

CONTROLLED CELL DEATH, PLANT SURVIVAL AND DEVELOPMENT

Eric Lam^{*†}

For plants to develop properly and survive, programmed cell death is an important response strategy to various internal and external cues. Morphologically, a key difference between programmed cell death of plant cells and apoptosis in animals is the absence of engulfment by neighbouring cells in plants. Recent genetic, molecular and biochemical approaches have begun to reveal interesting candidate regulators in plants that show both similar and new properties compared with their animal counterparts.

PLANT CELL BIOLOGY

HYPERSENSITIVE RESPONSE

A rapid plant programmed cell death that is orchestrated in and surrounding the initial site of plant pathogen entry that is typically associated with the activation of disease resistance.

TRACHEARY ELEMENTS

Specialized cells in the xylem of vascular plants that are responsible for the conductance of fluids as well as providing mechanical support.

**Biotechnology Center and the Department of Plant Science, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08901, USA.*

*†Department of Botany, The University of Hong Kong, Pokfulam Road, Pok Fu Lam, Hong Kong Special Administrative Region of the People's Republic of China.
e-mail: Lam@aesop.rutgers.edu; ericL@hkucc.hku.hk
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Eukaryotes such as plants, animals and yeast have all evolved ways of cellular suicide that are known as programmed cell death (PCD). In multicellular organisms such as plants and animals, the organized destruction of cells is important for forming body plans and specific organ shapes and for removing unwanted, damaged or infected cells^{1–4}. Two well-characterized model systems for the study of plant PCD are those of the HYPERSENSITIVE RESPONSE (HR), which is often observed during plant–microbe interactions, and the development of TRACHEARY ELEMENTS in the XYLEM of vascular plants^{5,6} (FIG. 1). A simplified depiction of the sequence of cytological events that take place during these forms of plant PCD is contrasted with those of apoptosis, the well-studied form of animal PCD (FIG. 2). The ‘clean’ process of apoptosis effectively contains the contents of the dead cell for removal by other cells and avoids activating an inflammatory response in animals. In the case of HR-associated cell death and the terminal differentiation of tracheary elements, the contents of the dying cells are not engulfed by other cells. In addition, the ‘corpse’ of the dead cell is held in place by the cell wall, and, for mature tracheary elements, the cell wall is reinforced during the early phase of PCD and carries out the essential function of mechanical support and transport after autolysis^{5,6}. These key characteristics therefore distinguish plant PCD from that of classic apoptosis and indicate that specialized features and pathways have probably evolved to control and execute the death programme in plant cells (BOX 1).

In addition to HR and tracheary element differentiation, other models of plant PCD such as those occurring during senescence, embryogenesis and ozone treatment have also been established and systematically characterized (see REFS 3,4,7 for more comprehensive reviews of the various forms of plant PCD). Recent genetic and molecular approaches have yielded many cellular components that, when mutated, can perturb plant–cell–death control under various conditions. The recent report of cell-free systems from *Zinnia elegans* and *Arabidopsis thaliana* that can be used to study nuclear changes during PCD could provide a much-needed assay for the biochemical characterization of key players in this process^{8,9}.

This article focuses on integrating information on the orchestration of cell death in plant cells, with emphasis on new results obtained from analyses of gene functions using mutants or transgene expression. This effort is especially timely given that the *A. thaliana* model system is now readily amenable to both forward- and reverse-genetic approaches, and that cell-biological markers for various subcellular compartments are well established for plants. So, the functional relevance of a given gene and the cytological events during cell death in plants can now be characterized with unprecedented ease.

Cytology of controlled cell death in plants
The cytological events that accompany cell-death activation have been documented comprehensively for several plant-PCD model systems. In PCD that takes place

during the transdifferentiation of MESOPHYLL cells into mature xylem cells in *Z. elegans* cell cultures⁶, vacuoles accumulate degradative enzymes and swell, and the cell wall is remodelled into a highly reticulated form. Nuclear and organelle DNA fragmentation closely follows the collapse of the vacuole during the autolytic phase at the end of this process^{10,11}. Chromatin condensation and other typical apoptotic morphologies are not seen (FIG. 2). This type of vacuole-directed-death process has also been documented during other plant developmental processes such as senescence and AERENCHYMