompatibility in *Papaver* could be an example of a non-vacuolar pathway. In this system, Ac-YVAD-CHO inhibitor treatments were ineffective at blocking PCD, suggesting that the enzymatic activity attributed to VPE was not required [25].

Mechanistically, little is known on the pathway that requires VPE or on the VPE mode of action towards vacuolar collapse. It has been suggested that VPE may activate a protein required for vacuole collapse [31, 32]. The significance of increased VPE expression during PCD is not clear either as background expression can be present in non-induced controls. There has been however some insights gained recently on the control of VPE expression during PCD. AtMPK6 is required for an increase in AtγVPE expression that correlates with heat shock-induced PCD in *Arabidopsis* leaves [33]. In addition, during the activation of the ER-PCD pathway in soybean cells using a tunicamycin treatment, two NAC transcription factors, GmNAC30 and GmNAC81, are induced by ER stress and directly activate the expression at least one VPE gene via a GmNAC81/GmNAC30 binding site (TGTGTT) in the VPE promoter sequence [34]. Finally, a novel role for VPE has been reported, where VPE mediates elicitor-induced stomatal closure by regulating NO accumulation in guard cells [35]. NO has been reported as a stress response and a PCD regulator in several experimental systems [36], but whether this role relates to the VPE PCD pathway is unknown.

The development of an activity labelling probe highly specific for all four *Arabidopsis* VPEs that can be used for in vivo imaging is an important advance that should allow to define more precisely the contribution of VPE to PCD and non-PCD

processes in various tissues [37]. For example, using this probe and vpe mutant plants, Misas-Villamil et al. [37] identified a role for VPE during compatible interactions that is possibly independent of PCD.

8.4 Phytaspase, a Serine Protease with Caspase-Like Activity, Promotes PCD

Phytaspase is a plant protease of the subtilisin-like family which was first identified when purifying a caspase-like activity in tobacco that produced a single cleavage in *A. tumefaciens* VirD2 protein at a TATD motif [17]. Phytaspase was then isolated by affinity chromatography from tobacco plant extracts and mass spectrometry analysis. Mutation analysis of the recombinant enzyme produced in tobacco showed serine dependence, confirming the designation of phytaspase as a subtilisin-like protease. The substrate specificity studies using a set of synthetic substrates suggested that the inhibitor Ac-VEID-CHO and VEID fluorogenic peptide were the preferred tetrapeptide sequences among those tested. YVAD, IETD and LEHD were also cleaved, while DEVD was not [38]. This cleavage profile illustrates that phytaspase has a distinct activity from other identified plant caspase-like proteases.

The effect of varying levels of phytaspase had on TMV and abiotic stress-mediated PCD confirmed its involvement in PCD. Phytaspase transient overexpression increased the number and size of lesions on TMV infected leaves, while in silenced transgenic lines, there were less severe symptoms compared to wild type [38]. The same PCD phenotypes were observed with PCD caused by abiotic stress in the form of sodium chloride stress or oxidative stress induced by the herbicide methyl-viologen. Phytaspase-overexpressing leaf discs required lower concentrations of either chemical to bleach, whereas leaf discs with phytaspase downregulation needed higher concentrations to bleach compared to wild-type plants [38].

An interesting development in the understanding of PCD regulation is that the mature phytaspase localised in the apoplast [38]. Western blot analysis, confocal microscopy and the application of the protein secretion inhibitor brefeldin demonstrated that the proenzyme underwent maturation before secretion into the apoplast. When PCD was triggered by methyl-viologen or TMV, a redistribution of phytaspase was observed with the protease being then detected inside the cell. Cycloheximide treatments inhibited de novo protein synthesis during PCD, but not phytaspase internalisation, suggesting a retrograde transport of the protease from the apoplast but not the cessation of phytaspase secretion [38]. Further research is needed to identify the exact point in PCD at which phytaspase is involved. So far, it appears that phytaspase is upstream of ROS production and cytochrome *c* release from mitochondria [38]. We can expect that the identification of the *Arabidopsis* orthologue will be crucial for understanding further the phytaspase pathway.

Phytaspase is possibly related to saspase, another subtilisin-like protease with caspase-like activity that is involved indirectly in victorin-induced cleavage of RuBisCo during PCD in oats (*Avena sativa*) [39]. Saspase, as phytaspase, appears to function in the extracellular space. Because the full sequence of saspase is not known, the extent of its phylogenetic relationship with phytaspase is not determined. Both phytaspase and saspase are not the only examples of serine proteases with caspase-like cleavage activity. In animal cells, the serine protease granzyme B cleaves caspase substrates [40]. Subtilisin-like proteases exist as large multigene families in plants [41], and in future studies members other than phytaspase may be identified with caspase-like activities.

8.5 Proteasome Can Both Promote and Prevent PCD, and Its PBA1 Subunit Has DEVDase Activity

Hatsugai et al. [42] discovered that adding proteasome inhibitors, β-lactone and APnLD-CHO, to *Arabidopsis* leaf extract strongly reduced caspase-3-like activity (DEVD). In addition, protein pull-down using the substrate analogue inhibitor biotin-DEVD-FMK identified the 20S proteasome β subunit 1 (PBA1) as having affinity for DEVD in line with what is known for this subunit in other organisms [42]. This observation was confirmed in a separate experiment where DEVD inhibitors competed the labelling of the PBA1 subunit by a PBA1 activity probe [43]. Further to this, Han et al. [44] purified a caspase-3-like activity from poplar xylem tissues, and MS analysis identified many proteasome subunit homologs in the purified fraction [44]. The caspase-3-like activity detected in plant extract is therefore at least in part due to proteasome activity.

The PBA1 subunit of the 20S proteasome is required for PCD induced as part of plant cell immunity and more specifically required for tonoplast fusion with the plasma membrane which constitutes an early step of the process [42]. These results may tie up with the observation that an inducible beta1-subunit of the proteasome acted as a positive regulator of ROS generation during HR induced by the elicitor cryptogein [45]. However, contrasting roles for the proteasome, either activating or suppressing PCD, have been reported in yeast. In this organism, the proteasome has a PCD promoting or PCD preventing role depending of the PCD scenario [46]. This appears to be the case in plants, as the proteasome was also described as a PCD suppressor in tobacco leaves because silencing the 20S proteasome α6 subunit *NbPAF* and the 19S regulatory subunit *NbRpn9* both promoted PCD [47]. So it could be concluded that the role of the proteasome in PCD is possibly more complex than its suggested caspase-3-like activity and the detection of caspase-3-like activity in plants encompasses a more complex situation than what is currently thought.

8.6 Subcellular Localisation of Caspase-Like Activities

Based on what we have described above, known proteases with caspase-like activity can have three distinct subcellular localisations: vacuolar, cytoplasmic and nuclear and extracellular. VPE is localised in lytic vacuoles of *Arabidopsis* leaves [48] and thought to be required for the process of vacuole rupture [16] that is required for the completion of vacuolar PCD [49]. The rupture of the tonoplast is rationalised as a step to release the hydrolases stored inside the vacuole, hydrolases, which include proteases that can both destroy the cell from within and destroy invading pathogens. In support of this mechanism in PCD, several cysteine proteases involved in plant PCDs have been showed to be present in vacuoles among them papain-like cysteine proteases [50].

Because of the importance of the vacuole in a number of plant PCD situations, one could have expected to find additional plant caspase-like proteases/activity residing inside the vacuole. Instead, caspase-3-like activity has been shown to match the subcellular location of the proteasome in the cytosol and the nucleus. This would fit the data obtained using micro-injection of a fluorogenic caspase-3 substrate (Ac-DEVD-AMC) in *Chara* cells, which detected a caspase-3-like activity in the cytosol and only weakly in the vacuole [51]. In another study using staurosporine-induced PCD and tobacco cell suspension, a FITC-VAD-fmk inhibitor localised in cytosol during the early stages of PCD and at a later stage primarily to the nucleus [52]. These observations have been confirmed with more details using *Papaver* pollen where self-incompatibility activates PCD to stop pollination. In this system, a fluorogenic substrate showed a DEVD activity to be both cytosolic and nucleic [53], compatible with the proteasome subcellular localisation.

The novel and unexpected localisation of phytaspase in the cell wall had not been reported by previous cell biology studies, possibly because in vivo caspase-6 substrates were not used at the time. Nevertheless, the presence of phytaspase in the cell wall implies that a role for this compartment during abiotic stress-induced PCD has been overlooked, a suggestion consolidated by, for example, the presence of the PCD-inducing peptide Grim Reaper in cell walls [54].

It remains unknown whether the proteases with caspase-like activities, of various phylogenetic origins and in various subcellular locations, feed into a unified pathway. But, because of their spatial separation, these proteases are unlikely to activate each other as described in the canonical caspase pathway of apoptosis, reinforcing the idea that the caspase pathway may not be the most suitable template to guide plant cell death studies. Other PCD proteases may be involved in inter-protease activation, or in conjunction with the proteases reviewed here, for example, this has been suggested for the papain-type cysteine endopeptidases with a KDEL signal peptide [55].

8.7 Conclusion

The inhibition of plant PCD using caspase inhibitors has resulted in significant progress in our understanding of the process. Protease activities, that cleave caspase substrates and are inhibited by caspase inhibitors, are central to many PCD processes in various experimental systems. The substrates used are however not caspase specific, and it is therefore not surprising that the corresponding plant proteases identified are not related to caspases. In the current state of our knowledge, we should therefore refrain from equating inhibition of plant proteases by caspase inhibitors as evidence of caspase analogue role in plant cells. What is certain however is that these proteases are required for the completion of plant PCD. Interestingly there is evidence that metacaspases, that have no caspase activity, may cleave proteins for which the animal orthologues are caspase substrates albeit using a different cleavage specificity [56]. However, this kind of data for a cleavage convergence is at present totally missing for proteases with caspase-like activity. At this stage there is no evidence that plant proteases capable of caspase-like activity have taken over or retained the precise function of a given animal caspase.

In the light of the different phylogenetic origins of the plant proteases identified with caspase-like activity, there are several possible hypothesis. One is that behind the divergence of plant and animal PCD lays a common ancestral mechanism of cell self-destruction that comprises cytochrome *c* release, protein cleavage of many targets and DNA fragmentation. A second hypothesis could be that there is a limited set of key proteins to disable in order to obtain an efficient disassembly of cells during PCD. In which case, plant and animal evolution may have homed in on the same targets recruiting unrelated proteases. This would imply the conservation of ubiquitous substrate cleavage sites that would be revealed by caspase inhibitors. Finally, it can be hypothesised that the proteolytic processes required for plant PCD may be totally unrelated to animal caspase-dependant PCD, the only point of convergence being the importance of proteases to cell dismantling. In summary, whatever hypothesis, or combination of hypotheses is correct, synthetic caspase inhibitors have been helpful as protease inhibitors to reveal some of the key proteases required for plant PCD. There is now a great need to use better tools such as KO lines and activity labelling probes to characterise plant PCD and to identify proteins that are in vivo substrates of these proteases.

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Chapter 9
Senescence-Associated Programmed Cell Death

Hilary J. Rogers

9.1 Introduction

Plant senescence is defined here as the process by which individual plant organs, or, in the case of annual plants, the whole individual, are programmed to die. Although death of the organ and its constituent cells is the final outcome of senescence, a key feature of senescence is the remobilisation of nutrients between organs that are no longer needed and other parts of the plant where nutrients can be used for growth or storage [1–3]. For example, the remobilisation of nutrients from leaves to the developing fruit in annual plants is important for optimal seed and fruit development. Leaf senescence is therefore an integral part of the ecological strategy of annual plants.

Programmed cell death (PCD) in the context of senescence is understood here to be distinct from developmental PCD, despite conservation of some cytological features. The latter generally relates to the removal of a small number of cells in an organ which is otherwise retained, such as the tapetum [4], nucellus [5], the suspensor [6], or the formation of holes in leaves of species such as *Monstera* or the lace plant *Aponogeton madagascariensis* [7]. These processes have, by some authors, been regarded as a form of programmed senescence [8]. This was based on the premise that there is no clear difference in the mechanisms of PCD occurring in isolated cells during early development and the larger-scale PCD occurring during whole organ senescence, and hence the terms are interchangeable. Conversely, it has been argued that the term PCD is appropriate both to the death of groups of cells during development and the PCD occurring at the end of senescence. In this chapter, I will consider PCD in the context of the regulation of whole organ senescence, focussing mainly on common mechanisms and signals but also assessing senescence

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and PCD in different organs to consider the commonality of mechanisms and signals. The regulation of senescence has been extensively reviewed (e.g. [1, 3, 9]) so only a brief overview will be presented here, trying to identify regulatory points relating to the later, PCD-related events and setting the context for senescence-associated PCD within the cell.

At the level of whole organ senescence, one of the key issues that have been widely discussed in this field is the distinction between the process of senescence and the process of cell death [3, 8, 10]. Are they a continuum, or are they distinct processes, regulated separately? Are they in fact opposing forces: senescence keeping the tissue alive until remobilisation is complete [11]? Clearly, remobilisation needs to be tightly coordinated to ensure that the structures required for nutrient mobilisation and transport are still functional while cell contents are broken down and cells enter PCD. A further difficulty in understanding senescence is that there obviously needs to be heterogeneity across organs at a cellular level to enable remobilisation to take place. For example, in apparently healthy petals in flowers at full bloom, electron microscopy reveals that many of the internal cells have already died [12]. This complicates studies, for example, of gene expression during senescence.

The timing of the remobilisation and hence PCD in the context of other developmental processes in the plant is also critical. Furthermore, species differ in the coordination of this timing. For example, in wheat where leaf senescence was disrupted by expression of the *ipt* gene driven by a senescence-induced promoter, leaves stayed green for longer, but seed yield was not increased. In contrast, transgenic tobacco, expressing the same transgene, produced a better yield of seed due to the extension of the photosynthetic period in the leaves [13]. Post-fertilisation floral senescence is also tightly regulated to remobilise nutrients towards developing seeds [14]. Hence, coordination of the initiation of senescence and ultimate PCD in different organs at the level of the whole plant is very important.

Another key issue is whether there is a senescence-associated PCD mechanism that is distinct from other forms of plant PCD and common to all senescent organs. The mechanisms of plant PCD have been studied in diverse systems such as xylogenesis, self-incompatible pollen responses, stress-induced PCD in cell cultures and the hypersensitive response [15] as well as developmental senescence. Are mechanisms of PCD conserved between senescence-associated PCD and PCD in these other systems or is PCD-associated senescence distinct? Furthermore, organ senescence is often classified as developmentally or stress induced. Numerous stresses cause premature leaf senescence thus removing leaves which are diseased or compromised [16]. So another question is whether the primary signal for senescence affects the PCD mechanism. Abscission is also a common feature in organ senescence [14, 17]. However, abscission of both floral organs and leaves can occur before the death of all the cells. So we can ask whether the PCD occurring in organs that are no longer attached to the plant is regulated in the same way as attached-organ senescence. An extreme example is that of postharvest biology. In a commercial setting, leaves and flowers are detached from the plant and kept in the cold and dark, often in a dehydrated state. Does this trigger the same type of PCD as developmental or attached-organ stress-induced senescence or even post-abscission

senescence? In this review, I will try to address some of these key questions and identify areas for future study. Our ability to understand senescence and senescence-associated PCD and control them would benefit both agricultural production and limit postharvest waste. Many key seed crops are monocarpic; hence, seed production is linked to resource remobilisation from the leaves. Delaying senescence through functional stay-green varieties can result in increased yield [18]. Postharvest fruit and vegetables are perishable items and require careful handling throughout the supply chain to avoid damage leading to rapid senescence-like processes. It is estimated that in India, for example, up to 30 % of fresh fruit and vegetables are lost through lack of an efficient cold chain [19]. It is therefore important that we identify the gaps in our knowledge of both pre- and postharvest senescence-associated PCD and the tools that can help us to fill them.

9.2 Induction of Senescence and Senescence-Associated PCD

If we accept that senescence-associated PCD occurs as the final stage of senescence and as a direct result of the induction of senescence, then the first question we can ask is whether there are common signals that induce the initiation and progression of senescence within or between different organs.

9.2.1 Signals and Mechanisms: How Much Conservation Is There?

Organ senescence occurs as part of the natural developmental programme of a plant. In some organs such as floral organs and seed pods, the lifespan of the organ is species specific [20, 21]. A distinctive feature of pod senescence is the very close coordination between seed development and pod/silique senescence, and floral senescence is often closely linked to pollination. This means that senescence in these organs is not influenced to a great extent by environmental stresses. Other organs such as leaves are responsive to abiotic and biotic stress signals that can initiate senescence in a single leaf or the whole plant [17]. An interesting question is therefore whether different signals can independently activate the same mechanism of senescence and PCD. At a gene expression level, this has been recently investigated with an ambitious exercise in which microarrays from experiments using 28 different methods for inducing senescence in *Arabidopsis* organs were compared [22]. Array data were taken from experiments on leaves, young seedlings, siliques, petioles and cell suspensions and included induction of senescence by plant growth regulators (ethylene, abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and brassinolide), high glucose/low nitrogen, a range of abiotic stresses (dark, cold, genotoxic, heat, osmotic, oxidative, wounding, salt, UV-B, ozone and heavy metal

stress), three pathogens (*Phytophthora infestans*, *Pseudomonas syringae* and *Botrytis cinerea*) as well as developmental senescence in leaves and siliques. An interesting finding was that although all the treatments induced symptoms of senescence, most of the treatments only shared less than 10 % of their up-regulated genes with developmentally induced senescence. Four treatments were identified as sharing most changes in gene expression (between one half and one third) with developmentally induced senescence: age-induced PCD in cell cultures, infection with *Botrytis cinerea* (48 h), high glucose/low nitrogen and dark stress (5 days). One feature these four induction methods share is that they were all imposed for longer periods of time. This suggests that the shared gene expression may relate to later events in senescence, perhaps associated to PCD rather than early induction of the senescence process. This was supported when time points within these treatments were analysed: more gene expression was shared with developmentally induced senescence at later time points. This suggests that though signals that initiate senescence differ between induction systems, the "executioner" genes are shared. This supports a model in which senescence-associated PCD mechanisms are common at least within similar organs.

9.2.2 Are Specific PGRs Associated with Senescence-Associated PCD?

Aside from the method of inducing senescence, another aspect of senescence control and its associated PCD are the internal triggers that initiate or regulate it. Plant growth regulators are clearly key coordinators of some types of developmental plant PCD. For example, PCD in cereal aleurone cells is induced by gibberellins and delayed by ABA [23]. However, the regulation of senescence-associated PCD by PGRs is much less clear-cut. Senescence seems to be regulated negatively by cytokinins and positively by the main stress hormones: abscisic acid (ABA), salicylic acid, (SA), jasmonic acid (JA) and ethylene (Fig. 9.1). Gibberellins and auxin also contribute to retarding senescence, and brassinosteroids may accelerate it although mechanisms are less clear [24]. An important question is whether growth regulators regulate only senescence initiation or also onset of PCD.

During senescence, cytokinin levels fall, and restoration of cytokinin levels by exogenous application delays senescence symptoms such as chlorophyll breakdown. An early study, over 40 years ago, showed that application of cytokinins to detached leaves delayed signs of senescence [26]. This is further supported by delayed senescence resulting from expression of the cytokinin biosynthesis *ipt* gene driven by the senescence-specific *SAG12* promoter in tobacco [13]. In addition, mutants in *ORE12-1* which encodes a cytokinin receptor AHK3 show delayed senescence. Phosphorylation of ARR2 by AHK3 is required for leaf longevity, and the mechanism of its action appears to be related to carbohydrate supply. ARR2 up-regulates extracellular invertase and hexose transporters resulting in increased sink activity [27]. This explains how the role of cytokinin in delaying senescence is

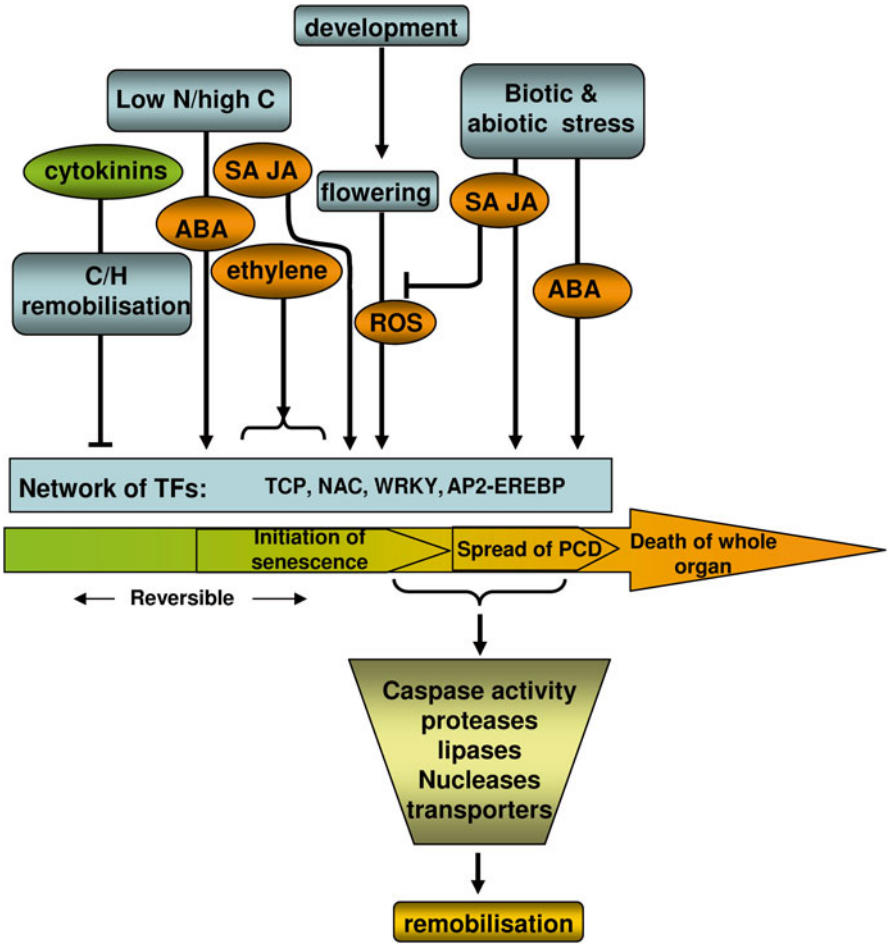

Fig. 9.1 Regulation of leaf senescence by developmental and stress signals, plant growth regulators and transcription factors. Adapted from Schippers et al. [24], Guiboileau et al. [1], and Fischer [9], integrating information on the levels of growth regulators and activation of genes from Breeze et al. [25] to attempt to infer timing of signals in relation to onset and spread of senescence-associated PCD

common to both photosynthetic organs such as leaves and non-photosynthetic organs such as flowers. Chang et al. [28] showed delayed floral senescence in petunia in transgenic plants expressing the SAG12::*ipt* construct. These data suggest that cytokinins are involved in the early stages of senescence in regulating the source/sink balance which tips the organ into the senescence programme.

Conversely, all the stress-related PGRs, ABA, SA, JA and ethylene, have roles as positive regulators of senescence [1]. ABA was shown to mediate leaf senescence induced by low nitrogen/high sugar suggesting a link to metabolic signalling [29, 30]. In several species, ABA rises during floral senescence, often quite late in the

senescence programme (reviewed in [21]), and it may play a particularly important role in the group of species in which ethylene is not a coordinator of senescence. Further analysis of whether ABA affects PCD in floral senescence would be useful.

SA mediates abiotic and biotic stress signals and affects gene expression during leaf senescence [29]. Reduction of SA levels through mutation of the SA biosynthetic gene *sid2* or in transgenic plants through expression of the SA hydroxylase-encoding gene *NahG* resulted in delayed yellowing, reduced necrosis [31] and extended leaf lifespan when flowering was induced [32]. Furthermore, expression of important senescence-associated genes (SAGs) such as *γVPE* [33] and the *WRKY6* [34] and *WRKY53* [35] transcription factors (TFs) was delayed. The effect of SA on SAGs is conserved across leaves, petals and siliques in *Arabidopsis* [21]. However, effects of SA on gene expression during developmental leaf senescence are not shared with dark-induced senescence indicating a divergent role [36]. SA also appears to have a role in autophagy (discussed below) and the timing of its responses suggests perhaps a role in the progression of senescence as well as its initiation [25]. Less is known about the role of SA in floral senescence although it appears to rise in *Lilium* post-anthesis [37]. Its role in floral senescence and PCD merits further investigation.

Methyl jasmonate (derived from JA) was originally identified through its activity in promoting senescence [38]. JA and methyl jasmonate mainly promote leaf senescence through their inhibition of photosynthesis [39]. One of the two key enzymes that degrade chlorophyll, CHLOROPHYLLASE1, is strongly up-regulated by JA [40]. At the same time, JA down-regulates Rubisco activase which catalyses the light activation of Rubisco and is required for photosynthesis [41]. However, JA also induces jasmonate-induced proteins (JIPs), some of which inhibit protein synthesis. Effects of JA and SA on leaf senescence are linked by their modulation of WRKY53 action. Early in senescence, *WRKY53* is induced by SA, while later as JA levels rise, *WRKY53* expression is down-regulated (Hiderhofer and Zentgraf [42]). Hence, the effects of JA appear to influence both early and later events during senescence.

Ethylene also regulates leaf senescence, and mutants in ethylene perception such as *etr-1* and *ein2/ore3* show delayed senescence [43]. However, sensitivity to ethylene is age dependent in leaves and thus may regulate the stage of commitment to the senescence programme rather than downstream PCD (Fig. 9.1). Ethylene is also a major regulator of floral senescence in some species (reviewed in [14, 20, 44]), again initiating and coordinating the senescence programme.

To what extent any or all of these PGRs regulate senescence-associated PCD is not clear. Based on changes in sensitivity to ethylene, Schippers et al. [24] suggested a model for leaf senescence by which there is a window of sensitivity to all the PGRs. Cytokinin and ethylene act early at a stage when the organ is not fully committed to senescence, while JA, ABA and SA act later in a window that is no longer sensitive to ethylene and in which senescence is now initiated [45, 46]. A comparison of JA, SA and ABA levels during leaf senescence [25] indicates that although patterns differ, all three PGRs show peaks of expression late in senescence and thus might be associated with PCD events. However, JA- and ABA-responsive genes are also up-regulated at the earlier stages of senescence suggesting a role for these PGRs both in the initial stages and later execution of senescence/PCD. The approximate

timing of PGR action and downstream TFs in relation to senescence progression and spread of PCD is summarised in Fig. 9.1, although clearly more data are needed to fully understand the regulation of these processes.

9.2.3 ROS as a Regulator of Senescence-Associated PCD

In *Arabidopsis* and other annual species, flowering is a trigger for leaf senescence under optimal conditions, although leaf age also independently regulates timing of senescence [24]. In both *Arabidopsis* and *Brassica napus*, bolting is associated with a transient rise in ROS which may be an important internal trigger in activating rosette senescence [47]. Furthermore, both leaf and petal senescence are associated with a rise in ROS (reviewed in [47, 48]). Developmentally induced PCD such as PCD in the abscission zone is also associated with a rise in ROS [49]. A fall in antioxidant capacity and rise in oxidative stress may thus be a component of the trigger in different forms of plant PCD. ROS levels are modulated through a complex network of ROS scavenging and ROS-producing enzymes, and changes in the balance of these enzymes occur during plant senescence. For example, both catalase and ascorbate peroxidase activities fall at an early stage of bolting in *Arabidopsis*, leading to a feedback amplification loop that results in an increase in H_2O_2 and activation of key TFs that initiate senescence in the leaves [47]. A very detailed analysis of transcriptional changes through leaf expansion and senescence [25] revealed that oxidative stress response genes are highly represented in the very early stages of leaf senescence. This supports a role for ROS both as an activator of senescence and senescence-associated PCD seen in other systems. Furthermore, recent evidence suggests that redox status may be important in activating PCD-related enzymes. For example, in developmentally induced PCD in spruce megagametophytes, production of H_2O_2 activates caspase-like proteins [50], and in ageing elm seeds, treatment with ascorbic acid delayed a rise in caspase-3-like activity [51]. A key parameter often missing though is the intracellular location of the ROS during PCD which has not been determined in most studies. However, a study of the effect of light and O_2 levels in harpin-induced PCD in *Nicotiana sylvestris* leaves concluded that a rise in ROS in the apoplast or in chloroplasts was not required for PCD, while changes in mitochondrial ROS may be important in coordinating the PCD programme [52]. It will be very interesting to get a full picture of the sources of ROS in senescence-induced PCD in different organs which so far is not complete (reviewed in [48, 53]).

9.2.4 Can We Identify TFs Regulating Senescence-Associated PCD?

The initiation of leaf senescence involves a massive reprogramming of gene expression. This reprogramming is mediated by a complex network of TFs [25] involving over 100 TFs of different families. The main families involved are the NAC, WRKY,

MYB, C2-H2 zinc finger, bZIP and AP2-EREBP families. NAC and WRKY TFs have been associated with senescence in several tissues including *Arabidopsis* leaves, petals and siliques [20, 21, 35, 54–57]. Direct evidence for TF control of senescence comes from mutants and over-expressing lines. For example, over-expression of *ANAC029* [58] and *ANAC092* [55], two important TFs involved in regulating senescence, results in premature senescence, while in their respective mutants, senescence is delayed, indicating that they are both positive regulators of senescence.

WRKY TFs are divided into three groups according to their structure, and the group III WRKY TFs are confirmed as senescence regulators. WRKY53 appears to act as a positive regulator of leaf senescence [35], whereas *wrky70* and *wrky54* mutants senesce early indicating that these TFs are negative regulators of senescence [56, 57]. All three of these group III WRKY TFs interact with another group III WRKY: WRKY30 by yeast 2-hybrid [56], suggesting that WRKY30 is an upstream regulator of the others. Furthermore, all four of these TFs are induced by SA, and both *WRKY53* and *WRKY30* are induced by ROS. This is probably a small part of a much more complex TF network, and these TFs may act quite early in the induction of leaf senescence. Numerous TFs are up-regulated before visible signs of leaf senescence including eight NAC domain and 4 WRKY TFs [25]. These TFs fell into two expression clusters: one group was up-regulated and then later down-regulated, whereas expression of the other group continued to rise into late senescence [25]. This latter group is likely to be more important in regulating the later stages of senescence including perhaps PCD events. WRKY TFs were overrepresented later in leaf senescence as were members of the large AP2-EREBP family of TFs. Members of both these TF families are induced by SA and JA [59], and AP2-EREBP TFs are also ethylene induced [60]. Thus, more focus on the roles of WRKY and AP2-EREBP TFs in regulating senescence-associated PCD may be fruitful. It will also be interesting to discover if the same networks of regulators operate in senescence of other organs or whether they differ.

The role of NAC TFs in leaf senescence has been recently reviewed [2] showing that this family of TFs plays an important role in integrating stress and developmental signals not only in *Arabidopsis* but also in crop species. Some of the NAC TFs may also be directly regulating expression of proteolytic enzymes. In soybean (*Glycine max*), two NAC TFs are associated with osmotic and ER stress-induced PCD [61]. Coordinately, they regulate common targets including a VPE gene [62], and expression of these TFs was associated with an increase in caspase-1 activity in soybean protoplasts. So in addition to regulating early stages of senescence, NAC TFs may also have a role in later events associated with PCD.

9.2.5 Regulation of Leaf Senescence-Associated PCD by MicroRNAs

Another level of control for senescence and senescence-associated PCD has been recently discovered in microRNAs. MicroRNAs (miRNAs) are small genome-encoded RNAs, approximately 21 nucleotides in length that regulate levels of target

transcripts by promoting their degradation or inhibiting their translation [63]. More than a thousand miRNAs have been discovered in plants, and they are involved in the regulation of many developmental processes including hormonal biosynthesis and signalling, pattern formation and biogenesis as well as responses to stress [64, 65]. It is thought that most TFs are under miRNA control [66]. Recently, at least two miRNAs regulating *Arabidopsis* leaf senescence have been identified. It will be interesting to see if these miRNAs also regulate senescence in other organs.

miR164 family members regulate the expression of *ANAC092* (*ORE1*), a positive regulator of senescence and cell death [67]. In triple mutants in which all three *miR164* family members are mutated, senescence-associated PCD was accelerated, including more rapid chlorophyll degradation and an accelerated increase in ion leakage, indicative of increased PCD. In *miR164* over-expressing lines, senescence-associated PCD is delayed. The regulation is complicated by the role of another TF: *EIN2*. *ANAC092* is up-regulated with leaf senescence by *EIN2* and negatively regulated by miR164. As leaves age, levels of miR164 decline, inhibited by *EIN2* thus resulting in *ANAC092* up-regulation. This has been described as a trifurcate feed-forward regulatory pathway [67].

Another miRNA controlling senescence is *miR319* [68]. This microRNA controls (*TEOSINTE BRANCHED1*, *CYCLOIDEA* and *PCF*) TCP TF genes which in turn regulate JA biosynthesis. In *jawD* plants, in which *miR319* is over-expressed, senescence is delayed, but this effect can be reversed by application of JA. However, a defect in JA alone such as that found in the *coi1* mutant does not affect senescence, suggesting that other factors are involved.

9.3 Mechanisms and Regulators of Senescence-Associated PCD

9.3.1 Cytology and Overall Mechanisms of Plant PCD

A central question relating to senescence-associated PCD is the cellular mechanism by which cell death is executed. PCD is now accepted as a rather broad term indicating active cell destruction and encompassing several distinct mechanisms of action. Four morphologically distinct forms of PCD are identifiable in eukaryotic cells: apoptosis, autophagy, oncosis and pyroptosis [69]. Additionally, PCD with necrosis-like morphology has been added to the list [70]. Of these, van Doorn et al. [15] suggest that only two can be identified in plants: vacuolar cell death and necrosis. Vacuolar cell death is defined as essentially autophagic PCD with a contribution to cellular destruction from collapsed lytic vacuoles. Necrosis is typified by early collapse of the plasma membrane and protoplast shrinkage. Furthermore, van Doorn et al. [15] suggest that PCD associated with development and senescence is by the vacuolar cell death route, whereas necrotic PCD occurs during abiotic stress-induced PCD. However, they also accept that some types of PCD such as those that

occur during the hypersensitive response, during the formation of starchy endosperm and during self-incompatible pollen–pistil interactions show features of both types of PCD mechanism. A key point of this classification is that other forms of PCD identified in animal cells such as apoptosis are not found in plant cells.

Vacuolar cell death as defined by van Doorn et al. [15] is generally associated with an increase of the vacuolar volume. Fusion of vesicles with the vacuole and invaginations of the vacuolar membrane are seen in electron micrographs, reminiscent of micro- or macroautophagy. Both are likely occurring in the same cells. For example, Toyooka et al. [71] suggested that in fact there may be two different autophagy-like mechanisms involved in PCD occurring during cotyledon senescence. Firstly, there is a process that resembles micropexophagy (microautophagy involving peroxisomes) in yeast. Starch granules are wrapped in small acidic vesicles and then transferred to a lytic vacuole. The second process resembles macroautophagy with the formation of autophagosomes. The final step in this form of PCD is rupture of the tonoplast delivering vacuolar hydrolases into the cytoplasm which degrade the contents. Other morphological features include nuclear fragmentation and the formation of actin cables while mitochondria and other organelles remain relatively intact until tonoplast rupture. Vacuolar cell death is further associated with expression of autophagic markers such as Atg8, acidification of the vacuole, cytoskeletal reorganisation and activation of VPEs. However, at least two mechanisms for vacuole-mediated PCD have been proposed [72]: one in which the vacuolar membrane collapses and the other in which it does not. The latter is associated with the hypersensitive response and involves fusion of the vacuolar membrane with the plasma membrane and delivery of lytic enzymes into the apoplastic space. Destructive vacuolar cell death is associated with developmental PCD and is similar to that described by van Doorn et al. [15].

9.3.2 Autophagy and Senescence-Induced PCD

Vacuolar PCD shows strong similarities to macroautophagy, a process that is well studied in yeast and animal cells and has recently been reviewed in plant cells [73]. The defining feature of macroautophagy is the formation of autophagosomes. These are double-membraned vesicles that enclose portions of the cytoplasm and deliver their content to the vacuole where it is degraded by lytic enzymes. Two pathways are involved which resemble ubiquitin conjugation systems. In yeast, Atg8 is cleaved by Atg4 and thus made available to bind Atg7. Atg8 is then transferred to Atg3 and finally to phosphatidylethanolamine. Atg7 also activates Atg12 and transfers it to Atg10 and finally to Atg5. These two proteins form a complex with Atg16 which is also required for autophagosome assembly. In *Arabidopsis*, homologous autophagy-related ATG genes have been identified to all of those found in yeast, with the added complexity that in yeast some components such as Atg8 are encoded by single genes while in *Arabidopsis* they comprise gene families of several members with differential expression patterns.

However, although autophagosome-like structures are visible in cells undergoing senescence-associated PCD, mutation of many of the autophagy-related genes (*ATG2*, *ATG4s, ATG5*, *ATG6*, *ATG7*, *ATG8s*, *ATG9*, *ATG10*, *ATG12s* and *ATG18a*) results in accelerated leaf senescence when plants are grown at normal nutrient levels [73]. Furthermore, Japanese morning glory (*Ipomoea nil*) mutants, in which autophagy genes were down-regulated, showed accelerated PCD symptoms in senescent petals [74]. This calls into question whether the autophagic machinery is responsible for senescence-associated PCD or whether it is associated with the remobilisation phase of senescence. Of course, it may act in both.

In leaves, autophagy-related genes are up-regulated surprisingly early before leaves are fully expanded and continue to increase in expression throughout senescence [25]. However, different ATG genes were up-regulated at different times: 14 were already up-regulated during leaf expansion, whereas *ATG7* was not up-regulated until levels of total protein and chlorophyll were declining in the leaves [25]. *ATG7*, *ATG8A ATG8B* and *ATG8H*, were coordinately up-regulated in the early stages of senescence. Since in yeast Atg8 and Atg7 are required in the final steps in the assembly of autophagosomes [73], the up-regulation of these genes in *Arabidopsis* leaves may activate senescence-associated autophagy. In pollinated *Petunia* petals, four *ATG8* homologues were up-regulated quite late, once wilting was already visible, and paralleled increases in ethylene, suggesting that ethylene may be regulating their expression [75]. Autophagy in *Arabidopsis* leaves also seems to be under hormonal control. ROS, SA, JA and ABA were all elevated in mutants of *ATG5* [76]. However, the accelerated leaf senescence associated with the *atg5* mutant was reversed in *sid2* and *NahG* plants but not in mutants that affect JA or ethylene signalling, indicating a regulatory role for SA. Furthermore, the SA analogue BTH induced the accumulation of autophagosomes. *ATG5*, *ATG8* and *ATG12* were up-regulated in all three *Arabidopsis* senescent tissues (petal, leaf and silique) studied by Wagstaff et al. [21], suggesting a common function across senescent organs. More sophisticated experiments using inducible RNAi constructs to down-regulate ATG genes at later stages in senescence may help to resolve whether these increases in expression later in senescence are required for PCD.

9.3.3 Other Vesicles in Senescence-Associated PCD

In addition to autophagosome-like vesicles, a number of other vesicles have been seen during plant PCD, many also in developmental and senescence-associated PCD (Table 9.1) which either transport cargo from the ER or cytoplasm to the vacuole or deliver their cargo directly to the cytoplasm following tonoplast rupture.

Ricinosomes were originally described from observations of seed endosperm in the castor oil plant *Ricinus communis* [77–79] and more recently in tomato anthers [80]. They have also been seen in endosperm and suspensor cells of quinoa (*Chenopodium quinoa*) acting as early markers for PCD [81]. Ricinosomes derive from the ER where they pick up their cargo of KDEL-cysteine proteases, and

Table 9.1 Vesicles associated with senescence and stress-induced PCD in plant cells

Vesicle	Tissue(s)	Derived from	Deliver cargo to	Other features + evidence	References
Ricinosomes	Seed endosperm, petals, tomato anthers	ER	Cytoplasm	Slightly smaller than glyoxysomes, develop as DNA is degraded during PCD; membrane has ribosomes; release cargo into cytoplasm on cytoplasmic acidification following tonoplast collapse *Cargo: KDEL proteases*	Schmid et al. [77, 78]; Greenwood et al. [79]; Senatore [80]; López-Fernández and Maldonado [81]
Autophagosomes	Starved tissues, senescent tissues, petals	ER	Vacuole	300–900 nm in diameter which can fuse directly with the vacuole or first engulfed by a smaller lysosome-like or endosome-like organelle, which begins the degradation of the contents, before eventual fusion with the vacuole *Cargo: cytoplasmic contents + enzymes, e.g. VEIDase*	Liu and Bassham [73]
KDEL vesicles (KV)	Seed	ER	Protein storage vesicles	200–500 nm in diameter, bud off ER, Golgi independent, separate transport to VmPE which goes via Golgi, KDEL protease cargo probably not processed in the KV *Cargo: KDEL proteases*	Toyooka et al. [71]

(continued)

Table 9.1 (continued)

Vesicle	Tissue(s)	Derived from	Deliver cargo to	Other features + evidence	References
Senescence-associated vesicles (SAVs)	Photosynthetic tissues only, senescence induced		Vacuole	700 nm in diameter located in the peripheral cytoplasm, around plastids, stain with cys pro and vacuole-specific stains, do not require AtAPG7 or SAG12, pH = 5.2 (more acidic than central vacuole = pH 6) induced by ethylene or dark-induced senescence, break down chloroplast components *Cargo: SAG12 and chloroplasts*	Otegui et al. [82]; Martinez et al. [83]; Carrión et al. [84]
ER bodies	Constitutive in epidermis, of young seedlings, induced in leaves by wounding, MeJA, suppressed by ethylene	ER	Vacuole	500 nm spindle shaped, surrounded by ribosomes; BGLU18 protein is localised in inducible ER bodies, while PYK10 protein (a different member of the same β-glucosidase subfamily) accumulates in constitutive ER bodies *Cargo: gamma VPE and RD21 proteases*	Hayashi et al. [85]; Ogasawara et al. [86]

(continued)

Table 9.1 (continued)

Vesicle	Tissue(s)	Derived from	Deliver cargo to	Other features + evidence	References
ATI1 bodies	Cotyledon, hypocotyl	ER	Vacuole	500–1000 nm without stress associated with ER; when stressed form spherical bodies, do not contain ER lumen (no mCherry-HDEL marker), and contain ATI1-GFP, but not Golgi, mitochondrial or peroxisome markers; transiently stain with autophagosome marker Atg8f, distinct from autophagosomes, seen in vacuole *Cargo unknown*	Honing et al. [87]

unlike autophagosomes, the cargo is delivered directly into the cytoplasm of cells undergoing PCD. Of relevance to senescence-associated PCD is that ricinosomes were also seen in senescent day lily (*Hemerocallis*) petals [77]. However, there have been no further reports of them in senescent tissues. Furthermore, Battelli et al. [88] did not see the KDEL protein expressed in *Lilium longiflorum* senescent petals associated with ricinosome-like vesicles, but did see the KDEL protease in the vacuole. In seeds of *Vigna mungo*, KDEL proteins were also seen inside KDEL vesicles (KV) [71]. These appear to be very similar to ricinosomes in size (200–500 nm) but deliver their cargo to storage vacuoles instead of the cytoplasm. So clearly further work is needed to establish whether ricinosome-mediated delivery of KDEL proteases to the cytoplasm post-tonoplast rupture is a conserved feature of senescence-associated PCD or whether delivery to vacuoles in senescence-associated PCD also occurs.

Senescence-associated vesicles (SAVs) are seen in senescent tobacco, *Arabidopsis* and soybean leaves [82–84] and form independently from the autophagosome-required gene *ATG7*. These are highly acidic vacuoles containing high cysteine peptidase activity, of similar size to ricinosomes (700 nm) but are located in the peripheral cytoplasm around plastids. They are only visible in senescent leaves and appear to contain the majority of cellular cysteine protease activity in senescent leaf cells [84]. They contain the senescence marker protease SAG12, and in fact all cells expressing *SAG12* contain SAVs; however, *SAG12* is not required for their formation. They are involved in breakdown of chloroplasts and are only found in senescent leaf cells that contain chloroplasts. They therefore may

be specifically required for chloroplast disassembly. However, *SAG12* is also expressed in non-photosynthetic senescent tissues such as petals [21, 89], so it will be interesting to discover whether SAVs are also important in non-photosynthetic senescent tissues.

Two forms of ER bodies containing different sets of β-glucosidases have been identified in *Arabidopsis*. They are surrounded by ribosomes, and one form was found constitutively in *Arabidopsis* seedling epidermal cells. On wounding or salt stress, another form of ER body is induced. The ER bodies fuse with each other and then with the vacuole [85, 86]. Of particular interest is that ER bodies carry precursors to γVPE and a stress-inducible protease RD21. It would be interesting to verify whether these vesicles are ever detected during senescence-associated PCD.

ATI1 bodies are formed in response to carbon starvation from the ER network [87]. These compartments are marked by ATI1 and ATI2, plant-specific proteins which associate with the autophagosome protein ATG8. ATI1 bodies are clearly distinct from ER bodies and also from Golgi, mitochondria and peroxisomes and do not contain ER-lumenal markers. They are also distinct from autophagosomes as they did not co-localise with ATG8f-marked vesicles [86]. Their destination is the central vacuole, and it is proposed that they may be carrying mis-folded proteins for destruction. It will be important to identify their cargo and also to discover whether they play a role in stress-induced or senescence-associated PCD.

9.3.4 Role of the Mitochondrion and Cytochrome c *Leakage*

The mitochondrion plays a central role in the regulation of animal cell apoptosis [90]. Pro-apoptotic members of the Bcl-2 family cause permeabilisation of the outer mitochondrial membrane and release of cytochrome *c* into the cytosol. Cytochrome *c* binds with Apaf1 promoting a caspase cascade which results in death of the cell [91]. Hence, cytochrome *c* leakage was searched for and found in many plant PCD systems [92]. In plant cells, cytochrome *c* leakage into the cytoplasm is categorised by van Doorn et al. [15] as a feature of necrosis, associated with abiotic stress-induced PCD. In support of this, it has been reported, for example, in heat-induced PCD in tobacco BY2 cultures [93] and cucumber cotyledons [94], whereas in pollination-induced senescence of petunia flowers, the release of cytochrome *c* did not occur [95]. It was reported however during induced ageing in elm seeds [51]. This induction method aims to simulate seed ageing or senescence, by the induction of accelerated ageing through elevated temperatures (controlled deterioration treatment). It therefore calls into question whether this is PCD by senescence or abiotic stress induction. On the other hand, leakage of cytochrome *c* to the cytoplasm was also noted during cotyledon senescence in mung bean (*Vigna radiata* [96]) and during tulip petal senescence [97] processes which are clearly developmental and not induced by abiotic stress. Hence, whether cytochrome *c* release is a key component of senescence-associated PCD mechanisms remains an open question. Analysis of cytosolic cytochrome *c* levels in many more classical organ senescence systems at

different time points is needed to resolve this question. Ideally, this should be done using techniques such as immunocytochemistry to visualise the cytochrome *c* levels spatially and correlate them with other PCD markers.

In animal cells, cytochrome *c* release can be triggered by increases in cytosolic calcium which activate the permeability transition pore (PTP), composed of VDAC and ANT. In plants, homologues to both VDAC and ANT are present, indicating that the PTP may be functional (recently reviewed by Diamond and McCabe [92]). VDAC expression is up-regulated in abiotic-induced PCD systems such as induction of PCD in pearl millet by salinity, drought, cold and SA [98]. However, in leaf senescence (eFP browser, [99, 100]) and ageing seeds [101], VDAC genes were either down-regulated or did not change in expression. This further supports a role for cytochrome *c* release, at least via the PTP, in abiotic stress induced but not senescence-induced plant PCD.

9.3.5 What Is the Role of the Endoplasmic Reticulum?

The endoplasmic reticulum (ER) performs an important role in the cell in monitoring protein folding, both in plants and other eukaryotes. In plants, ER stress can be induced by a range of abiotic and biotic stresses that result in an imbalance between the rate at which proteins are synthesised and the capacity of the ER to process them. If unfolded proteins accumulate in the ER, this triggers the unfolded protein response (UPR) [102]. Initially, the cell mitigates the stress by up-regulating ER chaperones, reducing the rate of translation and increasing the rate of degradation of mis-folded proteins. In plants, these processes are mediated by two pathways. One involves ER-membrane associated TFs (bZIP17 and bZIP28). The other requires a protein kinase: RNA-splicing factor IRE1. However, under prolonged ER stress, autophagy or PCD can be triggered. Autophagosomes were formed in response to ER-stress in *Arabidopsis* seedlings [103], and this process required *ATG18a*. Autophagosome production also required IRE1 indicating a dual role for IRE1 in protecting against ER-stress and also in activating autophagy. However how PCD is triggered by ER-stress is less clear [102]. One possibility is that this is also via IRE1. In animal cells, the ER-located Bax inhibitor-I (BI-1) binds to IRE1 and decreases its activity [104]. *BI-1* is conserved across eukaryotes, including plants, and seems to inhibit plant PCD [105–107]. Furthermore, it seems to play a role in mitigating ER stress [108]. BI-1 is highly up-regulated in senescent leaves (eFP browser, [100]) and is also up-regulated during silique senescence [21] although less so in petals. BI-1 is also linked with JA signalling: *atbi1-2* knockout mutants show accelerated MeJA-induced leaf senescence, induced by an increase in calcium levels [109] probably released from the ER into the cytoplasm. So this provides a significant link between the ER and senescence-associated PCD.

Recently, a key role for the ER in plant senescence-associated PCD has been proposed [110]. DMP1 is a membrane protein localised both to the ER and tonoplast. When expressed under its native promoter, which drives senescence-specific

expression, over-expression of DMP1 results in ER fragmentation, during both natural and dark-induced leaf senescence. The sequence of these membrane rearrangements indicates that ER disintegration precedes vacuolar collapse during senescence-associated PCD. So whether DMP1 and the ER are playing a regulatory role in senescence-associated PCD merits further investigation.

9.3.6 Proteolysis as a Regulator of Senescence-Associated PCD

Caspases are key regulators of animal apoptosis, and therefore extensive research was carried out to determine whether they had a role in the regulation of plant PCD [111]. It has been known for some time that caspase activity is associated with plant PCD; however, direct homologues of caspase genes have not been discovered in sequenced plant genomes. Attention was drawn to VPEs due to their caspase-1-like activity; however, unlike animal caspases that are located in the cytosol, VPEs are vacuolar. The *Arabidopsis* genome contains four VPE genes: α, β, γ and δ [72]. Mutation of all four VPEs in *Arabidopsis* inhibited PCD induced by the mycotoxin fumonisin B1 [112] and by infection with the mutualistic fungus *Piriformospora indica* [113]. Tonoplast rupture was also inhibited by mutation of all four VPE genes [112], and YVAD-ase (caspase-1-like) activity was abolished [112]. Only γVPE (AT4G32940) is up-regulated during leaf senescence (eFP browser, [100]), suggesting that it is this class that is most likely involved in senescence-associated PCD, although in *Arabidopsis* petals both γ- and βVPE genes were up-regulated [21]. Up-regulation of VPE genes has also been reported in petals of other species (carnation: [114]; daffodil: [115]; *Ipomea*: [116]). It will be interesting to see whether only γ- and βVPE genes are up-regulated in species other than *Arabidopsis* and critically identify their substrates.

According to van Doorn et al. [15], activation of VPEs is diagnostic of the vacuolar mechanisms of plant PCD. However, caspase activities are associated with a wide range of plant PCD systems including abiotic stress induced by heat (e.g. [93]), pathogens (e.g. [117]) and developmental processes during xylogenesis-associated PCD [118] as well as in petal senescence (e.g. [119]) and caspase-3-like activity in cotyledon senescence [96]. This suggests that if VPEs are indeed diagnostic of vacuolar cell death, then this mechanism is also contributing to PCD in these different PCD systems. However, caution must be exercised in the interpretation of these studies to infer that caspase activity is indeed involved in plant PCD. This is because in virtually all studies, substrates used to demonstrate caspase activity were synthetic tetra peptides containing the target sequence for mammalian caspases (such as YVAD as a target for caspase-1) and not whole proteins. These substrates are not fully specific for individual caspases [111] making the identification of specific caspase activities difficult.

Although the search for caspase genes in plant genomes was in vain, a related family of genes, metacaspases, were found [120]. Metacaspases are cysteine-dependent proteases falling into two structural classes (I and II). Although they do not have caspase activity, metacaspases have been associated with different forms of plant PCD including both abiotic (*AtMC8*, [121]; *AtMC4*, [122]) and biotic (*AtMC1* and *AtMC2*, [123]) stress-induced PCD in *Arabidopsis*, as well as embryogenesis-related developmental PCD [124] in *Picea*. Of particular interest in *Picea* is the finding that autophagy is required for death of the suspensor, and furthermore it lies downstream of a class II metacaspase gene which is also required for suspensor PCD [125]. In addition, if this metacaspase–autophagy PCD pathway is suppressed by silencing ATG gene expression or the class II metacaspase, there is a switch from vacuolar PCD to necrotic cell death.

In *Arabidopsis*, there are nine metacaspase genes, and a lack of phenotypes from single mutants suggests that there may be functional redundancy between the family members, making it difficult to verify their role in different forms of PCD. Of the nine *Arabidopsis* metacaspase genes, only *AtMC9* is highly expressed in senescence and PCD [126], and this gene is strongly up-regulated in older *Arabidopsis* petals (eFP browser, [100]). In their transcriptomic analysis of leaf senescence, Breeze et al. [25] found only two class II genes (*AtMC6* and *AtMC9*) were up-regulated but were different to those up-regulated during other forms of plant PCD in *Arabidopsis* (see above). Their up-regulation coincided with the up-regulation of the *ATG7* and *ATG8* genes suggesting that this may be an important time-point in the activation of senescence-associated PCD. AtMC9 is mainly localised in the nucleus [126] though GFP fusion protein was also detected in the cytoplasm, so it will be very interesting to understand the timing of its enzymatic activity during senescence-associated PCD.

Recently, caspase-3 activity has been associated in very different systems with the proteasome. Caspase-3 activity associated with xylem differentiation in *Populus* was derived from the 20S proteasome subunit [118]. Furthermore, a caspase-3 activity linked to the release of antibacterial proteins into the intercellular space during infection was associated more specifically with the PBA1 (β1) subunit of the 20S proteasome core [117]. The caspase-3 activity in both systems seems likely to be involved in tonoplast breakdown [117, 118]. It will therefore be interesting to map more precisely the timing and location of the caspase-3 activity during senescence-associated PCD to establish whether it plays the same role here. Of course, the crucial point is the detection of the substrates, which presumably are ubiquitinated prior to direction to the proteasome.

The proteasome also appears to have an important role as a means of removing negative and positive regulators of senescence. ORE9 which is an F-box protein [127] and ATE [128] which is part of the N-rule ubiquitination pathway are both positive regulators of *Arabidopsis* leaf senescence as mutants in these genes show delayed senescence. In contrast, a RING-type ubiquitin ligase *NLA* and the PUB-ARM E3 ubiquitin ligase *SAUL1* are negative leaf senescence regulators [129, 130]. The proteasome machinery has been implicated in PCD associated with the hypersensitive response, particularly the PUB-ARM proteins [131]. Hence, the

finding that *SAUL1* regulates senescence suggests a role for this protein also in senescence-associated PCD. Vogelmann et al. [132] showed up-regulation of leaf senescence-associated TFs such as *WRKY53*, *WRKY6 ANAC092* and *ANAC029* as well as premature PCD in *saul1-1* mutants. Detailed analysis of the timing of changes in gene expression revealed up-regulation of SA-associated genes. A role for SA in *SAUL1*-induced senescence and senescence-associated PCD was confirmed by the finding that the effects of *SAUL1* on senescence require PAD4, an enzyme required for SA signalling.

9.3.7 Downstream Effectors and Markers of Senescence-Associated PCD

It has long been known (e.g. [133]) that genes encoding degradative enzymes are up-regulated during senescence, and it is assumed that these are the effectors of senescence-associated PCD. The cysteine protease gene *SAG12* has emerged as a key marker for senescence, expressed as the first signs of leaf yellowing become visible [25] and somewhat earlier in petal senescence [89]. Later in leaf senescence, as yellowing becomes more widespread, a high proportion (44 %) of expressed genes are involved in metabolism, presumably involved in the degradation of cell contents and nutrient remobilisation [25]. Metabolic genes were also highly expressed in petal senescence in all species studied (reviewed in [20]). Another feature of gene expression in late leaf senescence is the reactivation of cytoskeleton-related genes. In yeast and mammalian cells, microtubules are required for efficient autophagy [134], so this late up-regulation of cytoskeleton-associated genes may further support for an autophagy-like model of senescence-associated PCD.

9.3.8 Chloroplasts During Senescence-Associated PCD

One important division in senescing organs is between photosynthetic organs where chloroplasts play a key role in ROS generation and are a source of photosynthate, and non-photosynthetic organs which lack functional chloroplasts. In photosynthetic organs, senescence can be considered as a form of starvation which is imposed by a withdrawal of nutrients that are redirected to other parts of the plant. For example, many seed pods such as those of legumes and siliques in *Brassica* species are photosynthetic organs that undergo a senescence programme which has parallels with that in leaves. Chloroplasts are dismantled resulting in yellowing, and nutrients are transferred to the developing seeds [135]. Alternatively, a reduction in photosynthetic capacity may be a result of reduced light levels, reduced water availability or disease and stresses which tip the balance towards senescence.

Chlorophyll degradation is a key marker of the senescence of photosynthetically active tissues. Genes associated with chlorophyll breakdown are switched on relatively early, and the morphology of plastids changes to a form termed "gerontoplasts". This process is reversible, and Thomas et al. [10] proposed that this is the stage that should be called senescence, whereas PCD starts after this, once a point of no return has been reached (Fig. 9.1). Although reversibility has been demonstrated in some systems, including cells in which downstream processes associated with PCD-like expression of *SAG12* have been initiated [136], it is in fact relatively rare [8]. Thomas et al. [10] suggested that the presence of functioning chloroplasts is a key feature maintaining life of the cell and that a reversal of senescence and early PCD processes requires chloroplast function. A second peak in expression of chlorophyll breakdown genes late in senescence [25] may regulate the final breakdown of chlorophyll–protein complexes and the cessation of photosynthesis during the process that leads to irreversible cell death.

More recently, it has been shown that the reduction in size and number of chloroplasts occurring as a result of dark-induced senescence requires functional autophagy genes [137]. Furthermore, both chlorophyll and Rubisco were seen in the vacuole indicating that chloroplasts were being transported to the vacuole for degradation via autophagy. However, stromal proteins have also been seen in senescence-associated vesicles (SAVs) [83] which do not require a functional autophagic machinery [82]; hence, it would seem that at least two independent mechanisms are involved in chloroplast breakdown during senescence-associated PCD.

9.3.9 Progression of Senescence-Associated PCD Across Organs

One of the difficulties in studying the gene regulation of senescence-associated PCD is the asynchrony of PCD within an organ. In leaves, PCD often starts at the tip of the leaves (Fig 9.2a) seen as the areas that first yellow or turn brown in both dicots and monocots [3]. Staining with trypan blue that specifically stains dead cells shows the increase in dead cells with time during developmental leaf senescence [2]. In many autumnal leaves, the loss of chlorophyll is evidently delayed around the major veins. In common with leaves, senescence and PCD in cotyledons are not uniform across the organ. Thus, the first signs of PCD, using TUNEL staining as a marker, were seen in the margins of cotton cotyledons [138] followed later by the centre. The asynchrony of PCD is also evident in other organs such as siliques and petals. In petals, the loss of internal cells is remarkable in flowers that still appear in full bloom or early senescence (Fig 9.2b).

In organs other than leaves, a key point is the coordination of senescence and PCD between different cell types and related organs. In pods, for example, specialised sets of cells perform specific functions such as dehiscence to release the seeds [139]. The walls of *Arabidopsis* siliques comprise inner and outer epidermal cells enclosing three mesophyll layers and adjacent to the inner epidermis a layer of cells

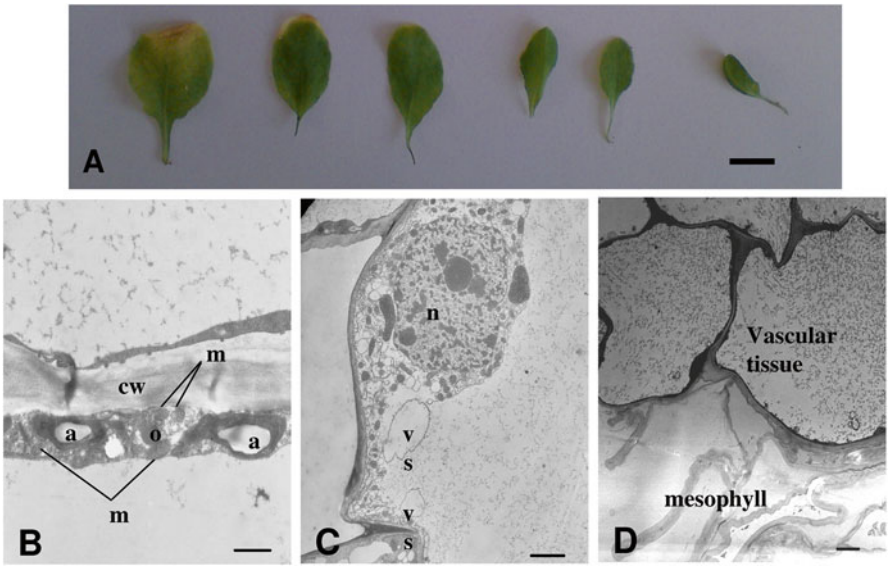

Fig. 9.2 Heterogeneity of PCD across tissues in *Arabidopsis* leaves (**a**) and *Lilium longiflorum* senescent tepals (**b**–**d**). (**a**) Leaves from 10-week-old *Arabidopsis* plants: yellowing and browning typically starts at the tip of the leaf and spreads downwards (scale bar = 5 mm); (**b**) at loose closed bud stage, *L. longiflorum* tepal mesophyll cells are round in shape and rich in amyloplasts, without signs of autolysis. Magnification 10 K, scale bar, 1 μm; (**c**) at open flower stage, epidermal cells show a diffuse cytoplasm with vesiculation. Magnification 3.2 K, scale bar, 3 μm; (**d**) in early senescence (as tepals become translucent and show signs of browning). Intact vascular tissues and a completely collapsed mesophyll layer. Magnification 2 K, scale bar, 3 μm. Nuclei (n), vacuole (v), cell wall (cw), plastids (p), mitochondria (m), amyloplasts (a), vesicles (vs) and oleosomes (o). Electron microscopy images of *L. longiflorum* tepals are reproduced from R. Battelli PhD thesis. See Battelli et al. [119] for further details and images of tepal senescence in *L. longiflorum*

that become the encocarp. Cells of the inner epidermis start to undergo PCD first, while the endocarp cell walls thicken, and the cytoplasm of inner epidermal cells disappears completely by the time the siliques are fully yellow but well before dehiscence [21].

In many flowers, pollination triggers senescence and ultimate PCD (reviewed in [20]); however, PCD is regulated within the individual organs and cell types. In pistils, senescence starts in the transmitting tract shown by early cellular deterioration and activation of expression of the senescence marker gene *BNF1* [140]. *BNF1* is then activated in the stigma followed by the ovules. In the absence of pollination, pistils do senesce, although the timing of this event is species specific. In some species such as pea [141], this occurs after just 3 days, while flowers can last for months in orchids [14]. A key indicator of pistil senescence is a loss of responsiveness to GA_3 which is able to induce parthenocarpic fruit development [140]; this in turn is regulated by ethylene which seems to be produced by the ovules [142]. The coordination of pistil senescence with petal senescence also seems to vary. In some species, such as almond, the stigma remains receptive even when petals are already

senescing [143], while in others such as the economically important grass *Leymus chinensis*, the stigma remains receptive to pollen for only 3 h [144]. So another area in need of further research is the coordination of PCD within and between related senescing organs.

9.3.10 Senescence-Associated PCD On and Off the Plant

If remobilisation to other plant organs is the defining feature of senescence, then postharvest senescence or off-the-plant senescence must be categorised separately. However, both terminate in PCD; thus, it is of interest to review the progression and final stages of the process. Postharvest storage of plant material such as leafy vegetables and flowers results in deterioration which closely resembles physiological features of on-the-plant senescence including protein degradation, leaf yellowing or petal wilting (e.g. [20, 145]).

Perhaps surprisingly, processes very similar to those evoked during developmental or stress-induced senescence on the plant are also activated in organs that are detached from the plant. Even a leaf that has been ingested by a ruminant activates similar biochemical and cellular processes as those activated during senescence [146] including chlorophyll and protein breakdown, increase of protease and nuclease activity and increasing membrane leakage indicating progressive PCD. Both *SAG12* and *γVPE* were up-regulated postharvest in broccoli [145], suggesting similar PCD processes.

The majority of studies on petal senescence are performed with detached flowers, and remarkably few studies have compared on- and off-the-plant senescence. Arrom and Munné-Bosch [37] did show differences in endogenous growth regulator levels; however, these were difficult to interpret. An interesting study on PCD in detached petals [147] indicates that nuclear morphology in abscised petals resembles more closely the effects of dehydration-induced PCD than developmental PCD in petals. Differing morphologies of petal PCD were found in different species [148] in which either DNA fragmented within the nucleus or membrane-bound chromatin-containing bodies indicated nuclear fragmentation. Further detailed comparisons of the cytology of petal PCD on and off the plant in abscising and non-abscising species would be interesting, to determine whether the vacuolar/autophagic mechanism is operating in all cases.

Other forms of senescence such as fruit and seed senescence necessarily occur off the plant. The duration of seed viability is a species-specific character which is also affected by environmental effects such as temperature and humidity [149, 150], and viability declines with age. Several studies have shown that seed ageing is associated with nucleic acid degradation (e.g. [151, 152]) and increased oxidative stress resulting in lipid peroxidation (e.g. [153]). Recently, a transcriptomic survey of artificially accelerated pea seed ageing showed that an early change, prior to loss of viability, is up-regulation of genes related to PCD including BI-1, oxidative stress

and protein ubiquitination [101]. Markers of ER-stress such as BiP were also up-regulated. A reduction in the expression of antioxidant-related genes was also reported in other systems (e.g. sunflower seeds [154]). In a controlled deterioration treatment experiment with elm seeds [51], changes in ROS were also associated with PCD markers. Furthermore, a rise in caspase activity and cytochrome *c* release could be blocked by treatment with ascorbic acid, suggesting a link between oxidative stress and PCD-linked caspase activity.

9.4 Future Prospects and Tools Needed

One of the difficulties in deriving mechanisms applicable across senescence systems is a lack of comparable analyses in different organs within the same species. At a molecular level, these have been attempted comparing different organs within model species such as *Arabidopsis* [21] and ornamentals such as wallflowers [89]. Given new perspectives on the cytological mechanisms of senescence-associated PCD [15], it would be very useful to revisit well-studied senescence systems such as leaves and petals as well as the less studied senescence systems such as other floral organs within the same species to establish common mechanisms.

Another issue that remains to be resolved is the spatial and temporal development of PCD through individual organs. There is a need to study gene expression and signal perception and transduction at a cellular rather than an organ level to understand how PCD is being regulated within the cell and across the organ. The use of transgenic lines expressing fluorescent proteins fused to markers for cell types and cell status may help to resolve this.

The input signals that start the senescence process seem to operate through overlapping growth regulators and downstream TFs. More careful comparisons of different induction systems such as that carried out by the PRESTA project (http://www2.warwick.ac.uk/fac/sci/lifesci/research/presta/) examining the transcriptomic patterns induced by different stress treatments provide a valuable resource. This needs to be expanded since comparisons of existing transcriptomic data carried out in different labs with different parameters run the risk of being misleading. Unravelling networks of TFs is telling us a lot about upstream regulation of senescence and PCD; however, even downstream genes may have multiple input signals from several TFs, so the network may be very complex. TF networks will also need to be integrated with an understanding of epigenetic mechanisms and posttranslational protein–protein interactions to get a full picture of the network.

However, next-generation sequencing opens the way to study these mechanisms beyond the well-studied model organisms into crop species. This is especially needed in horticultural crops that are represented by so many different species and varieties. These provide perishable fresh produce, and potential benefits include improving nutritional quality while reducing waste in support of a healthy diet and food security.

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Chapter 10
The Hypersensitive Response in PAMP- and Effector-Triggered Immune Responses

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10.1 Introduction

Every cell in a plant has been preprogrammed to be capable of cell death when desired or necessary. Other chapters in this book detail many different aspects of programmed cell death (PCD) as part of growth, development, and adaptation to changing environments. In this chapter, we will examine the central role of PCD as a defense mechanism against biotrophic pathogens. Plants, in the absence of a circulating and adaptive immune system, have provided every cell with the capability to detect a wide range of foreign microorganisms and to sacrifice itself and those cells nearby upon detection of a potential attack for the benefit of the rest of the

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plant. The PCD during the hypersensitive response (HR) presents the classic cell death signatures of DNA laddering, caspase-like proteolytic activity, and cytochrome *c* release from the mitochondria [1, 2]. In addition, autofluorescence of cell walls and cell contents, a halt in cytoplasmic streaming, the loss of cortical microtubules, and the appearance of particles in the vacuole have also been described [3]. Although bursts of cytotoxic signaling molecules are produced that injure both host and pathogen cells, the HR has been shown to be a form of PCD which requires cells to be both metabolically and transcriptionally active to proceed and is consequently not classed as a form of necrotic cell death.

In this chapter, we will avoid mentioning much more about generic plant PCD mechanisms as these will be undoubtedly discussed at length in other chapters. We will portray the many types of molecules that microorganisms possess that can be detected by plants, through the many families of plant proteins that play a critical role in pathogen perception and in which subcellular locations they initiate signaling. We will describe events surrounding pathogen detection, important cell signaling events that lead to inducible defenses, and PCD, which are critical for determining the outcome of plant–pathogen interactions. We will provide an enticing glimpse into the evolutionary battles of defense and counter defense that continues between adapted plant pathogens and their host plant species, highlighting the multiple levels at which pathogens try to steal control of host cells.

The first challenge that an invading pathogen faces when it lands on the plant is breaking though preformed barriers, such as the waxy cuticle and cell wall, in addition to surviving in the presence of secondary metabolites and any antimicrobial compounds constitutively present in the tissues of a particular plant species [4]. The presence of preformed structures and chemicals can present successful obstacles to infection and can be partly responsible for all members of a plant species possessing resistance to all isolates of a pathogen species, termed "nonhost" resistance [4].

10.2 PAMPs and PAMP-Triggered Immunity

If a microbe can survive and breakthrough the preformed barriers, the next challenge is to sequester the nutrients it needs from the plant and begin to grow on the plant surface or intercellularly by entering through wounds or stomata. However, all microbes, whether pathogenic or beneficial, possess an array of exposed structures and chemicals that the plant can recognize as "nonself" molecules. Examples of characteristic structures that are recognized by plants include β-glucan [5], chitin [6], and ergosterol [7] from fungi and lipopolysaccharide (LPS) [8], the translation elongation factor EF-Tu [9], and flagellin from Gram-negative bacteria [10, 11]. The original definition "pattern-associated molecular patterns (PAMPs)" describes these molecules as highly conserved within a class of microbes that have an essential function in microbial fitness or survival [12, 13]. Some PAMPs are also required for virulence. For instance, a *Pseudomonas syringae* pv. *tabaci* flagellin mutant that evades recognition also displayed reduced virulence *in planta* due to reduced

motility [14]. In addition, as these conserved molecules may also occur in non-pathogenic and symbiotic microorganisms, the alternative term microbe-associated molecular pattern (MAMP) is sometimes preferred [15].

PAMPs/MAMPs are recognized by receptors, known as pattern recognition receptors (PRRs), which are most commonly associated with the plasma membrane [16]. PRR activation by PAMP recognition displays similarities in subsequent signal transduction mechanisms between animal and plants, such as generation of reactive oxygen species (ROS) and MAP kinase activation that leads to differential gene expression [17]. Perception of PAMPS/MAMPS and the subsequent downstream induction of defenses are known as PAMP-triggered immunity (PTI) [18]. The detection of certain PAMPs can also induce an HR-like cell death, although, presently, there are more PAMPs studied thus far which do not induce cell death following perception. PTI is more commonly associated with induction of a range of antimicrobial compounds and cell wall thickening, known as callose depositions, in the vicinity of the detected PAMP [17]. PTI is considered to be the primary layer of inducible defense against pathogenic intruders in the zigzag model [18]. The zigzag model is currently the favored means of describing the coevolution of molecular defense and counter defense mechanisms that shape many plant–pathogen interactions and will be referred to throughout this chapter.

Bacterial flagellin, for example, is recognized as a PAMP by leucine-rich repeat (LRR) domains of the receptor FLAGELLIN-SENSING 2 (FLS2) in *Arabidopsis* [19] and by the Toll-like receptor (TLR5) in vertebrates [20]. Both receptors, however, recognize highly conserved, but different peptide fragments of flagellin [21, 22]. This suggests that although PAMP perception systems of plants and animals share similarities, they may have evolved independently via convergent evolution [23]. FLS2 is a plasma membrane-localized receptor-like kinase (RLK) that detects apoplastic flagellin through its extracellular leucine-rich repeat (LRR) domain [24–27]. The recognition of flagellin by FLS2, and particularly the active twenty two amino acid peptide-derivative flg22, is one of the best studied PAMP response in plants to date, and so we will describe this later as our principle model for PAMP-triggered immunity (PTI) [15, 24–26]. A second PAMP in the flagellin protein of *P. syringae* populations has been recently identified, termed flgII-28 [28]. Interestingly, the recognition of flgII-28 seems to be limited to Solanaceae plants and in an FLS2-independent manner, indicating that another receptor may be employed.

Another highly potent bacterial PAMP is the elongation factor EF-Tu. It is one of the most abundant bacterial proteins released from dying bacteria during plant colonization [29, 30]. In the same way a peptide of flagellin is perceived by FLS2, an N-acetylated peptide comprising the N-terminal 18 amino acids termed elf18 is specifically recognized in *Arabidopsis*. The corresponding receptor, elongation factor Tu receptor (EFR), is a Brassicaceae-specific PRR and belongs, like FLS2, to the RLK family [15].

Besides broadly conserved PAMPs, like flagellin and EF-Tu, some studies have shown that some PAMPs are more narrowly conserved such as a 13-amino acid surface-exposed fragment from a cell wall transglutaminase, named Pep13, which is only conserved among *Phytophthora* species [12].

Within seconds after flg22 perception in plants, FLS2 forms a complex with the regulatory LRR-RLK BRI1-ASSOCIATED KINASE 1 (BAK1), which is also known as SOMATIC EMBRYOGENESIS RECEPTOR-LIKE 3 (SERK3). Phosphorylation of both FLS2 and BAK1 occurs immediately, which is critical for downstream PTI responses [31]. Both FLS2 and BAK1 have been shown to interact with the plasma membrane-associated RLCK (receptor-like cytoplasmic kinase) BOTRYTIS-INDUCED KINASE 1 (BIK1) [32]. Intriguingly, FLS2 undergoes ligand-induced endocytosis and subsequent degradation after treatment with active flg22 [33], and subsequent de novo receptor synthesis resensitizes cells for a new round of stimulus perception [34]. In turn, BIK1, which positively regulates flg22 calcium influx, directly interacts with and phosphorylates plasma membrane-borne NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), in a calcium-independent manner [35]. In addition, another BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) negatively regulates cell death, activation of constitutive defense responses, and activation of MITOGEN-ACTIVATED PROTEIN KINASE 4 (MPK4) [36]. It appears both flg22 and elf18 trigger a conserved set of BAK1-dependent signal transduction events and defense responses in *Arabidopsis*, suggesting a common signaling pathway exists downstream of both receptor proteins [37]. RBOHD has also been demonstrated to interact with both receptor kinases EFR and FLS2 [38]. PTI studies extended to other plant species have also shown many PAMP responses to be regulated by BAK1 such as *Phytophthora infestans* elicitin INF1 in *Nicotiana benthamiana* [39]. BAK1 belongs to the SERK family that contains five LRR-RLK proteins that are able to bind to multiple and in some cases the same RLKs [40]. Interestingly, BAK1 was first characterized as essential for activating the brassinosteroid (BR) hormone receptor RLK, BRASSINOSTEROID-INSENSITIVE 1 (BRI1) [41, 42].

PTI signaling after receptor activation is known to activate mitogen-activated protein kinase (MAPKs) phosphorylation signaling cascades, production of reactive oxygen species (ROS), and transient elevation of cytosolic calcium ion levels which in turn induce transcriptional changes to coordinate antimicrobial defenses such as stomatal closure [17, 43]. These MAPK cascades have a similar structure to other eukaryotic MAPK-based signaling. In response to a stimulus, a MAPK kinase kinase (MAPKKK) phosphorylates, and thus activates, a MAPK kinase (MAPKK) which in turn phosphorylates a MAPK. When MAPKs are activated, they phosphorylate specific downstream transcriptional activators which lead to activation of cellular responses. It is predicted that the *Arabidopsis* genome contains 20 MAPKs, 10 MAPKKs, and more than 80 MAPKKKs [43].

Several MAPKs involved in plant defense response have been identified, but organizing them into clear pathways involving upstream receptors MAPKKKs, MAPKKs, and MAPKs and the downstream factors has remained challenging. In *Arabidopsis*, a complete cascade induced by flagellin has been identified (see Supplemental Fig. 10.1). A MAPKKK named AtMEKK1 activates the MAPKKs, AtMKK4, and MKK5 which in turn dephosphorylate AtMPK3/AtMPK6 which phosphorylates WRKY22/WRKY29 transcription factors [44]. An example of a target gene, rapidly upregulated after flg22 treatment in *Arabidopsis*, is FRK

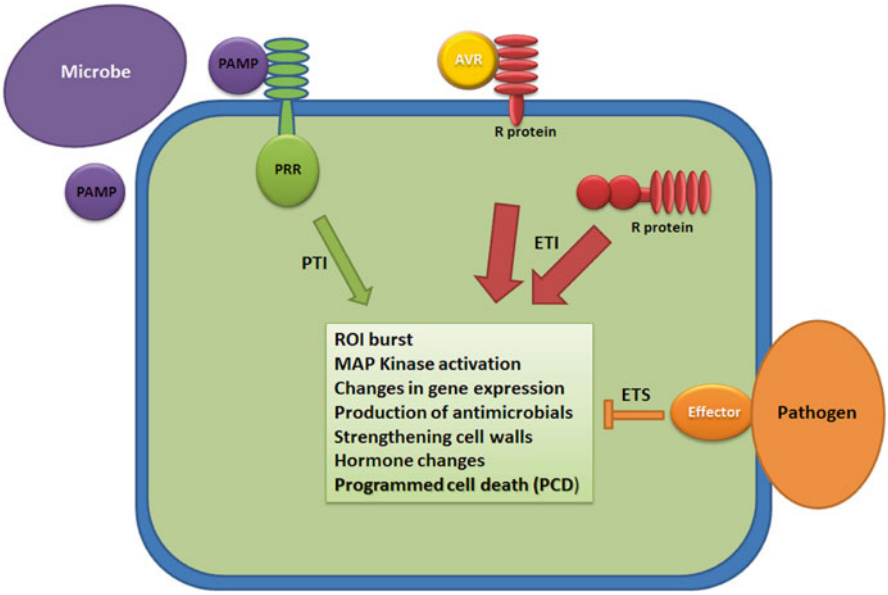

Fig. 10.1 Overview of plant immune responses. Pathogen-/microbe-associated molecular patterns (*PAMP*) are recognized by pattern recognition receptor (*PRR*); avirulent (*AVR*) effectors are recognized directly or indirectly by resistance (R) proteins inducing effector-triggered immunity (*ETI*); effectors have evolved to suppress defense responses in host plants, in a process known as effector-triggered susceptibility (*ETS*). Both PTI and ETI can result in programmed cell death (*PCD*) but also induce other signaling events described in the *box* which can occur independently of cell death

(FLG22-INDUCED RECEPTOR KINASE) [44]. However, more recently, it was suggested that AtMEKK1 may play a structural role in AtMPK4 signaling, independent of its protein kinase activity following experiments using a kinase-impaired version of AtMEKK1 [45]. MPK3 and MPK6 are closely related proteins that show a high level of functional redundancy. Both MAPKs are key regulators of a diverse set of processes also activated by other PAMPs such as elf18 and chitin. The H2O2-activated MAPK cascade also shares MPK3 and MPK6 activation with the flg22 pathway but activates different target genes [46].

The MAPKKK of the flagellin cascade, AtMEKK1, also initiates a different MAPK cascade. Bimolecular fluorescence complementation (BiFC) analysis showed that AtMEKK1 interacts with AtMKK1 and AtMKK2, two closely related MAPK kinases, which activate AtMPK4 after flg22 perception [47]. Intriguingly, and similarly to the mpk4 and mekk1 mutants, *Atmkk1 Atmkk2* double mutant seedlings accumulate high levels of H_2O_2, display spontaneous cell death, constitutively express pathogenesis-related (PR) genes, and exhibit pathogen resistance. This alternative AtMEKK1-activated cascade appears to negatively regulate innate immune responses, including cell death. Furthermore, activation of some endogenous MAPKs by expression of a constitutively active form, such as MKK4 or

MKK5, leads to HR-like cell death [47]. ROS production will be discussed in more detail later. See Fig. 10.1 for an overview of plant immune responses.

10.3 Overcoming PAMP Perception

According to the current paradigm, PAMPs are invariant surface structures that are extremely difficult for microbes to alter without decreasing their own fitness. However, since recognition by plants exposes microbes to host defenses, pathogens must adapt to avoid recognition of any PAMPs. This could be brought about by manipulating the PAMP itself as shown in flagellin studies which provided insight into PAMP plasticity, whether it is changes in PAMP expression [48], sequence variation [49], or posttranslational modifications [50]. However, many host-adapted symbionts and pathogens have evolved to secrete additional proteins that are targeted to host plant cells to perturb the PTI signaling after PAMP perception has occurred or have evolved to disguise the presence of PAMPS.

It is a commonly held theory that well-adapted plant pathogens and symbionts have evolved methods to evade or overcome the PTI response in host plants in order to successfully prolong colonization [51]. One of the most common strategies deployed by adapted pathogens is to secrete small proteins, known as effectors, into the apoplastic space between cells and/or delivering them or targeting them inside plant cells [52]. One of the key roles effectors play in disease development is to suppress PTI, and consequently every species of plant-interacting microbe has evolved at least one system to secrete effector proteins. Each individual pathogen isolate has a unique combination of effector proteins, thought to have coevolved through interactions with particular host plant proteins.

Effectors are proteins and secondary metabolites generated by pests and microbes that need to directly interfere with the plant to enhance colonization of a host. This state of susceptibility of the plant is known as effector-triggered susceptibility (ETS) in the zigzag model [48]. Apoplastic-targeted effectors include cell wall-degrading enzymes (CWDEs), cysteine-rich proteins, and toxins which are generally more important for necrotrophs than biotrophs and hemibiotrophs [53, 54]. Apoplastic effectors from biotrophs have more refined roles in manipulation of the plant host such as suppressing the plant's early PAMP responses. Some apoplastic effectors function to mask the presence of PAMPs, for example, the lysine motifs (LysMs) containing effector Ecp6, from the fungal plant pathogen *Cladosporium fulvum*, which disguises the presence of chitin fragments [55]. The effector Pep1, from *Ustilago maydis*, binds to and inhibits a host peroxidase (POX12) to suppress the production of ROS and callose deposition at penetration sites [56]. However, apoplastic effectors are in danger of being recognized by either plant PRRs as PAMPs or by *R* genes with LRRs oriented extracellularly [57].

Many pathogen effectors functionally characterized thus far have been shown to enter plant cells to manipulate intracellular plant mechanisms and processes. Since the focus of this review is not effectors and effector functions, we will focus on a

few examples of effectors that suppress PTI induced by flg22 perception. AvrPtoB from *Pseudomonas syringae* pv. *tomato* DC3000 is translocated into plant cells, has E3 ligase activity, and binds many host proteins with kinase domains, including the PRRs FLS2 and CERK1 [58–60], and the co-receptor BAK1 [61]. In addition, AvrPtoB's ubiquitin ligase activity inhibits immunity-associated PCD induced by several R proteins [59]. Further evidence suggests that AvrPtoB acts as a general eukaryotic cell death suppressor [62]. Another bacterial effector, AvrPphB from *P. syringae* pv. *phaseolicola*, has cysteine protease activity and targets PBS1 (*avrPphB* SUSCEPTIBLE), BIK1, and PBL1 (PBS1-like) which are important regulators of some PRRs [63]. Another *P. syringae* pv. *tomato* effector, known as HopA1, is a phosphothreonine lyase and targets some of the key MAPKs in flg22 responses including MPK3, MPK4, and MPK6 [63–65]. The RXLR effector Avr3a from *Phytophthora infestans* targets the ubiquitin ligase CMPG1 which plays a role in regulating the cell death induced by PAMP elicitin INF1 in *Nicotiana* species and other recognition events that signal from the plasma membrane [66, 67]. Despite some of the abovementioned examples, enzymatic activities of most pathogen effectors have been difficult to identify because many have no sequence or structural similarity to known protein domains.

10.4 Effector Recognition by Resistance Genes

Plants possess a large number of resistance (R) proteins that scan the intercellular and intracellular environments for effectors and signs of effector activity [68]. Any secreted pathogen effectors are consequently at risk of being recognized in the plant by a cognate R protein which triggers PCD in the form of the HR. This PCD and other associated inducible defenses are now also known as effector-triggered immunity (ETI) from the zigzag model and are believed to deny biotrophic pathogens the nutrients and conditions required for growth, thus preventing further infection spread [17, 18, 69]. The region of cell death is under tight genetic control to restrict cell death to the infection site and prevent the spread of unnecessary or disease-associated cell death (see supplemental film). The HR PCD is carried out by enzymes, such as cathepsins, caspases, and metacaspases, which are conserved with other types of PCD and with PCD in other organisms [69]. The type of PCD shown to be most important during the HR involves autophagy (an intracellular process for the degradation of cytosolic macromolecules and organelles in the lysosomes or vacuoles) [70, 71]. The ETI response uses many signaling mechanisms conserved with the PTI response, but signaling occurs with more speed and causes more severe immune responses that typically result in PCD. However, it should be considered that PTI and ETI are highly interconnected and cannot always be described as separate mechanisms as both recognition mechanisms can lead to PCD of tissue at the site of attempted infection [69, 70].

An effector known to be recognized by an R protein usually becomes known as an avirulence (AVR) protein (Table 10.1). Harold Henry Flor first described a

Table 10.1 Avirulent effector proteins, host targets, and resistance proteins in plant–pathogen interactions

Pathogen	AVR protein	Host	R protein	Recognition	Target	Guarded by R protein	References
Melampsora lini (flax rust fungus)	AVRL567	*Linum usitatissimum* (flax)	L5, L6, L7	Direct	?	N/A	[72]
Magnaporthe grisea (rice blast fungus)	AVR-Pita	*Oryza sativa* (rice)	Pi-ta	Direct	?	N/A	[73]
Ralstonia solanacearum (bacterial wilt)	PopP2	*Arabidopsis thaliana* (thale cress)	RRS1-R	Direct	?	N/A	[74]
Xanthomonas oryzae (bacterial blight)	AvrRxo1	*Zea mays* (maize)	Rxo1	Direct	?	N/A	[75, 76]
Pseudomonas syringae (bacterial speck)	HopZ1a	*Arabidopsis thaliana*	ZAR1	Indirect	ZED1	ZED1	[77]
Pseudomonas syringae	AvrB, AvrRPM1, AvrRpt2	*Arabidopsis thaliana*	RPM1, RPM1, RPS2	Indirect	RIN4	RIN4	[78, 79]
Pseudomonas syringae	AvrPto	*Solanum lycopersicon* (tomato)	PRF	Indirect	PTO BAK1 FLS2 EFR RIN4	PTO/RIN4	[80] [81] [82] [83]
Pseudomonas syringae	AvrPphB	*Arabidopsis thaliana*	RPS5	Indirect	PBS1 BIK1 PBL1	PBS1	[63] [84] [85]
Cochliobolus victoriae (victoria blight fungus)	Victorin	*Arabidopsis thaliana*	LOV1	Indirect	TRX-h5	?	[86]

Phytophthora infestans (late blight, oomycete)	AVR3a	*Solanum tuberosum* (potato)	R3a	Indirect	CMPG1	?	[66, 67, 87, 88]
Phytophthora infestans	PiAVR2	*Solanum tuberosum*	R2	Indirect	BSL1	BSL1	[89, 90]
Cladosporium fulvum (tomato leaf mold fungus)	AVR2	*Solanum pimpinellifolium* (currant tomato)	Cf-2	Indirect	RCR3 (pim)	?	[91]
Globodera rostochiensis (potato cyst nematode)	Gr-VAP1	*Solanum pimpinellifolium* (currant tomato)	Cf-2	Indirect	RCR3 (pim)	?	[92]
Phytophthora infestans	EPIC1 EPIC2B	*Solanum lycopersicon* (tomato)		Not recognized	RCR3 (pim)	N/A	[54]
Xanthomonas campestris pv. *Vesicatoria* (bacteria)	AvrBs3	*Capsicum annuum* (pepper)	Bs3	Indirect	UPA20 UPA7 UPA16	Upa box in promoter of Bs3	[93–95] [96] [97]

"gene-for-gene" concept which explained that a single pathogen effector would trigger an HR in a host plant which expressed the cognate *R* gene [98]. It was conceived that effectors would interact with the R proteins in a manner akin to ligands with receptors as in hormone signaling. Subsequently, much scientific effort was spent identifying and cloning pairs of *AVR* and *R* genes. Some of the first were shown to interact directly confirming the notion that R proteins were direct receptors for AVR proteins, and this was incorporated into Flor's concept (see first 3 examples in Table 10.1).

An R protein detects the presence of a particular effector and is under high evolutionary selection pressure to coevolve with pathogens in natural environments [99]. Each individual pathogen isolate secretes a unique combination of effector proteins; in some cases, hundreds are known to be upregulated during infection on a host plant. There are large numbers of effectors that any plant could encounter from various pathogen sources which drive evolution of a relatively large number of *R* genes. Consequently, every plant species/cultivar possesses a diverse and unique range of different classes of *R* genes [18, 100]. On the other hand, pathogens have to keep evolving to evade the detection of any of their essential effectors in plants. This drives fast birth and death rate of alleles of both the effector genes in pathogen races and *R* genes in plant cultivars and wild species.

10.5 Guard Hypothesis

In many cases, research has struggled to show any direct interaction of AVR and R proteins. In addition, the genomic era highlighted that the large number of effectors in pathogen genomes was far greater than the number of *R* genes in plant genomes to protect them from every potential encounter on a gene-for-gene basis. Subsequently, the "guard hypothesis" was conceived [18, 101], suggesting that AVR proteins are recognized by their cognate R protein when the effector manipulates a host target protein during its role in promoting disease (see supplemental Fig. 10.2). Effectors are thought to be detected indirectly by their action/activity on host target proteins, distinguished as unfamiliar modifications by R proteins from the plant's own regulatory mechanisms. The target, or guardee, is therefore presumed to have virulence function for the pathogen, i.e., the effect of the AVR protein on the guardee contributes to pathogen fitness [102]. The R protein may recognize "modified self" if the target is altered by the AVR protein. Alternatively, the R protein may be dislodged from, or recruited to, the guardee upon AVR protein binding, triggering defense.

The guard hypothesis model also provides a solution to the perceived lack of *R* genes in plant genomes. Plants would require fewer R proteins when detecting changes to key regulators of major pathways. Furthermore, one guard R protein could potentially detect attack by multiple effectors which target the same host

protein: for example, AtRIN4 (RPM1 INTERACTING PROTEIN 4) is targeted by three unrelated effectors, AvrB, AvrRPM1 (RESISTANCE TO PSEUDOMONAS SYRINGAE SSP. MACULICOLA 1), and AvrRpt2, from *P. syringae* (Table 10.1). To achieve immune suppression, AvrB and AvrRPM1 induce phosphorylation of RIN4 to increase its activity [103], and phosphorylation of RIN4 is guarded by resistance protein RPM1 [78, 79] triggering the HR. AvrRpt2 has a different effect on the target, resulting in cleavage of RIN4 into separate domains that can suppress PTI [104]. This cleavage of RIN4 is monitored by the resistance protein RPS2 [79]. A fourth effector, $HopF2_{Pto}$, has also been shown to interact with RIN4 [105] and to suppress the RPS2-mediated HR. This is indicative of effector-triggered susceptibility 2 (ETS2), where pathogens evolve a further effector set to combat ETI.

Pathogen virulence targets, or guardees, have been used as tools to identify key points of weakness in plant defense responses and may represent "hubs" that many pathogens target to suppress immunity. The oomycete *P. infestans* secretes the effector protein PiAVR2, triggering a HR in *Solanum tuberosum* when resistance protein R2 is present [89]. Again, recognition is indirect and involves the phosphatase BSL1 [90]. When PiAVR2 binds to its host target BSL1, the resistance protein then interacts with BSU LIKE PROTEIN1 (BSL1), resulting in an active immune response. R2 appears to guard BSL1 and is responsive to changes induced by effector binding. BSL1 is an orthologue of BSU1 (*bri1* SUPRESSOR1) in *Arabidopsis*, involved in brassinosteroid (BR) signaling. An overactive BR pathway antagonizes PTI [106, 107]; therefore, pathogens have much to gain by its manipulation.

Other points of cross-talk exist between PTI and the brassinosteroid signal transduction pathway—one of these is the receptor-like cytoplasmic kinase BIK1. This is a negative regulator of BR signaling, but a positive regulator of PTI signaling [108], and is one of several cytoplasmic kinases in *Arabidopsis* targeted by the *Pseudomonas syringae* effector AvrPphB, a cysteine protease. These kinases are cleaved by AvrPphB, inhibiting PTI responses usually triggered by flg22, elf18, and chitin [63]. One of the cleaved targets, PBS1, is another example of a guardee, monitored by the resistance protein RPS5 and resulting in a HR when conformational change is detected [84].

Effector proteins may also act to inhibit host protein activity, for example, AVR2 from *C. fulvum,* which functions as a protease inhibitor, suppressing activity of the cysteine protease RCR3 (REQUIRED FOR *C. fulvum* RESISTANCE 3) in *Solanum pimpinellifolium* [91]. The interaction of effector and target is recognized by the receptor-like protein Cf-2, triggering a HR and immunity. Cysteine proteases are thought to have antimicrobial activity; thus, it is of benefit to the pathogen to suppress them [91]. A nematode effector has recently been shown to target the same protein; Gr-VAP1 of *G. rostochiensis* modifies the active site of RCR3, triggering Cf-2-mediated PCD [92]. This highlights the ability of guard proteins to potentially recognize not only multiple effectors but multiple pathogens.

10.6 Evolution, Structure, and Function of Resistance Genes

Currently there are five major classes of resistance genes in plants based on the presence of a limited number of conserved structural domains. One group contains a distinctive intracellular serine/threonine kinase (STK) domain, such as PTO, which requires the partner, PRF [81]. Another group, which could be classed with PRRs, is characterized by extracellular LRR domains, a transmembrane region, and either an intracellular STK domain or a short region without known motifs. An example of this class would be Cf-2 from tomato (Table 10.1) [92]. Yet the vast majority of plant *R* genes cloned to date belong to the nucleotide-binding (NB) leucine-rich repeat (LRR) class of immune receptors [101], which are thought to be the largest group of genes in the plant kingdom [109]. Potato (*S. tuberosum*) has 755 genes NB-LRRs found to date [110], while the model plant *A. thaliana* has 151 genes [111] and the cereal maize (*Zea mays*) has only 95 genes [112]. NB-LRRs are also represented in the basal land plants with the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* having 25 and 2 NB-LRRs, respectively [113, 114]. When compared with the human complement of 22 NB-LRR genes, it is clear that the most impressive and extensive expansion of the NB-LRRs has occurred in the angiosperms [115]. Structurally, NB-LRRs are modular proteins made up of well-defined domains, some known to be involved in regulating PCD in animals. Although plant NB-LRRs resemble animal NB-LRR immune receptors in structure and function, it is thought that this has occurred through convergent evolution rather than a shared common ancestor [114].

The NB domain occurs in several different protein families including ATPases and G proteins, and its high conservation in the largest class of *R* genes suggests that nucleotide-binding state is important for their function. The core nucleotide-binding fold is part of the larger NB-ARC domain, named after the first three proteins it was identified in: human apoptotic protease-activating factor-1 (APAF-1), plant R proteins, and *Caenorhabditis elegans* death-4 protein (CED-4) [116]. Intriguingly, APAF-1 and CED-4 are also known to be involved in regulating PCD in animals [116]. In fact, the NB domain of some R proteins can be sufficient for host cell death responses [117]. The NB-ARC domain in many *R* genes has been shown to be a functional ATPase domain [118]. Several other domains help make up the NB-ARC domain, including the kinase 1a, (or P-loop domain), 2, and 3a domains, as well as other short conserved motifs with as yet unknown functions such as Gly–Leu–Pro–Leu (GLPL) [101]. Mutations in this region can result in either loss-of-function or autoactivation where PCD is initiated in the absence of a pathogen or AVR protein [81, 119, 120]. Evidence from the 3D structures of APAF-1 and CED-4, various loss-of-function mutations of *R* gene NB-ARC domains, and homology modeling techniques revealed that the plant NB-ARC domain forms an ADP-bound [121] globular nucleotide-binding pocket, which is closed when the protein is inactive [122].

The NB-LRR family can be subdivided into two groups dependent on the domain present at the N-terminus. TIR-NB-LRRs (TNLs) have N-terminal domains with

homology to the *Drosophila* toll and human interleukin-1 receptor (TIR), while some CC-NB-LRRs (CNLs), but not all, have a predicted coiled-coil (CC) domain within the protein's N-terminus [123]. Some NB-LRRs which fall into the CNL class of receptors are often referred to as non-TIR-type NB-LRRs (nTNLs) as it is difficult to identify specific structural domains in the N-termini of these proteins [124]. Interestingly, TNL and nTNLs are evolutionary distinct as they group into two monophyletic clades when the phylogeny is based on the NB-ARC domain [114]. Representatives of both TNLs and CNLs are found in *Physcomitrella patens*, evidence that both NB-LRR groups are evolutionary ancient [114]. However, TNLs have been lost from the genomes of monocotyledonous plants but are present in basal angiosperms and gymnosperms, suggesting that cereals dispensed with TNLs for plant immunity but retained and expanded their complements of CNLs [125].

NB-LRRs genes are distributed throughout the plant genome but tend to cluster together, for example, 76 % of NB-LRRs are distributed through 92 clusters in the potato genome [110]. Cluster size can vary, with some clusters containing more than 10 NB-LRRs and can contain either CNLs or TNLs exclusively, or a combination of both [126]. The diversity of NB-LRR complements of different plant species has been produced by various means including gene and genome duplications, gene conversions, unequal crossing over, and ectopic recombination [126]. NB-LRR numbers appear to be regulated by "birth and death" process by which new NB-LRRs evolve by diversifying selection, while some are lost and others are retained [127], thus preventing any fitness cost to the host [128]. NB-LRRs genes can be broadly classed as either fast- or slow-evolving, referred to as type I or type II genes, respectively. Type I genes frequently show sequence exchanges among homologs which can result in extensive chimeric structures, while type II genes are thought of as independently evolving and highly conserved within populations or species as gene conversions are relatively rare in these genes [129]. Using *Arabidopsis* as an example, it appears that the majority of NB-LRR genes classed as type I belong to multigenic families or are found in clusters, while those genes classed as type II are more likely to be found isolated in the genome [130].

The TIR and CC domains are thought to be involved in signaling of the R protein. CC domains are well known for their role in homo- and heterodimerization of eukaryotic transcription factors, and similar coiled-coil domains may promote protein–protein interactions. Indeed, the CC domain of MLA10 which provides powdery mildew resistance in barley was found to be a compact, rod-shaped structure made up of two identical CC-subunits. It is thought that the shape of the CC domain allows a large surface for inter-protein interactions [131]. The TIR domain's similarity to Toll and IL-1R from animals suggests the TIR plays a role in signaling. The structure of the TIR domain of L6 has been revealed to be globular-shaped, consisting of two monomeric units which results in a defined fold in this protein domain [132]. Interestingly, the TIR and CC domains of some NB-LRRs have proven to be sufficient for cell death induction as in L6 from flax and MLA10 from barley, respectively [131, 132]. However, MLA10 also requires the short NB linker region as well as the CC domain for downstream signaling.

The LRR domain has long been associated with recognition specificity of NB-LRRs and PRRs. Extensive mutational analyses of some R proteins has highlighted that the LRR domain has a high degree of tolerance for substitutions that drive the evolution of recognition specificity [120, 133, 134]. Interestingly, if the LRR of BRI1 is fused to the STK domain of Xa21, the resulting protein signals in a brassinosteroid-inducible manner in rice cells [42]. Nevertheless, the variability within the LRR domain of NB-LRR proteins has proved difficult for 3D structure modeling and prediction. Much of what is known about the structure of LRR domains of NB-LRRs comes from the structure of leucine-rich repeats within a porcine ribonuclease inhibitor [135] and subsequent homology modeling of different LRR structures held in protein databases. LRRs are thought to be compact, horseshoe-shaped structures when inactive [117]. Diversity studies of RPS2 suggest that the LRR sequence can also determine interactions with other host factors and so may not only play a role in recognition [136].

10.7 Activation and Function of NB-LRRs

After discussing the structure and function of the individual domains, what do we know about how R proteins actually function to trigger PCD? One theoretical model for the structure of a full-length NB-LRR proposes an electrostatic interaction between the NB-ARC domain and the N-terminus of the LRR domain, which stabilizes the protein and encourages it to remain in a closed conformation [117]. Residues thought to be involved in this interaction appear to be conserved in NB-LRRs, suggesting that this pattern of folding is important in the function of these proteins. The C-terminus of the LRR domain is thought to extend from the folded protein like an antenna and from this position is able to function in pathogen sensing. The model then predicts that the remaining domain, either a CC or a TIR domain, interacts with both the NB-ARC and LRR domains at the same time giving rise to a tightly folded, auto-inhibited protein which is able to remain responsive to pathogen invasion (Fig. 10.2).

Upon perception of a pathogen, either directly or via a change in the cellular environment, the LRR domain of an NB-LRR is thought to transduce a signal to the other domains within the protein, resulting in a lifting of auto-inhibition. As mentioned previously, when the NB-LRR protein is in an inactive state, the NB-ARC domain is bound by ADP. Upon activation by pathogen perception, the NB-ARC domain is bound by ATP, and the entire protein switches to an open conformation [117]. This conformational change in the protein would potentially uncover previously buried residues within the protein, thus allowing these newly exposed portions to interact with other proteins (Fig. 10.2). Although it is well known that *R* genes can confer resistances to various pathogens, it is still little understood how NB-LRR proteins are able to bring about pathogen resistance in the host.

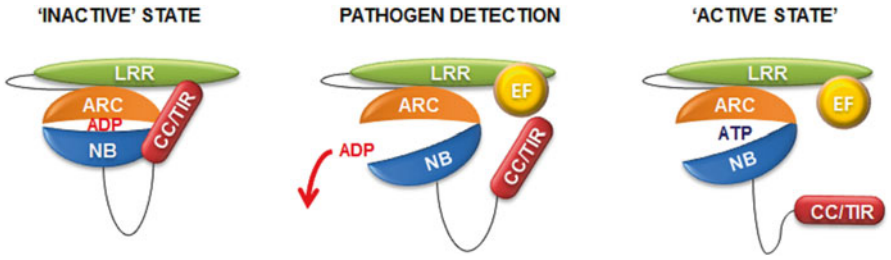

Fig. 10.2 Proposed model for the protein structure of a full-length NB-LRR. Electrostatic interactions hold the NB-ARC domain and the N-terminus of the LRR domain together in a closed conformation. The C-terminus of the LRR domain extends from the folded protein like an antenna and functions in pathogen sensing. The CC/TIR domain interacts with both the NB-ARC and LRR domains, resulting in a tightly folded, auto-inhibited protein able to respond to pathogen invasion. Upon direct or indirect pathogen perception, the LRR domain transduces a signal which lifts auto-inhibition from the NB-LRR protein. The inactive, ADP-bound NB-ARC domain becomes ATP-bound, and the protein adopts an open, active conformation, leaving the NB-LRR able to establish defense signaling (Modified from [122])

10.8 Chaperones of NB-LRR Genes

Tight regulation of R proteins by the host is essential to prevent inappropriate signaling and induction of PCD in the absence of a pathogen and to date a small number of R protein-interacting molecular chaperones have been characterized. The most well-known family of molecular chaperones is the heat shock protein family, and one subclass of these proteins, the cytosolic HEAT SHOCK PROTEIN 90 (HSP90) protein family, plays a particularly important role in plant disease resistance [137–139].

One of the first studies implicating HSP90 in R protein interactions was a genetic screen identifying mutants impaired in *RPM1*-mediated resistance to the *P. syringae* effector AvrRpm1 [139]. Single amino acid substitutions in the ATPase domain of HSP90 were found to be responsible for reducing the steady-state levels of RPM1 in non-challenged plants. Subsequent structural analysis of HSP90 revealed a protein with three domains: an N-terminal ATPase domain (ND), a middle domain (MD) implicated in protein binding, and a C-terminal dimerization domain (CD). Dimerization of two HSP90 proteins has been revealed to be essential for HSP90 function [137].

The highly conserved SGT1 (SUPPRESSOR OF G2 ALLELE OF SKP1) protein has also been found to be an essential component in the regulation of most NB-LRR proteins [140]. It is involved in numerous cellular processes including ubiquitination and kinetochore assembly, as well as the maturation of R proteins, and it has been shown that accumulation of the potato R protein, Rx, was reliant on the interaction between SGT1 and HSP90 [138, 141]. Similar to the structure of HSP90, SGT1 consists of three domains: the tetratricopeptide repeats (TPR), the CHORD (cysteine- and histidine-rich domains)-containing protein, and SGT1

domain (CS) and the SGT1-specific domain (SGS), with the SGS domain providing the connection between SGT1 and the LRRs of R proteins. Another protein co-chaperone required for R protein regulation is RAR1 (REQUIRED FOR MLA12 RESISTANCE), which is composed of two CHORD domains. CHORD1 and CHORD2 of RAR1 are both zinc-binding, with each domain binding to two zinc atoms, giving rise to the structure of the protein [142]. It is thought that together HSP90, SGT1, and RAR1 form a complex which mediates the stabilization, maturation, and regulation of R proteins.

The HSP90–SGT1–RAR1 ternary complex is thought to be initiated when the CHORD1 domain of RAR1 binds to an ND domain of one HSP90 protein of a HSP90 dimer [143]. This leads the CHORD2 domain of RAR1 to be in closer proximity to the ND domain of the second HSP90 protein in the dimer, allowing binding to occur [143]. The binding of RAR1 to both proteins within the HSP90 dimer theoretically holds the dimerized proteins in an open conformation, allowing the CS domain of SGT1 to bind to the CHORD2 domain of RAR1, thus altering the conformation of SGT1 and boosting its affinity for NB-LRR proteins which are now brought into this protein complex [138]. The binding of RAR1-SGT1-R protein to the HSP90 dimer is thought to allow ATP hydrolysis of the ATP-binding pocket of HSP90. Along with the release of ADP, mature R proteins, SGT1 and RAR1 are able to dissociate from the protein complex [138] (see Supplemental Fig. 10.3).

While binding with HSP90, it is known that SGT1 can simultaneously bind with the SKP1P-CDC53P-F BOX (SCF) E3 ubiquitin ligase subunit Skp1 via its TPR (TETRATRICOPEPTIDE REPEAT) domain [144]. This finding has led to proposals that SGT1 could link plant disease resistance with the activity of E3 ubiquitin ligases, suggesting that ubiquitination may play a part in the regulation of NB-LRR proteins [143].

10.9 Localization of R Proteins

Upon effector-induced activation of NB-LRR resistance proteins, downstream signaling culminating in defense reactions is commonly associated with extensive transcriptional reprogramming. Until some years ago and consistent with this observation, the theory of plant resistance proteins accumulating in the nucleus was widely accepted, but recent studies have revealed that, in fact, the requirement of nuclear localization for activation of the HR may be the exception rather than the rule. Three different subcellular localizations, where plant resistance proteins activate defense responses, can be distinguished: nucleus, nucleus/cytosol, and other subcellular compartments except the nucleus. Some examples for each pattern are described below:

1. *Nucleus*: In the presence of the powdery mildew effector AvrA10, the barley CC-NBS-LRR resistance protein MLA10 activates defense responses by trans-

locating into the nucleus, where it interacts with both WRKY transcriptional repressors and the transcriptional activator MYB6 [145, 146]. The *Arabidopsis* TIR-NBS-LRR resistance protein RPS4 is required to accumulate in the nucleus in order to mediate the HR in the presence of its cognate effector AvrRps4 [147–149].

2. *Nucleus/cytosol*: RPS4 is also a good example for showing that a single resistance protein may activate distinct signaling pathways in the cytoplasm and nucleus, indicating the importance of coordinated nucleocytoplasmic trafficking. Bacterial growth is inhibited, but RPS4-mediated HR is blocked when AvrRps4 is forced to accumulate in the nucleus, and sequestration of the effector in the cytosol significantly impairs RPS4-mediated resistance but has little impact on the RPS4-mediated HR [149]. The potato CC-NBS-LRR resistance protein Rx on the other hand, which mediates recognition of the potato virus X coat protein (PVX CP) and localizes to both the nucleus and the cytosol [150, 151], is not activated in the presence of forced nuclear PVX CP accumulation [152]. Moreover, it was observed that sequestration of Rx in the nucleus impairs its function, while forced cytosolic accumulation enhanced its function [153].
3. *Subcellular compartment*: For some plant NB-LRR proteins, other subcellular localizations are required which likely reflect the localization of the corresponding pathogen effector proteins. The *P. syringae* effector AvrPphB, for example, is targeted to the plasma membrane (PM) after myristoylation of an N-terminal motif [154]. AvrPphB is detected by RPS5 which is also acylated at its N-terminus and localizes to the PM [155, 156]. Interfering with the PM localization by targeted mutation of the predicted acylation sites disrupts the RPS5-mediated HR [156]. Despite lacking predicted acylation motifs, RPM1, another plant NB-LRR protein, is also most likely activated on the PM, where its corresponding effector AvrB and co-activators RIPK and RIN4 localize [103, 157, 158]. Beside the PM, NB-LRR proteins have also been localized to other endomembrane locations. For example, the flax rust resistance proteins L6 and M localize to the Golgi apparatus and the tonoplast, respectively [159]. Additionally, the recognition of the *P. infestans* effector Avr3aKI by the potato resistance protein R3a and subsequent signal initiation is likely to occur at endocytic vesicles. In the presence of recognized effector derivatives, R3a relocates from the cytoplasm to endosomal compartments, which is required for the defense activation [88].

10.10 HR Signaling Following Pathogen Recognition

Following pathogen recognition via PTI or ETI reactive oxygen species (ROS), reactive nitrogen species (RNS) and the downstream phytohormone salicylic acid (SA) are produced. These signaling molecules are common to both immune responses and are associated with HR induction.

10.11 ROSProduction During the HR

The link between ROS and the HR was first established by Doke who showed that an avirulent but not a virulent isolate of *P. infestans* induced superoxide production rapidly in potato prior to the onset of the HR [160]. This oxidative burst is biphasic, with a transient rise in ROS ~30 min following pathogen inoculation followed by a second rise which continues until PCD is triggered [161, 162]. The ROS produced include the superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2).

Several sites of ROS production exist within the plant cell including chloroplasts, mitochondria, and peroxisomes. Recent observations suggest that ROS generated in these organelles are important for the HR. In mammals, mitochondria play an important role in PCD; however, in plants, there is little evidence that mitochondria are involved. Flavodoxins are electron transport proteins found only in photosynthetic organisms such as cyanobacteria but not in higher plants. Transgenic plants expressing a plastid-targeted flavodoxin produce low levels of ROS compared to untransformed control plants when challenged with a nonhost pathogen. As a consequence, the HR was inhibited in these flavodoxin transformants, although the induction of other defense responses associated with the HR such as PR gene induction and SA synthesis remained intact [163]. These results are consistent with previous studies that the HR can be uncoupled from pathogen resistance [164]. Peroxisomes provide a rich source of H_2O_2 through the glycolate oxidase (GOX) reaction. Silencing of *GOX* in *N. benthamiana* delayed the onset of the HR in response to a nonhost pathogen and ETI [165].

An O_2^- burst in the mitochondrial matrix of tobacco was found to precede the HR, in response to an avirulent but not virulent strain of *Pseudomonas syringae* pv. *maculicola*. The generation of this mitochondrial O_2^- burst was shown to be most likely mediated via alternative oxidase (AOX) activity [166].

However, the majority of evidence implicates an NADPH oxidase as the main source of ROS in plants, analogous to the enzyme generating the oxidative burst during the mammalian immune response. These integral plasma membrane proteins generate O_2^- in the apoplast which is rapidly dismutated to the more stable molecule, H_2O_2. The RBOH family is key enzymatic subunits of the plant NADPH oxidase. Of the ten members of the AtRBOH gene family in *Arabidopsis* [167], AtRBOHD is the major source of ROS generation during the HR. However, the double mutant *atrbohD*/*atrbohF* shows that both AtRBOHD and AtRBOHF are required to mediate a full oxidative burst [168].

ROS-scavenging pathways determine the magnitude, lifetime, and distribution of oxidative signals modulating cell death. These include superoxide dismutase (SOD), which scavenges superoxide radicals whereas; H_2O_2 is removed through the catalytic action of ascorbate peroxidase (APX) and catalase (CAT). The isozymes of these enzymes have different compartmentalization and H_2O_2 affinities indicating different roles in H_2O_2-scavenging and ROS signaling; APX isoforms are chloroplastic and cytosolic with micromolar H_2O_2 affinity, whereas CAT has millimolar

H_2O_2 affinity and isoforms are found in the cytosol, peroxisome, and mitochondria [162, 169]. The high capacity of CAT to turnover H_2O_2 (~6 million molecules of H_2O_2 to H_2O and O_2 per min) suggests a key antioxidant role. The *Arabidopsis* genome contains three homologous catalase genes (*CAT1*, *CAT2*, and *CAT3*); CAT2 and CAT3 represent the major enzymatic activity in vegetative tissues. Genetic studies have revealed that CAT2 and CAT3 interact with the zinc finger protein LESION STIMULATING DISEASE 1 (LSD1) to regulate the HR in response to avirulent *P. syringae* [170].

10.12 Nitric Oxide (NO) Production During the HR

Nitric oxide (NO) is an important signaling molecule, due to its reactive nature and small size which means it can diffuse across membranes. NO is produced in response to avirulent pathogen challenge and is pivotal for HR induction. RNS include the radical $NO^{\bullet}$, the nitrosonium ion (NO^{+}), the *nitroxyl* anion (NO^{-}), and ONOO– formed from the reaction of NO with ROS. Occurring immediately prior to the onset of the HR, NO production is concomitant with the generation of ROS [171, 172] and shows a biphasic pattern of production similar to that of ROS. An initial NO burst occurs a few minutes after infection followed by a second sustained increase in NO for several hours. For example, in tomato, challenge with the powdery mildew fungus, *Oidium neolycopersici*, caused a rapid NO burst in both susceptible and resistant cultivars, but sustained NO production was only observed in the resistant tomato cultivars. This NO production occurred simultaneously with ROS production followed by HR cell death of penetrated epidermal cells, preventing further pathogen growth [173]. Similarly, infection of barley with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* resulted in a transient NO burst in epidermal cells preceding the HR [174]. NO production is also required for the progression of the HR response to *P. syringae*, mediating defense against these bacterial pathogens [171, 175].

NO production in mammals is primarily a function of NO synthase activity (NOS). The inducible NOS isoform, iNOS, is part of the mammalian immune response producing NO from an L-arginine substrate. A homolog of the mammalian NOS which also uses L-arginine as a substrate was found in the unicellular green algae *Ostreococcus tauri* [176]. However, the search for NOS in higher plants has remained elusive, despite several studies that support L-arginine-dependent NO production reminiscent of a NOS activity [177–179].

There are alternative routes for NO production in higher plants. Nitrate reductase (NR) is a key enzyme involved in nitrogen assimilation, catalyzing the transfer of electrons from NADPH to nitrate to produce nitrite [180]. However, NR can also catalyze the NADPH-dependent reduction of nitrite to NO [181]. Cytosolic NR is emerging as an important NO source in plants with a role during pathogen defense. For example, in *Arabidopsis*, cytosolic NR is encoded by *NIA1* and *NIA2*, and *nia1/*

nia2 double mutants failed to produce NO and to develop an HR following challenge with avirulent strains of *P. syringae pv. maculicola* (*Psm*) [175].

NO can also be formed in the acidic environment of the plant apoplast, synthesized via the nonenzymatic reduction of nitrite by reducing agents such as phenolics or ascorbic acid [182]. Scavenging NO is key to regulate NO signaling and the onset of the HR. NO can be eliminated by oxidation to NO_3 following the formation of an oxidized form of Hb (methaemoglobin). Estimates of PCD using electrolyte leakage in response to an avirulent *P. syringae* strain demonstrated that *GLB1*-suppressed lines had higher NO production and enhanced cell death, whereas overproduction of *GLB1* suppressed NO production and reduced PCD [183]. Cellular NO levels can also be regulated by the turnover of RNS derivatives, for example, ONOO–, which can in turn be detoxified by peroxiredoxins [184].

10.13 S-Nitrosylation

A stable and mobile NO reservoir is formed in the cell via the interaction of NO with the major intracellular antioxidant glutathione (GSH) to form *S*-nitrosoglutathione (GSNO). GSNO can subsequently transfer its NO group to specifically modify other cellular thiols to form *S*-nitrosothiols. Cellular GSNO homeostasis is controlled by nitrosoglutathione reductase (GSNOR), an enzyme conserved in bacteria, animals, and plants [185, 186]. *S*-nitrosothiol formation is a posttranslational modification called S-nitrosylation which can alter protein structure and activity. An increasing number of S-nitrosylated proteins have been reported, several of which have roles in the HR including AtRBOHD [187], peroxiredoxin IIE [184], and the salicylic acid-binding protein (SABP3) [188]. For example, *SABP3* is a chloroplast carbonic anhydrase (CA) that reversibly converts CO_2 to bicarbonate but may also have an antioxidant activity. S-nitrosylated SABP3 was identified from *Arabidopsis* tissue undergoing the HR, and this was found to inhibit both its SA binding and its CA activity. Therefore, SABP3 S-nitrosylation may be part of a negative feedback loop to limit the HR [188]. Interestingly SABP3 was also identified as a candidate for tyrosine nitration by ONOO–.

10.14 NO and ROS Interplay

There is considerable interplay and cooperation between RNS and ROS signaling to trigger and regulate the HR progression [171, 189, 190]. An appropriate balance between ROS and NO production is required for HR induction. For example, in soybean cell suspensions, high NO levels require a corresponding ROS burst generating H_2O_2 to induce cell death [191]. Antisense APX lines with a reduced capacity to scavenge H_2O_2 show enhanced PCD symptoms in response to the NO donor sodium nitroprusside (SNP) [192], suggesting that high NO and H_2O_2 levels are

needed to promote PCD. Furthermore, APX is S-nitrosylated during PCD, inhibiting its activity and promoting increasing cellular H_2O_2 levels [193].

AtRBOHD is also S-nitrosylated, reducing its NADPH oxidase activity and blunting ROS production and the progression of the HR [184]. This AtRBOHD S-nitrosylation may govern a negative feedback loop to limit the HR. Interestingly, the NO donor SNP was found to induce the transcript levels of *ZmRBOHB* and *ZmRBOHD* in maize, suggesting that NO controls NADPH oxidase function at several nodes and may initially promote the production of ROS [194].

The production of NO and ROS together can lead to the reaction of NO with superoxide (O_2^-) to generate the highly reactive peroxynitrite ion (ONOO–). Although ONOO– levels increase during the HR in *Arabidopsis* plants challenged with avirulent *P. syringae* pv. *tomato* [195], it does not appear that ONOO– promotes PCD in plants as it does in animals [191]. NO and ONOO– inhibit the activity of the major H_2O_2 scavenging enzymes CAT and APX in plant cells [196]. The accumulation of ONOO– induces the tyrosine nitration of proteins the levels of which have been found to increase the HR. Again this posttranslational modification influences protein structure and activity [184, 197]. The plastid-localized peroxiredoxin IIE (PrxIIE) can detoxify both H_2O_2 and ONOO–. During the HR, S-nitrosylation of PrxIIE has been demonstrated [184], inhibiting PrxIIE activity and leading to increases in cellular H_2O_2 and ONOO– levels. Therefore, the S-nitrosylation of PrxIIE during the HR in turn regulates the extent of tyrosine nitration mediated by ONOO–. Accumulating evidence is now emerging of the intricate interplay of RNS and ROS, regulating the accumulation of the other and modulating the progression of the HR.

10.15 The Role of Salicylic Acid in the HR

Resistance to biotrophic pathogens generally involves SA-dependent signaling pathways and culminates in the HR at the site of pathogen attack [18]. SA accumulation is induced by downstream signaling following ROS production from NADPH oxidase [198]. In fact ROS and SA can act synergistically together to promote the HR [199, 200]. It has been demonstrated that SA inhibits the respiratory chain in mitochondria by disrupting the electron flow leading to ROS generation further driving the HR [201]. The application of SA to plants promotes PCD, for example, SA potentiates cell death in pathogen-treated soybean suspension cells and several lesion mimic mutants display elevated SA levels or related SA signaling phenotypes [202].

In addition, runaway cell death in the lsd1 mutant is triggered by ROS, SA, and following inoculation with an avirulent pathogen [203]. Furthermore, the breakdown of H_2O_2 by catalase (a SABP) from tobacco is inhibited by the binding of SA [204]. Therefore, these studies indicate that SA and ROS act together in a self-amplifying feedback loop.

NO also positively impacts SA production in response to pathogen challenge [186, 190]. *Arabidopsis glb1* mutants lacking the function of the NO-oxidizing hemoglobin GLB1 accumulated higher levels of SA in response to *Pst* AvrRPM1 [184]. The chloroplast is an important source of SA where it is synthesized from chorismic acid by an isochorismate synthase (ICS1). SA is exported to the cytosol by EDS5 (ENHANCED DISEASE SUSCEPTIBILITY5) which functions as a multidrug and toxin extrusion-like transporter [205]. The production of ROS in the chloroplast is also regulated by SA [206]. A link between the chloroplast and the HR is supported by findings that in several plant–pathogen systems, SA accumulation and development of the HR are light dependent. For example, *Arabidopsis* plants inoculated with an avirulent strain of *P. syringae* in the dark are unable to accumulate SA or undergo HR development. Furthermore, SA applied in the dark failed to induce the HR in response to the turnip crinkle virus (TCV) in *Arabidopsis* (reviewed in [207]).

Recently, the regulatory switch that controls PCD in response to SA was elucidated and found to involve the NPR (NON EXPRESSOR OF PATHOGENESIS RELATED GENES 1) family, with NPR1 being central. SA levels are high around pathogen infection sites where PCD is induced. Under these conditions, NPR3 binds SA, and this mediates NPR1 breakdown, releasing the suppression that NPR1 has on PCD. In sites distal from pathogen infection, SA binds NPR4 (which has a much higher affinity for SA), blocking the proteasome-mediated degradation of NPR1 promoting cell survival [208]. NPR1 therefore acts as a master switch in terms of SA-mediated PCD. NPR1 is itself regulated by the redox state of the cell. The cysteine residues of NPR1 form intermolecular disulfide bonds forming NPR1 oligomers in the cytosol. It is interesting that the redox modification S-nitrosylation promotes NPR1 oligomerization. Following the reduction of NPR1, these oligomers are disrupted, and monomers are able to enter the nucleus and regulate defense-related gene expression [209].

Finally the HR not only restricts pathogen growth at the site of pathogen attack but perpetuates the generation of long-range signals, mediated by ROS and SA, to induce systemic acquired resistance priming the plant for resistance to secondary infection [210–212]. For a brief overview of the role of the signaling molecules ROS, NO, and SA during the HR, see Fig. 10.3.

10.16 Summary

We have described here some of the important processes that trigger PCD during plant defense responses to biotrophic pathogens. The actual mechanisms of PCD during PTI and ETI share many similarities with other types of PCD in plants and with PCD in other eukaryotes. Unfortunately, there is so much relevant and interesting information that we did not have space to include here. Scientists are continuing to work together toward building a comprehensive picture of the signals, changes in gene expression and protein interactions that regulate PCD during plant–pathogen

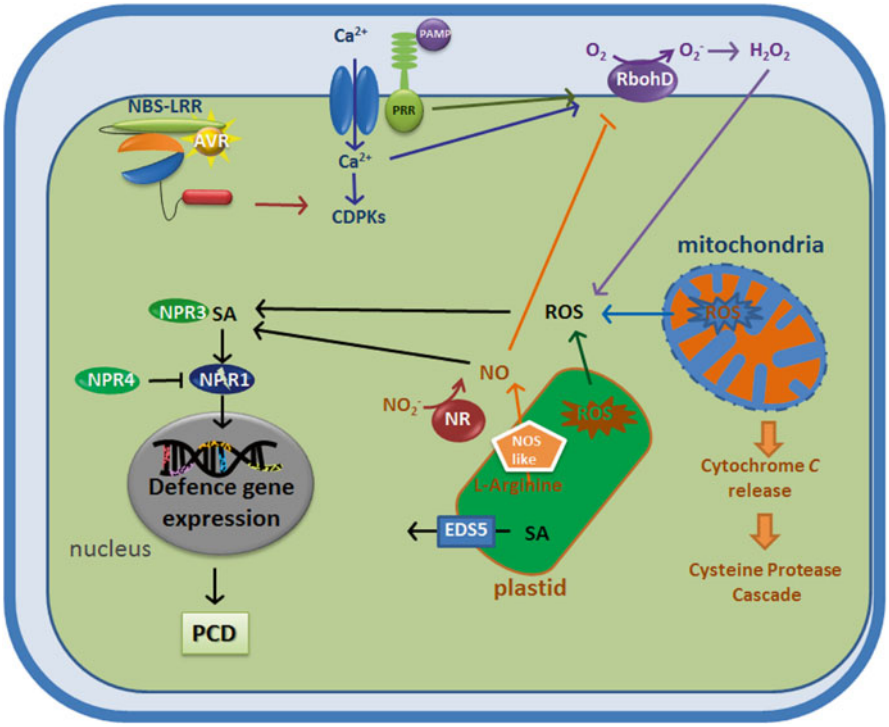

Fig. 10.3 Brief overview of the role of the signaling molecules ROS, NO, and SA during the HR. The recognition of avirulent (AVR) effector activity in plants by NBS-LRRs leads to calcium (Ca^{2+}) influxes across the plasma membrane (PM) that activate CALCIUM DEPENDENT PROTEIN KINASES (CDPKs). Calcium signaling is thought to activate the PM bound NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), which generates superoxide O^{2-} that undergoes dismutation to hydrogen peroxide (H_2O_2) which is both toxic to pathogens and also initiates PCD during the HR. There are several other sites for ROS production within the plant cell including chloroplasts and mitochondria. NADPH oxidase can also be activated by other signals such as those from RECEPTOR-LIKE CYTOPLASMIC KINASEs (RLCK), e.g., BOTRYTIS-INDUCED KINASE 1 (BIK). An increase in the permeability of the mitochondrial membrane and subsequent cytochrome C release can be caused by common cellular stress signals including ROS, elevated calcium levels, or inhibition of electron transport. Cytochrome *C* release activates the cysteine protease cascades that can lead to PCD. L-arginine-dependent NO production has been detected in plants reminiscent of a mammalian nitric oxide synthase (NOS)-like activity. NO can also be produced from nitrite via cytosolic nitrate reductase (NR), an emerging important NO source during the HR. Nitric oxide regulates the production of ROS via RBOHD by S-nitrosylation of the enzyme inhibiting further ROS production and PCD progression. An appropriate balance between ROS and NO production is required for successful HR induction. Both ROS and NO positively impact on SA production and signaling, together regulating the HR response. SA is synthesized in the chloroplast and exported to the cytosol by EDS5 (ENHANCED DISEASE SUSCEPTIBILITY5). NPR3 binds SA mediating NPR1 breakdown, releasing the suppression that NPR1 has on PCD. Conversely, SA binds NPR4 with higher affinity, blocking the proteasome-mediated degradation of NPR1 promoting cell survival

interactions. Currently, there is still a lot to learn about what determines which cells live or die. We hope we have provided a tantalizing glimpse of the molecular warfare that can result in PCD during plant–pathogen interactions.

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Chapter 11
To Live or Die: Autophagy in Plants

Brice E. Floyd, Yunting Pu, Junmarie Soto-Burgos, and Diane C. Bassham

Abbreviations

4E-BP1	eIF4E-binding protein 1
AML1	*Arabidopsis* Mei2-like 1
ATF6	Activating transcription factor 6
ATG	Autophagy-related
EBP1	ErbB-3 epidermal growth factor receptor binding protein
ER	Endoplasmic reticulum
HR	Hypersensitive response
IRE1	Inositol-requiring enzyme-1
LST8	Lethal with Sec Thirteen 8
MAMP	Microbe-associated molecular pattern
PAMP	Pathogen-associated molecular pattern
PAS	Phagophore assembly site
PCD	Programmed cell death
PE	Phosphatidylinositol
PP2A	Protein phosphatase 2A
PtdIns3K	Phosphatidylinositol-3-kinase
RAPTOR	Regulatory-associated protein of TOR
ROS	Reactive oxygen species
S6K	Ribosomal p70 S6 kinase
SA	Salicylic acid
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
TOR	Target of rapamycin
UPR	Unfolded protein response
VPE	Vacuolar processing enzyme
VPS	Vacuolar protein sorting

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11.1 Introduction

During the lifetime of a plant cell, its constituents are turned over to remove damaged components, recover nutrients and energy tied up in macromolecules, and eliminate production of destructive reactive oxygen species (ROS) from damaged chloroplasts, mitochondria, and peroxisomes. While short-lived proteins tend to be degraded via the proteasome in the cytosol or by proteases within organelles, much of the degradation of macromolecules and larger structures occurs by the process of autophagy, literally defined as "self-eating" [1]. Upon activation of autophagy, cellular components are transferred into the vacuole through a unique vesicle trafficking pathway and are degraded by lytic enzymes therein. The breakdown products are then transported back into the cytoplasm for reuse by the cell.

Several types of autophagy have been described in plant cells, including the conserved macroautophagy and microautophagy pathways and a third, possibly plant-specific, pathway termed mega-autophagy. Most of the available molecular information relates to macroautophagy, while the other pathways have been described morphologically. In microautophagy, invagination of the tonoplast to form intravacuolar vesicles leads to direct engulfment of cytoplasmic constituents and tonoplast components into the vacuole [2]. This has mostly been studied during developmental transitions, including the conversion of protein storage vacuoles to lytic vacuoles during seed germination in legumes [3–5] and during petal senescence [6, 7]. Interestingly, a biosynthetic pathway related to microautophagy has been described in wheat seeds, in which storage proteins are delivered to protein storage vacuoles via engulfment of ER-derived protein bodies by the vacuole [8, 9].

Mega-autophagy has been classed as an autophagy pathway as, like micro- and macroautophagy, it results in degradation of cellular contents by vacuolar enzymes. However, unlike the more typical autophagy types, it does not involve uptake of material into the vacuole. Instead, permeabilization and then rupture of the tonoplast leads to release of vacuolar hydrolases into the cytoplasm [10]. Also in contrast to other autophagy pathways, mega-autophagy invariably leads to cell death rather than cell remodeling or stress tolerance [11]. Because of this fundamental difference in mechanism compared with other autophagy types, it is sometimes referred to as autolysis rather than autophagy.

Macroautophagy occurs at a low basal level to maintain proper cell function, but can be upregulated by stress conditions and developmental cues. Upon initiation, a membrane structure known as a phagophore forms and begins to engulf material to be degraded (Fig. 11.1). This phagophore expands by the addition of membrane from various sites within the cell until it finally closes to form a double-membrane vesicle called an autophagosome that contains the autophagic cargo [12]. The completed autophagosome is transported to the vacuole, in a process that may involve the cytoskeleton [13], where the outer membrane fuses with the tonoplast. The inner membrane with its contents is released into the vacuole as an autophagic body, and attack by vacuolar hydrolases leads to its breakdown and recycling.

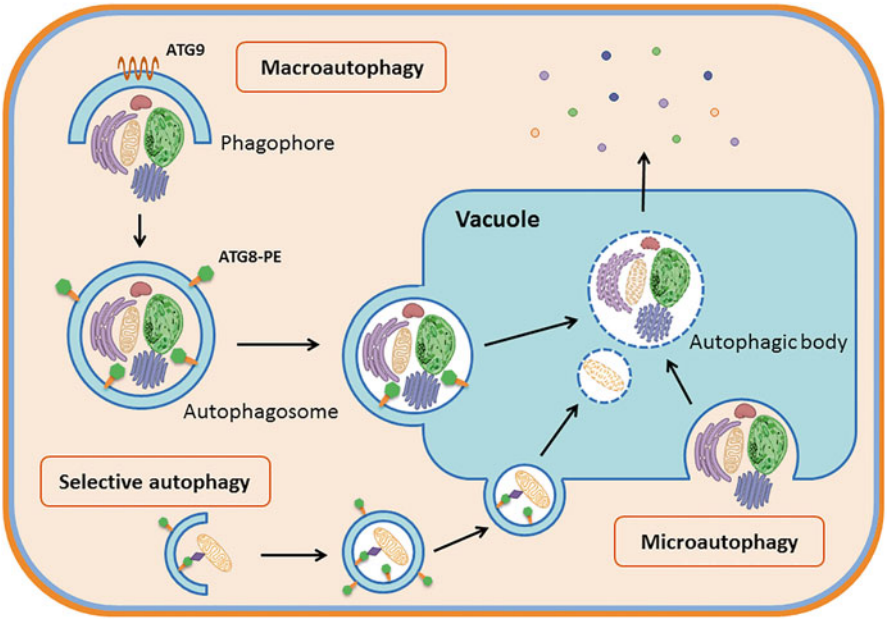

Fig. 11.1 Major autophagy pathways in plants. When macroautophagy is triggered, the formation of a double-membrane cup-shaped structure called the phagophore is initiated with the aid of ATG9 and begins to surround organelles or protein aggregates to be degraded. Closure of the phagophore forms a double-membrane vesicle called an autophagosome. ATG8 is required for autophagosome formation and expansion. ATG8 is anchored to the autophagosome membrane by conjugation to phosphatidylethanolamine (PE) and thus is used as a marker to visualize autophagosomes. Autophagosomes carry the cargo to the vacuole, where the outer membrane of the autophagosome fuses with the vacuolar membrane, releasing the inner membrane and the cargo into the vacuole as an autophagic body. Autophagic bodies are degraded in the vacuole to small molecules and exported back to the cytosol for reuse. Selective macroautophagy is a specialized form of macroautophagy in which a receptor (*purple diamond*) recognizes both a specific organelle or protein and also ATG8. The specific cargo is thus recruited to the autophagosome and delivered to the vacuole for degradation. In microautophagy, the tonoplast directly engulfs the cargo, forming an autophagic body that is degraded inside the vacuole

Autophagy has been best studied in plants as a response to stress conditions that enables cell survival by recycling of nutrients or clearance of cellular damage. Paradoxically, there are many examples in which autophagy also functions in programmed cell death by extensive degradation of cell components, allowing the nutrients and energy within to be reclaimed by the plant [14]. While this process has sometimes been termed autophagic cell death, in many cases autophagy merely accompanies cell death rather than being a direct cause. The mechanisms controlling this switch in function from cell survival to cell death are mostly unknown, although studies in animal cells are just beginning to elucidate these pathways [15]. In this chapter we review the pathway, functions, and regulation of autophagy in relation to its role in cell survival and cell death during stress and development.

11.2 Molecular Mechanisms of Autophagy

While several types of autophagy have been described morphologically in plants [2], significant molecular information is available only for macroautophagy, which we will discuss here. The macroautophagy pathway can be divided into several steps: induction, autophagosome formation, cargo selection and packaging, vesicle fusion, and breakdown [16].

11.2.1 Induction

At least three complexes are involved in the induction of autophagy, the target of rapamycin (TOR) kinase complex [17], phosphatidylinositol-3-kinase (PtdIns3K) complex [18], and autophagy-related (ATG)1/ATG13 complex [19]; control of autophagy will be discussed in the section on regulation.

11.2.2 Autophagosome Formation

Autophagosomes are formed from the expansion of a membrane core, termed a phagophore. The phagophore assembly site (PAS) is the proposed site for autophagosome initiation [20] and is defined in yeast as the place where almost all of the ATG proteins transiently colocalize [21]. Autophagosome formation requires the recruitment of two conjugates, ATG12–ATG5 and ATG8–PE, to the PAS. ATG12–ATG5 and ATG8–PE are generated by ubiquitin-like conjugation systems described below. Both conjugation systems have been reconstituted in vitro in *Arabidopsis* [22]. The ATG9 cycling system, PI3K complex, and ATG1 complex have also been implicated in autophagosome biogenesis [23–25]. This section will discuss the two ubiquitin-like systems and the PAS.

11.2.2.1 ATG12–ATG5 Conjugation System

ATG12 and ATG5 are covalently and irreversibly linked through an isopeptide bond between the C-terminal glycine of ATG12 and an internal lysine residue of ATG5 [26–28] (Fig. 11.2). Two enzymes, ATG7 and ATG10, are involved in this ubiquitination-related conjugation system. ATG7, acting as a ubiquitin-activating-like enzyme (E1), activates ATG12 by the hydrolysis of ATP, resulting in a thioester bond between the C-terminal glycine of ATG12 and a cysteine residue of ATG7 [29, 30]. The C-terminal glycine of ATG12 is transferred to a cysteine of ATG10, which functions as a ubiquitin conjugating-like enzyme (E2), and a new thioester bond is formed, releasing ATG7 [31]. An isopeptide bond is then formed between the

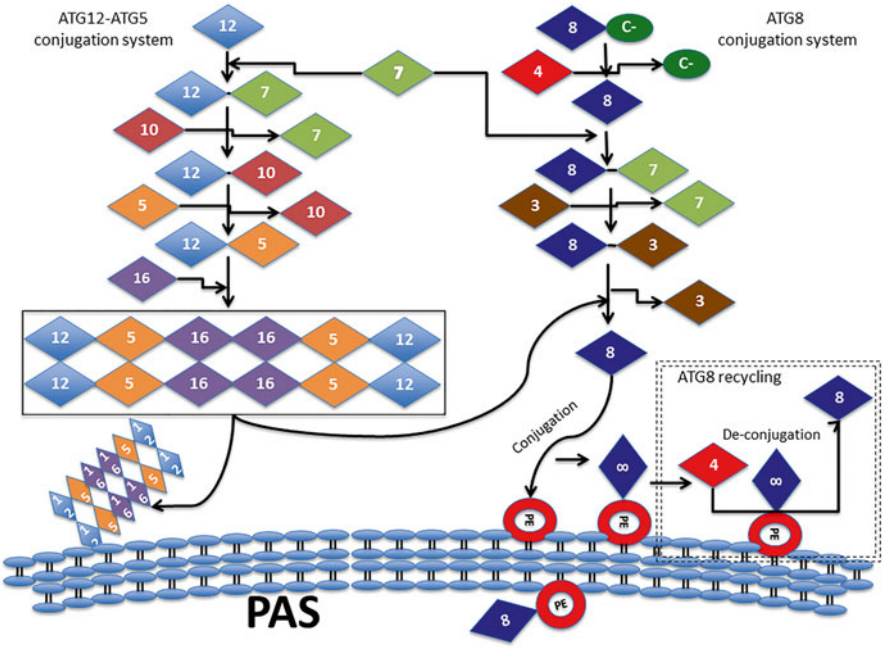

Fig. 11.2 Two ubiquitin-like conjugation systems function in autophagosome formation. (*Left*) The ATG12–ATG5 conjugation system is depicted. ATG12 binds to ATG7 and then is transferred to ATG10. Finally ATG12 is covalently conjugated to ATG5 to form the ATG12–ATG5 complex. This complex interacts with ATG16 and forms a tetramer, which is recruited to the PAS. (*Right*) The ATG8 conjugation system is depicted. First the ATG4 protease cleaves the C-terminus of ATG8, and ATG8 binds to ATG7. Next, ATG8 is transferred to ATG3. Once ATG8 dissociates from ATG3, it is conjugated to phosphatidylethanolamine (PE) in the PAS. The ATG12–ATG5•ATG16 complex may act as an E3-like enzyme during the conjugation of ATG8 with PE. Since the protease ATG4 can cleave the ATG8–PE adduct, this reaction is reversible, freeing ATG8 into the cytosol. ATG proteins are depicted as *diamonds with their respective number inside*

ATG12 C-terminal glycine and the amino group of a lysine in ATG5, releasing ATG10 and producing the ATG12–ATG5 conjugate. This conjugation is essential to the formation of autophagosomes [32]. The additional autophagy protein ATG16 self-oligomerizes and interacts with ATG5 within the ATG12–ATG5 conjugate, forming a tetrameric complex [33].

11.2.2.2 ATG8–PE Conjugation System

During autophagosome formation, ATG8 is conjugated to PE, a membrane lipid (Fig. 11.2). In this process three enzymes are involved: ATG4, ATG7, and ATG3. ATG4, a cysteine protease, first removes the C-terminus of ATG8, leaving a glycine exposed at the C-terminus [34]. ATG7, an E1-like enzyme, activates ATG8 by

covalently linking the exposed glycine of ATG8 to an active cysteine in ATG7. Once ATG8 is activated, it is transferred to an active cysteine residue in ATG3 through an E2-like mechanism [35]. ATG8 is then conjugated to a PE adduct and recruited to the autophagosome membrane [36]. The ATG12–ATG5–ATG16 complex has been suggested to function as an E3 enzyme and catalyze the conjugation step [37–39]. Unlike ATG12–ATG5 conjugation, the lipidation of ATG8 is reversible. The ATG4 protease can also deconjugate the ATG8–PE complex, recycling ATG8 and thus generating a conjugation cycle. This is essential for autophagosome expansion and the normal progression of autophagy [27, 34, 40].

11.2.2.3 Phagophore Assembly Site

Fine mapping of ATG protein localization during autophagosome formation suggested that ATG proteins have individual roles and distinct locations during autophagosome expansion [21]. The origin of the double membrane of the autophagosome is not well understood. Recent work has shown that in mammals autophagosomes can form at the endoplasmic reticulum (ER)–mitochondria contact site [41] and that ER exit sites as well as ER–Golgi intermediate compartments are important for this process [42–46]. This therefore gives insight into one of the possible membrane sources and initiation sites for autophagosome formation. In addition, it had been shown that the Golgi complex, endosomes, and plasma membrane may supply lipids for autophagosome biogenesis [47–50]. Unlike in animal cells, which have multiple sites at which phagophores can initiate, yeast have a single PAS, and ATG9 vesicles have been suggested to bring membrane to this location [51]. Overall, it seems that during autophagy new double-membrane vesicles form de novo with multiple membrane sources, rather than budding from pre-existing organelles [52].

The integral membrane protein ATG9 is recruited to the PAS with the help of a PtdIns3K complex at early stages of phagophore formation and may play a role in the nucleation and recruitment of other ATG components such as the ATG12–ATG5–ATG16 complex and ATG8–PE adduct [20]. In yeast, around three ATG9 molecules are required for one round of autophagosome formation [51]. Another important complex in ATG9 cycling and autophagosome formation is the ATG18–ATG2 complex. ATG18 binds to both ATG2 and to phosphatidylinositol-3-phosphate generated at the PAS, thus recruiting it to the PAS [53].

A recent study showed that the BAR domain protein, SH3P2, has a role in autophagosome formation in *Arabidopsis*. This protein is associated with the PtdIns3K complex and also interacts with ATG8. It is localized to the PAS and actively participates in fusion events during the formation of autophagosomes. It is likely that SH3P2 promotes expansion or maturation of the developing autophagosome membrane [54].

11.2.3 Cargo Selection

Although autophagy is generally considered to be a nonselective process, certain proteins can be delivered to the vacuole via autophagy at a rate that suggests they are selectively targeted [55, 56]. p62 and mNBR1 are major selective autophagy receptors in mammals, targeting ubiquitin-modified proteins and protein aggregates for autophagy [57–61]. A functional ortholog that appears to be a hybrid of p62 and mNBR1 has been discovered in *Arabidopsis,* termed NBR1 [62], and in tobacco, termed Joka2 [63]. NBR1 and Joka2 both bind to ATG8 isoforms. Additionally, NBR1 binds to ubiquitin-modified proteins for selective autophagy. A recent study proposed that NBR1 targets ubiquitinated protein aggregates under stress conditions, which are likely to consist of damaged or denatured proteins [64].

11.2.4 Vesicle Fusion

After completion, autophagosomes move to and fuse with the vacuole. The timing of this process is critical. Incomplete formation of the double-membrane autophagosome prior to fusion with the vacuole would result in the cargo remaining in the cytosol. In both yeast and animals, the machinery needed for vesicle fusion includes soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins [65]. In yeast, additional components have been shown to be required for fusion, including the vacuolar protein sorting (VPS)-C complex and Mon1-Ccz1 [66–74]. One SNARE important for autophagy is VTI1, a v-SNARE required for vacuolar trafficking. Several VTI1 homologs are present in *Arabidopsis* [75]. One of these homologs, VTI12, is thought to have a function during autophagy [76]. When the autophagosome finally fuses with the vacuole, the inner membrane of the autophagosome is released into the lumen of the vacuole as an autophagic body (Fig. 11.1).

11.2.5 Vesicle Breakdown

After release of the autophagic body into the vacuole lumen, it is degraded and its contents recycled (Fig. 11.1). In order for the degradation process to occur properly in yeast, the vacuole needs to be acidic and the activity of the vacuolar hydrolases Pep4 and Prb1 is required [77]. ATG15 is a lipase that is involved in this breakdown process and seems to function in the intravacuolar lysis of the autophagic body [78, 79]. ATG22 acts after the degradation process is complete to mediate the efflux of amino acids back to the cytoplasm [80]. In plants, it has been suggested that hydrolases are present inside the autophagic body from its formation, allowing degradation to begin before fusion with the vacuole; additional work is needed to confirm this [14, 81].

11.3 Autophagic Cell Death

Programmed cell death (PCD) in animals can be classified into three known pathways, apoptosis, necrosis, and autophagy. Apoptosis, also called type I cell death, has been the most studied form of cell death since its discovery in 1842 by Karl Vogt through developmental analysis. This was followed a century later by electron microscopy evidence describing the process in 1972 [82, 83]. The classic apoptosis pathway involves the mitochondrial-dependent activation of a family of cysteine proteases known as caspases. This causes a caspase activation cascade ultimately resulting in degradation of the nucleus as can be observed by chromatin condensation, DNA laddering, and nuclear fragmentation. Remaining cellular contents are contained in plasma membrane-enclosed blebs and engulfed by other cells. Apoptosis can also function independently from mitochondria and caspase signaling and instead respond, for instance, to ROS or DNA damage [84–86]. Necrotic PCD occurs as a response to mechanical damage or overwhelming stress, is activated rapidly, and can involve receptor-interacting proteins as well as caspases [84, 85]. Necrosis was originally believed to be accidental cell death, but more recent evidence suggests that it is programmed, at least in some cases [87]. During necrosis, cellular swelling and plasma membrane rupture occur by which cytoplasmic contents are lost [86].

Autophagy is a cellular pathway used under normal conditions to turn over cell components and maintain homeostasis. However, when highly upregulated it can also function during cell death, in which the cell's components are degraded extensively. Autophagic PCD, also called type II cell death, involves vacuolization of the cytoplasm on a large scale. Cytoplasm-containing vesicles, formed during autophagy, fuse with lytic organelles such as the lysosome or vacuole and are degraded [84, 85]. It is important to note, however, that the term "autophagic PCD" only describes autophagy as being present during cell death and not necessarily as the cause of cell death.

Early work in cell death studied each mode of cell death in isolation from other cell death mechanisms. However, accumulating evidence suggests that there is considerable overlap and signaling crosstalk between the different cell death pathways and how they are regulated [84, 85]. It is important to take into account this overlap when forming new conclusions in cell death research.

11.3.1 Autophagy in Cell Survival and Cell Death in Non-plants

In metazoans, autophagic PCD is essential for some developmental processes and can also lead to activation of other cell death pathways [88]. It is believed that in many organisms autophagy can function either in a pro-survival or a pro-death role, depending on the circumstances, as *atg* mutants can show phenotypes indicative of either increased cell death, such as neurodegeneration, or decreased cell death, such as enhanced cancer progression [89].

Autophagy as a pro-survival pathway has been studied extensively in yeast. Many *atg* mutants were identified initially due to their loss of viability under nitrogen starvation conditions. *atg* yeast cells undergoing nitrogen starvation are unable to maintain adequate amino acid and protein synthesis levels due to loss of protein degradation and recycling [90]. Upon starvation, wild-type cells upregulate respiratory pathways and ROS scavenging proteins, while *atg* mutants fail to do this, leading to ROS accumulation and deficient respiration [91]. Autophagy is therefore required for upregulation of starvation-induced proteins and may be important in maintaining mitochondrial integrity during N starvation. Dysfunction of mitochondria is the principal cause of cell death in yeast *atg* mutants, together with reduced protein synthesis and ROS accumulation, illustrating a pro-survival role for autophagy during nutrient starvation.

Autophagy can also function as a pro-death pathway either as the cause of cell death or by assisting in cytoplasmic clearance during other forms of cell death. Inhibiting caspase activity, which is essential for apoptosis, can induce autophagic cell death suggesting that autophagy can function in a pro-death role [92]. Autophagy can also lead to cell death by induction of apoptosis [93]. Recent work has found autophagy to be involved in both oncogenesis and tumor suppression in cancer. Autophagy's role in tumor cell death is further reviewed by [94, 95].

11.3.2 Autophagic Programmed Cell Death in Plants

Plant PCD pathways differ from those of metazoans, but as in animals, autophagy is also believed to function in PCD in plants under certain conditions [96]. During autophagic PCD in plants, also referred to as vacuolar cell death, organelles and cytoplasmic materials are taken up into autophagosomes and trafficked to the vacuole for degradation. The vacuole size increases and it eventually lyses, releasing lytic enzymes into the cytoplasm to further cell death (Fig. 11.3) [97]. Finally, the plasma membrane and cell wall are also degraded. Apoptosis as defined in animals, however, has not been shown to occur in plants. To date, there is limited evidence for an apoptosis pathway that involves mitochondria in plant development, and many of the animal apoptosis regulators are absent from plant genomes [101–104]. Furthermore, plants do not appear to have caspases similar in sequence to those found in metazoans. Also, plants lack the phagocytic cells that engulf and degrade cell debris following apoptosis, suggesting that autophagy and the endomembrane system may have a more prominent role in programmed cell death in plants. Plants do, however, have some features associated with apoptosis. For example, metacaspases seem to serve similar functions in plants as metazoan caspases [105]. Caspase-like activity and cytochrome c leakage from plant mitochondria have also been reported, indicating that apoptosis-like processes may occur in plant cells [106, 107]. Programmed cell death in plants has been difficult to classify using definitions formed in animal systems, and a discussion on the classification and of the types of programmed cell death in plants can be found in [96]. The classification shown in Fig. 11.3 is one way to describe plant PCD types, but is not universally accepted and other classifications are possible.

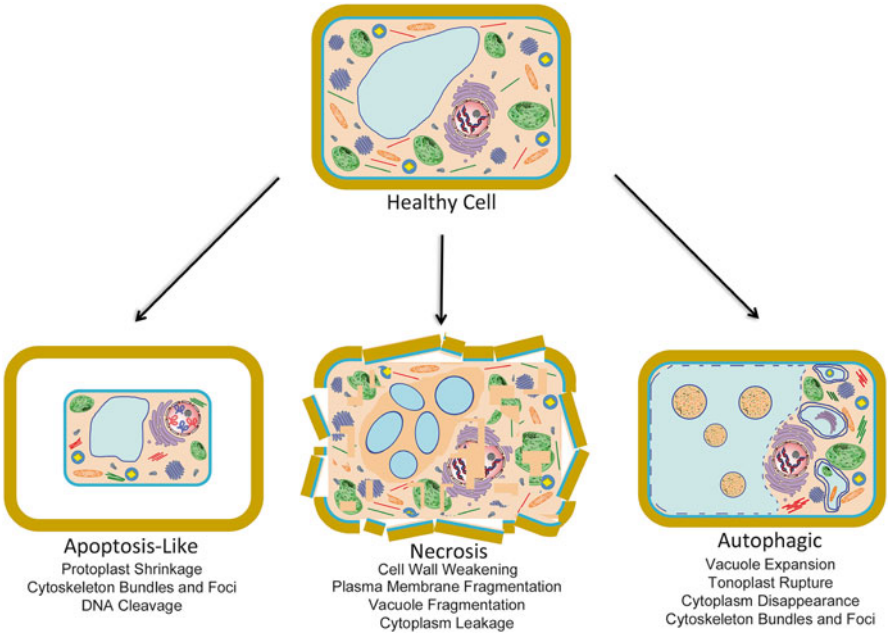

Fig. 11.3 Classes of programmed cell death described in plants. *Apoptosis-like PCD* involves shrinkage of the plasma membrane away from the cell wall while maintaining plasma membrane and vacuole membrane integrity. DNA cleavage into smaller fragments as well as condensation of the cytoskeleton into bundles and foci also occurs. Apoptotic cells in animal systems eventually break up into apoptotic bodies, but this does not occur in plants. *Necrosis* occurs as a result of severe cell stress and trauma or through programmed mechanisms. It involves cell and organelle swelling followed by breakup of organelles, plasma membrane, and cell wall. *Autophagic PCD* can be characterized by vacuolization of the cell on a large scale. The cytoskeleton thickens into bundles, organelles aggregate, and larger vesicles and vacuoles begin to form in the cytoplasm. Vesicles accumulate in the vacuole containing cytoplasm and degraded organelles. The vacuole membrane eventually ruptures, releasing lytic enzymes into the cytoplasm and furthering cell death [97–100]

Two major evolutionarily conserved forms of autophagy have been described in plants, microautophagy and macroautophagy. Microautophagy's contribution to cell death is not yet clear [108]. Macroautophagy, on the other hand, has the strongest links to metabolism and cell death and represents the best characterized autophagic mechanism [109]. A third type of autophagy, termed mega-autophagy, has been proposed to better describe the morphology of cells undergoing what appears to be autophagic PCD in plants [110]. Mega-autophagy is thought to occur later during autophagic PCD through permeabilization of the vacuolar membrane. This releases large amounts of vacuolar hydrolases into the cytoplasm leading to degradation of cytoplasmic contents. Tonoplast permeabilization and rupture seems to be common in plant cell death and has been observed during senescence, phloem cell development, aerenchyma formation, root cap cell formation, and xylem differentiation

[111–113] as reviewed by van Doorn and Woltering [110]. Unlike micro- and macroautophagy, mega-autophagy does not rely on membrane-bound vesicles to traffic cellular components to the vacuole and is therefore often termed autolysis rather than autophagy.

The vacuole is essential during autophagic cell death in plants. Not only is the vacuole the ultimate destination of autophagic vesicles, but it also houses vacuolar processing enzymes (VPEs). VPEs are cysteine proteases responsible for the activation of inactive protein precursors that function within the vacuole [114]. VPEs differ in protein sequence from metazoan caspases involved in cell death, but they have similar structural motifs, activities, and self-activation pathways [11, 101, 114, 115]. VPEs are involved in cell death through destruction of the vacuolar membrane and the release of hydrolytic enzymes to the cytoplasm [114, 116]. Such internal destruction is useful in plant cells as they are surrounded by cell walls, preventing phagocytosis of cell remnants by neighboring cells. The activity of VPEs and related proteins shows a conserved role for caspases and caspase-like activity during programmed cell death in metazoans and plants.

11.4 Physiological Roles of Autophagy

11.4.1 Developmental Autophagic Cell Death

One of the major roles of autophagy in a developmental process in plants is in senescence. The concepts of senescence and organism death are different between animals and plants. Somatic mutation, telomere attrition, and the molecular costs of repair and maintenance of an organism serve as a mark for senescence in animals [117]. However, in plants these death factors are less significant to the life span of the plant. Autophagy-related regulatory networks integrated with nutrient signaling may have a dominant part to play in the developmental senescence process [117]. Some theories in animal systems suggest that aging and progressive deterioration is a result of an imbalance between the energy demands of reproduction and growth and the cost of repair and maintenance [118]. Such a theory for senescence may not apply to plants since plants are material and energy rich [119]. Autophagy, and the growth and autophagy regulator TOR (see Sect. 11.5), may be key players in senescence and death within plants, as TOR is a point of convergence for energy status, nutrient availability, mRNA translation, autophagy, and cell longevity [117].

Autophagy is implicated in senescence of the whole plant. Modulation of senescence requires incorporation of multiple environmental and developmental signals to determine the time of senescence [117, 120]. *atg9* [23], *atg7* [121], *atg18* [122], and *atg5* [123] mutants all show an early senescence phenotype, suggesting a role for autophagy in control of senescence. However, the early senescence phenotype observed in autophagy mutants seems to be due to increased levels of the plant hormone salicylic acid (SA) rather than the lack of autophagy. SA is involved in plant

growth and development as well as plant defense responses [124]. Interestingly, lowering endogenous levels of SA in *atg* mutants, either through blocking biosynthesis or by enzymatic breakdown of SA, eliminates the early senescence phenotype under nutrient-rich conditions [123]. Autophagy has also been shown to degrade chloroplast components during senescence through the degradation of RuBisCo-containing bodies, reducing both the size and number of chloroplasts [125, 126], thus contributing to the leaf senescence process.

In addition to senescence, multiple examples of developmental programmed cell death in plant tissues have been described, some of which may involve autophagy [110]. These include the formation of xylem and vascular vessels, embryo development, pollen growth down the style, stigma hair cell elimination, weak shoot death, and leaf shape. In all cases vacuole size increases and various organelles disappear. The tonoplast then ruptures along with the plasma membrane, and sometimes the cell wall is degraded [110]. Microscopic observation of cell morphology suggests the involvement of autophagy, but the extent to which cell death results from autophagy or from other cell death pathways (apoptosis-like and necrosis) is difficult to determine. Perhaps the best evidence for autophagy as a cause of cell death is in the embryo suspensor of Norway spruce, in which a metacaspase activates autophagy, leading to vacuolar cell death [127]. Clearly observing autophagy as defined in animals (double-membrane cytoplasmic vesicles and the presence of an autolysosome) during developmental PCD has been more difficult in plants.

The temporal processes of plant PCD have been studied using the lace plant (*Aponogeton madagascariensis*), illustrating a role for both macroautophagy and mega-autophagy in developmental PCD [128]. During PCD, actin filaments thicken into larger bundles that eventually disappear during late PCD. Mitochondria and chloroplasts form groupings and also aggregate in the vacuole as cell death progresses. Organelle aggregate formation increases, and the aggregates are surrounded by phospholipid bilayers and found within the vacuole. During later stages of cell death, more vesicles appear within the cell, which may be attributed to autophagy. Using electron microscopy, double-membrane vesicles were rare, but single-membrane vesicles containing cytoplasmic material were commonly seen fusing with the tonoplast. Furthermore, live cell imaging also showed vesicles containing organelles. Late PCD involved tonoplast rupture, nuclear shrinkage, plasma membrane collapse, and cell wall disappearance [128].

Only a few examples exist in which autophagy has been shown to be required for the PCD process during plant development, often through the study of *atg* and related mutants. For example, the role of autophagy during xylem tracheary element differentiation was studied using *atg5* and *rabGTPase* mutants [129, 130]. Rab proteins regulate many steps in endomembrane transport and have been found to function in autophagy and autophagic PCD [131, 132]. It was found that ATG5 and RabGTPases are involved in PCD and differentiation of tracheary elements and that RabG3b localizes to autophagic structures. This also implicated RabG3b as being involved in plant autophagy and autophagic PCD.

11.4.2 Autophagy as an Immune Response

Organisms such as metazoans, which have specialized immune cells, largely rely on that system to mount inflammatory immune responses to protect cells and ultimately the organism from pathological abnormalities. Plants, however, lack the specialized immune cells that are found in animals. Instead each cell can activate immune responses to protect itself from invaders.

Fungi, bacteria, and viruses are all known to cause cell death in plants. Plant cells possess a multilayered system for dealing with microbial pathogens. The first layer involves detection of pathogens by sensing pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) through receptors in the plant cell membrane. Upon detection, a signaling cascade initiates PAMP-triggered immunity responses including ethylene production, mitogen-activated protein kinase activation, callose deposition, cell wall thickening, production of antimicrobial proteins, and immune marker gene expression [133–135]. To evade PAMP and MAMP-triggered immunity, pathogens can inject effectors into plants that manipulate host machinery for pathogen benefit [133]. As a counter to effectors, plants have evolved another layer of defense to recognize effector modifications of host target proteins via host surveillance proteins (termed resistance (R) proteins). R-mediated defenses often include localized PCD known as the hypersensitive response (HR) to limit spread of the pathogen [133]. HR is a defense mechanism upon tissue compromise, whereby the invading microorganism is inhibited by a combination of a layer of dead cells, local production of antimicrobial compounds, and induction of systemic acquired resistance in the host. If successful, the host is non-susceptible to the invading pathogen [110].

Autophagy activation in response to infection in plants has been proposed to have contradictory roles both in pathogen resistance (pro-death) and in restricting spread of plant HR PCD (pro-survival) [132, 136]. In mammalian cells, autophagy functions as an antiviral and antimicrobial pathway [137]. The physiological function of autophagy during pathogen infection in plants is still under investigation, and it is unclear if and when autophagy might serve a pro-death vs. a pro-survival role. Virus-induced silencing of the autophagy genes *BECLIN1*, *PI3K/VPS34*, *ATG3*, *and ATG7* in tobacco resulted in unrestricted HR PCD beyond the site of tobacco mosaic virus (TMV) infection [136], whereas in control plants, PCD was restricted to the infection site. This illustrates the importance of autophagy in restricting HR PCD to pathogen infection sites, implicating autophagy in the regulation of HR PCD. Larger lesions were observed in *BECLIN1*-silenced plants and were visible sooner after infection than in control plants. There was also an increase in accumulation of TMV at the site of infection in *ATG*-silenced plants, suggesting that *ATG* genes may play a role in virus replication or movement between cells [136].

The *Arabidopsis* BECLIN1 homolog ATG6 was also found to be required for limiting pathogen-induced HR PCD following infection with the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) [138]. *ATG*6 anti-

sense plants showed greater spread of HR PCD as well as increased disease susceptibility. Contrasting results were found in *Arabidopsis* autophagy knockout lines [135]. Pathogen infection with *Pst* DC3000 of both *atg7* and *atg9* mutants resulted in cell death being constrained to the site of infection and no death beyond the primary HR lesion even after 15 days. In some cases HR PCD was suppressed in *atg* mutants, suggesting that autophagy contributes to the death process. This is supported by the analysis of RabG3b, the overexpression of which resulted in increased autophagic structures and expanded, accelerated cell death when challenged with the fungal toxin FB1 or with *Pst* DC3000. Findings suggest that RabG3b is a regulator of autophagy during the HR PCD response to pathogens in addition to developmental processes and that autophagy acts as a pro-death HR mechanism following infection [132].

The contradictory reports of autophagy mutant phenotypes during pathogen infection may be explained by hormone signaling. The phytohormone SA hyperaccumulates in *atg* mutants such as *atg5,* which has a threefold higher SA level than wild-type plants [123]. *Pst* DC3000 infection in *atg5* leaves resulted in the spread of chlorotic cell death, with loss of chlorophyll and photosynthetic capacity, whereas in control plants it was limited to the site of infection. This phenotype was stronger in older leaves than younger leaves hinting at a role of SA since older leaves of *atg5* mutant plants were higher in SA than younger leaves [123]. Interestingly, spread of chlorotic cell death in *atg5* double mutants with reduced SA (*atg5xsid2*, *atg5xnpr1*) was reduced compared with single *atg5* mutants; SA signaling is therefore required for the chlorotic cell death observed in *atg5* [123]. Autophagy thus seems to negatively regulate cell death via the SA pathway. The involvement of SA in the regulation of autophagy as well as experimental design may help to explain the differences in findings between Liu et al./Yoshimoto et al. (pro-survival) and Hofius et al. (pro-death) [123, 133, 135, 136]. SA levels and leaf age may be critical in determining phenotypes, as both senescence and the hypersensitive HR response involve SA signaling. Hofius et al. [135] used 3–4-week-old plants, whereas others used older plants, and young 3–4-week-old plants showed only small increases in SA compared to older plants [123].

Importantly, autophagy can also serve different roles depending on the lifestyle of the pathogen. Necrotrophic pathogens kill their hosts by the secretion of toxins, while biotrophic pathogens secrete effectors to manipulate host machinery [139]. During necrotrophic pathogen infection, autophagy is activated in both infected tissue and surrounding areas. Furthermore, *atg* mutants are hypersensitive to infection [134, 140]. Autophagy also seems to be involved in preventing accumulation of high ROS levels following necrotroph infection via clearance of damaged organelles [134]. In summary, autophagy serves an anti-death, pro-life function by controlling widespread cell death and can also function in a pro-death mechanism during HR PCD [132, 141]. It is unclear if the dual roles of autophagy function independently or simultaneously to elicit an HR response and contain PCD to infected cells.

11.4.3 Autophagy in Abiotic Stress Responses

While autophagy as a response to abiotic stresses is perhaps the best studied aspect of autophagy function in plants, there is relatively little evidence for its function in cell death in these conditions. Autophagy was initially shown to be activated in response to nutrient starvation in suspension cell cultures from several plant species [142–145] and later in whole plants [23, 121, 122, 146]. Under these conditions it is primarily thought to be a nonselective process and to function in the recycling of nutrients when nutrients are severely limiting to allow essential protein synthesis to continue, to generate other critical biosynthetic precursors, and to enable the citric acid cycle to function by providing alternative substrates [2].

Autophagy also functions in a variety of other abiotic stresses in plants. It is activated during drought [147], oxidative [148, 149], salt [147], ER [150], and heat [64] stresses and probably acts to degrade damaged, denatured, and aggregated proteins under these conditions, for example, oxidized proteins during oxidative stress [148, 151], ER fragments during ER stress [150, 152, 153], and protein aggregates during heat stress [64].

Analysis of mutants, mainly in *Arabidopsis*, has allowed the contribution of autophagy to plant tolerance of stresses to be assessed. Mutants with defects in the autophagy pathway are more sensitive to multiple stresses, including the majority of those mentioned above [154], indicating that activation of autophagy under these conditions plays an important role in plant survival. The relationship between plant survival and cell survival vs. cell death under these stress conditions is often unclear, but autophagy is generally considered to act in a pro-survival mode during abiotic stress.

A few exceptions exist in which cell death via autophagy has been examined during abiotic stress. In the unicellular green alga *Micrasterias denticulata*, salt stress causes organelle degradation by autophagy accompanied by DNA breakdown indicative of cell death [155]. Similarly, in the salt-tolerant *Arabidopsis* relative *Thellungiella halophila,* high salt concentrations cause both autophagy and cell death [156]. Heavy metal toxicity can also lead to both autophagy and cell death in plants, probably due to oxidative stress [157]. In all of these cases, however, it is not clear whether autophagy is responsible for the cell death observed or merely accompanies cell death activated via an alternative mechanism to reclaim nutrients. More detailed mechanistic analyses are needed to clarify this issue.

11.5 Regulation of Autophagy

11.5.1 The TOR Signaling Pathway

The TOR signaling pathway is a key pathway for autophagy regulation and is probably the best studied autophagy regulatory pathway identified so far in plants. In addition to autophagy, it is involved in the regulation of plant growth, stress

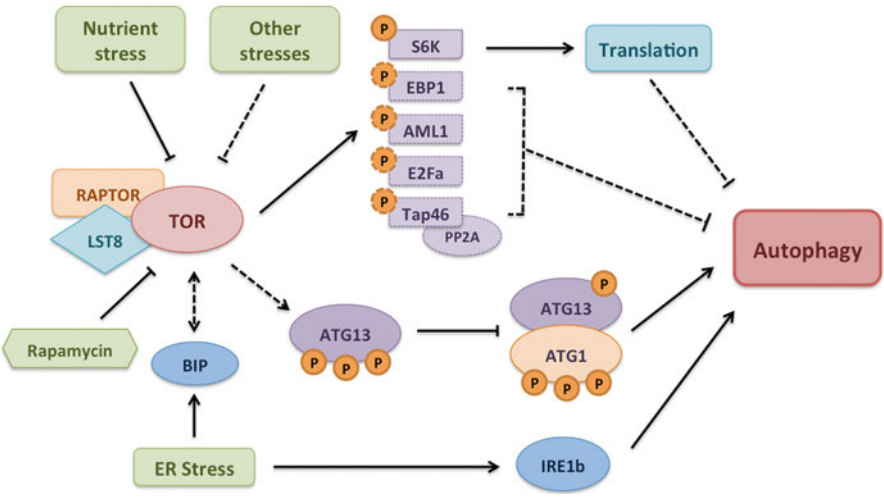

Fig. 11.4 Potential regulatory pathways for autophagy in plants. Autophagy can be activated in plants by nutrient, salt, osmotic, oxidative, and ER stress. TOR kinase is a key regulator of autophagy. The TOR complex, composed of TOR, RAPTOR, and LST8, senses nutrient stress signals and negatively regulates autophagy. S6K is a substrate of the TOR kinase complex and regulates mRNA translation. Several additional proteins have been suggested to be TOR kinase substrates, including EBP1, AML1, E2Fa, and the PP2A interactor Tap46. However, it is unclear whether these substrates further regulate autophagy. TOR may regulate the ATG1/ATG13 complex, a major activator of autophagy. In yeast, under nutrient-rich conditions ATG13 is hyper-phosphorylated, reducing its association with ATG1 and leading to inhibition of autophagy; whether this also occurs in plants is not known. IRE1b is a regulator of autophagy during ER stress. BIP is an ER stress response gene and may be a link between ER stress and the TOR complex for autophagy regulation. *Dashed arrows* and *lines* suggest possible functions that remain to be confirmed [1, 152, 160]

resistance, mRNA translation, and cell wall formation [158, 159]. The major known component in the TOR signaling pathway in plants is the TOR complex itself, which is composed of TOR and its binding partners RAPTOR (regulatory-associated protein of TOR) and LST8 (Lethal with Sec Thirteen 8) (Fig. 11.4). Genes encoding TOR complex homologs have been found in many plant species including *Arabidopsis*, rice, maize, and soybean and also in the model green alga *Chlamydomonas reinhardtii*. TOR is a highly conserved serine/threonine protein kinase that is inhibited by rapamycin via rapamycin binding to the FRB (FKBP12 and rapamycin binding) domain of TOR and FKBP12 protein [161]. Two distinct types of TOR complex exist in yeast and animals, TORC1 and TORC2, differentiated by distinct binding partners and functions [162, 163]. While TORC2 controls spatial cell growth, TORC1 modulates temporal cell growth by regulation of translation, and it negatively regulates autophagy [160, 162]. In plants, only TORC1 component homologs have been identified [164], and this complex is therefore referred as the TOR complex here. The plant TOR complex contains TOR and two binding partners, RAPTOR, which recruits substrates and presents them to TOR for phosphorylation [165–167], and LST8, which stabilizes the TOR complex [168, 169] (Fig. 11.4).

TOR signaling functions via phosphorylation of substrates by the TOR complex [170, 171]. Ribosomal p70 S6 kinase (S6K) has been definitively identified as a substrate of TOR in plants [172, 173]. Several additional proteins have also been suggested to be substrates of the TOR complex, including (a) Tap46, which is phosphorylated by TOR in plants and interacts with protein phosphatase type 2A (PP2A), a regulator of autophagy in yeast [174]; (b) *Arabidopsis* Mei2-like 1 (AML1), which interacts with RAPTOR in vitro [175]; (c) ErbB-3 epidermal growth factor receptor-binding protein (EBP1), whose expression is correlated with that of TOR [158, 176]; and (d) transcription factor E2Fa, which is phosphorylated by TOR in vitro, immunoprecipitates with TOR, and is involved in glucose sensitivity in root meristems [177].

TOR, the catalytic subunit within the TOR complex, is a PtdIns3-related kinase based on its sequence, although it functions as a protein kinase. *TOR* is well conserved in yeast, animals, and plants and is widely expressed in embryos, endosperm, and primary meristems in *Arabidopsis* [164]. Disruption of *TOR* is embryo lethal [158]. Studies in yeast and animals show that TOR controls cell growth and protein synthesis in response to amino acids and growth factors during nutrient signaling, and it activates translation initiation by two regulatory pathways [163]. TOR phosphorylates S6K to activate translation of 5′ terminal oligopyrimidine tract mRNAs [178]. It also deactivates 4E-BP1 (eIF4E-binding protein 1) through phosphorylation to in turn activate the initiation factor eIF4E and the translation of mRNA containing a 5′ untranslated region [179].

In plants, TOR is involved in the control of growth, development, and life span in response to nutrient and light energy status [177, 180, 181]. Downregulation [180] or inhibition [181] of TOR kinase in *Arabidopsis* causes slower growth, decreased nutrient uptake, and accumulation of storage molecules, amino acids, and TCA cycle intermediates, indicating a role in the regulation of carbon partitioning and primary metabolism. A function for TOR in glucose-induced root growth has been demonstrated, via phosphorylation of the cell cycle-regulating transcription factor E2Fa [177]. Inhibition of TOR delays phase change, flowering, and senescence, thus extending the *Arabidopsis* life span, and overexpression causes opposite phenotypes [181], indicating that TOR can regulate life span in plants. Transcripts encoding catabolic proteins, including those involved in autophagy, increase upon inhibition of TOR [180, 181], confirming the role of TOR as a negative regulator of catabolism.

RAPTOR is also highly conserved in eukaryotes. Two *RAPTOR* genes have been identified in *Arabidopsis*, *RAPTOR A* and *RAPTOR B*. *RAPTOR B* has a much higher level of expression than *RAPTOR A* [166]. In yeast and animals, RAPTOR recruits substrates such as S6K and 4E-BP1 and presents them to TOR to be fully phosphorylated [165, 182]. In *Arabidopsis*, disruption of *RAPTOR* leads to severe defects in plant growth and development [166, 167]. RAPTOR has been shown to interact with S6K in response to osmotic stress signals, suggesting a role for RAPTOR in the TOR signaling pathway and plant stress responses [172].

LST8 is suggested to be a binding partner of TOR and to stabilize the TOR complex [168, 169]. Two genes encoding LST8 have been identified in *Arabidopsis*,

LST8-1 and *LST8-2*, with *LST8-1* being much more highly expressed. Mutation of *LST8* results in defects in plant growth, flowering, and metabolic adaptation to long days, similar to plants with reduced *TOR* expression, suggesting an important role for LST8 in plant growth regulation [169].

In addition to its role in growth regulation, the TOR signaling pathway negatively regulates autophagy in plants [183]. When *TOR* transcript level was reduced by RNA interference in *Arabidopsis*, this led to constitutive autophagy [183]. Some autophagy-related *ATG* genes were upregulated, including *ATG9* and *ATG18a* [158, 183]. The regulation of autophagy by the TOR signaling pathway depends on *ATG18a*, a gene required for autophagosome formation, indicating that the observed autophagy requires the classical autophagy components [183]. TOR has also been shown to function as a negative regulator of autophagy in the model green alga *Chlamydomonas reinhardtii* [184]. In maize, the ortholog of TOR has been suggested to be involved in growth regulation, with S6K as substrate, although it is not clear whether TOR plays a role in the regulation of autophagy in maize [185, 186].

In yeast, the TORC1 complex regulates the ATG1–ATG13–ATG17 complex in response to nutrient availability. Upon starvation, ATG13 is dephosphorylated, activating ATG1 and inducing autophagy [16, 19]. In plants, the components downstream of the TOR complex remain poorly studied, but it is likely that they also include the ATG1 complex, which has been shown to be a regulator of autophagy in *Arabidopsis*.

11.5.2 ATG1/ATG13 Kinase Complex

ATG1 and ATG13 are components of the core autophagy machinery in yeast and function within a kinase complex, in which ATG1 is the catalytic subunit. This kinase positively regulates autophagy in response to nutritional status [160, 187]. In yeast, TOR hyper-phosphorylates ATG13 under nutrient-rich conditions, which decreases its affinity for ATG1, preventing their association and thus repressing the induction of autophagy. Under starvation conditions, inactivation of TOR leads to dephosphorylation of ATG13, enabling ATG1 to associate with ATG13 and activating autophagy [188, 189]. The ATG1/ATG13 complex activates autophagy via several subsequent steps, including the engagement of ATG9 in autophagosome formation and decoration of the phagophore with ATG8 and the VPS-34/ATG6/ATG14/VPS15 lipid kinase complex [190].

The ATG1/ATG13 kinase complex in yeast consists of ATG1, ATG13, ATG17, ATG29, and ATG31, but only ATG1 and ATG13 homologs have been identified in plants, including *Arabidopsis*, rice, maize, and soybean, each with different numbers of homologs of each gene [191]. ATG13 has been reported to be absent from the *Chlamydomonas* genome [192], although it is possible that a more divergent functional homolog takes its place. Four *ATG1* homologs are present in *Arabidopsis*, including three full-length *ATG1* genes, *AtATG1a*, *AtATG1b*, and *AtATG1c*, and a truncated version of *ATG1*, *AtATG1t*, which contains the kinase domain but not the

regulatory domain and seems to be plant specific [25, 191]. Two *ATG13* genes have also been identified in *Arabidopsis*, *AtATG13a* and *AtATG13b* [191].

Recent research on the ATG1/ATG13 kinase in *Arabidopsis* suggests that its function is conserved with that of other species [25]. ATG1a and ATG13a are reversibly modified phosphoproteins, and their phosphorylation is associated with nutritional status. ATG1a is hyper-phosphorylated during starvation, while ATG13a is hypo-phosphorylated, suggesting that the ATG1/ATG13 kinase complex is a regulator of autophagy in response to nutrient conditions [25].

A double mutant in *ATG13a* and *b* has the typical autophagy-related phenotypes of early senescence and hypersensitivity to nutrient deprivation. Biogenesis of autophagosomes or deposition of autophagic bodies is arrested in the *AtATG13* mutant upon nitrogen starvation, but ATG8 lipidation and ATG5–ATG12 conjugation are not, suggesting that the induction of the phagophore is independent of the ATG1/ATG13 kinase complex, but the complex may be essential for autophagosome closure or delivery to the vacuole [25]. The protein levels of ATG1a and ATG13a are strongly regulated by nutrient status, and their turnover is linked to autophagy. ATG1a associates with autophagic bodies and is delivered to vacuole for degradation, suggesting that the ATG1/ATG13 complex is a substrate of autophagy, and a negative feedback mechanism may exist to reduce ATG1/ATG13 complex levels after induction of autophagy [25].

Taken together, the available information suggests that the ATG1/ATG13 kinase complex is a regulator of autophagy in plants and meanwhile is also a target of autophagy in response to nutritional status. A feedback mechanism may therefore exist to regulate the amount of the complex and in turn to regulate autophagy in response to nutritional status.

11.5.3 IRE1

Autophagy is induced in plants under multiple stress conditions, including nutrient deprivation [27, 28, 121, 122], salt and drought stress [147], heat stress [64], oxidative stress [148], and ER stress [150, 153].

ER stress is triggered when the amount of unfolded or misfolded proteins exceeds the capability of the degradation system in cells [193, 194], leading to a homeostatic response called the unfolded protein response (UPR) to assist proper folding or degradation of misfolded proteins. The ER folding machinery consists of a variety of molecular chaperones and other factors that assist in correctly folding polypeptides. One of the molecular chaperones in the ER is the binding protein BiP, a heat shock protein 70 that assists protein folding in the ER lumen by binding to nascent proteins when they enter the ER [195]. As addressed above, TOR has been shown to be a negative regulator of autophagy in *Chlamydomonas* [184]. A recent study in *Chlamydomonas* revealed that BiP is a link between ER stress and the TOR signaling pathway [196, 197]. However, whether the TOR signaling pathway is involved in autophagy regulation during ER stress is unknown.

The UPR is initiated by ER sensors located on the ER membrane. Inositol-requiring enzyme-1 (IRE1) is an ER sensor that activates a UPR signaling pathway in yeast [198, 199]. Another two ER sensors are found in mammals, activating transcription factor 6 (ATF6) [200] and protein kinase RNA-like endoplasmic reticulum kinase (PERK) [201]. In plants, only IRE1 and ATF6 have been identified as ER sensors [202, 203], and only the IRE1 signaling pathway appears to be linked to regulation of autophagy [150, 153, 204].

IRE1 is highly conserved and functions as both a kinase and a ribonuclease [198, 199, 205]. Two *IRE1* homologs have been identified in *Arabidopsis*, *IRE1a* and *IRE1b*, and they seem to have some distinct roles [202, 206, 207]. When ER stress is triggered, IRE1 is activated by oligomerization and autophosphorylation [208]. Activated IRE1 splices an mRNA encoding a membrane-associated basic leucine zipper transcription factor (bZIP60) in *Arabidopsis*, in an analogous manner to yeast and mammals [209]. Spliced *bZIP60* mRNA is then translated, producing an active protein that is translocated into the nucleus and upregulates UPR genes such as *BIP* [207, 210]. Defects in IRE1 cause enhanced cell death and inhibition of secretory pathway protein degradation upon ER stress, suggesting that IRE1 also plays a role in additional ER stress pathways [211]. In addition to *Arabidopsis*, IRE1 and bZIP60 homologs have also been found in other plant species and show similar splicing mechanisms. In rice, one IRE1 homolog has been found, *OsIRE1* [212], and two *bZIP60* homologs have been found, *OsbZIP74* and *OsbZIP50* [213, 214]. In maize, one *bZIP60* homolog has been found, *ZmbZIP60* [215, 216].

IRE1 is suggested to be a link between ER stress and autophagy in plants [150, 152, 153]. When ER stress is triggered by dithiothreitol (DTT) or tunicamycin, a mutant defective in *ire1b* is unable to form autophagosomes, suggesting *IRE1b* is required for the induction of autophagy under ER stress. Interestingly, mutations in either *IRE1a* or the only identified IRE1 target mRNA *bZIP60* have no effect on autophagy upon ER stress [150]. The ribonuclease function of IRE1 therefore may not be involved in autophagy regulation. As mentioned above, IRE1 also functions as a protein kinase, suggesting that the kinase function of IRE1b may be the key for autophagy regulation. In animals, the c-Jun N-terminal kinase pathway acts downstream of IRE1 to activate autophagy, but there is no evidence for the existence of this pathway in plants [217]. Further research on IRE1b may reveal the mechanism of autophagy regulation by IRE1 under ER stress.

11.6 Future Perspectives

While the components required for autophagy and the conditions under which it is activated in plants are known in some detail, many questions remain. A major question that has gained a great deal of attention in recent years is the source of membrane for the formation of autophagosomes. Surprisingly, a large number of different organelles and trafficking pathways seem to contribute membrane to the forming phagophore, with the ER playing a prominent role [12, 41]. The integration of these

pathways and membrane sources to generate autophagosomes in response to different stimuli remains an important area of study.

Autophagy can be either a nonselective or selective process. It is unknown if selective autophagy plays a role in plant programmed cell death. However, multiple examples of selective autophagy are known in eukaryotes [56, 218–221] and autophagy can target ribosomes, ER, nuclear portions, peroxisomes, mitochondria, protein aggregates, and other cell components. The possible role of selective autophagy in plant cell death, and its potential cargo, is a key research question for the near future.

Additional work is also needed to clarify the relationship between the role of autophagy in cell survival and PCD during environmental stress, development, and pathogen infection. A good model is the function of autophagy during ER stress. It is suggested that autophagy plays a role in cell survival during ER stress, which could otherwise lead to cell death [150, 204, 222]. Apoptosis has been suggested to be the pathway for cell death in response to ER stress, but given the difficulties in classifying cell death in plants, autophagy may also contribute to the cell death process under severe stress conditions. The relationship between autophagy and pathogen defense also needs to be explored, in particular to understand the connection with SA signaling during the HR. In animal cells intracellular pathogens can be degraded by autophagy through a process known as xenophagy [223]. Whether or not pathogens are degraded by a similar mechanism in plants, especially considering that most plant bacterial pathogens are extracellular, is not understood.

The mechanism of regulation of autophagy in response to the many conditions and developmental signals that activate the pathway is likely to be a fruitful area of research for the future. While TOR is a major evolutionarily conserved regulator of autophagy, the majority of the upstream factors found in animals that connect perception of the autophagy-activating stimulus to TOR activity are absent from plant genomes. This indicates that alternative mechanisms exist for regulating TOR function in plants; identification and characterization of these pathways is key to increasing our understanding of autophagy regulation in plant species.

In summary, autophagy in plants contributes to PCD during development, stress, and pathogen defense by degrading cell components and enabling their recycling for use by the plant. The extent to which autophagy is a cause of cell death, how it interacts with other known PCD pathways, and the mechanisms by which its activity is regulated remains to be determined and should be a fruitful area for future research.

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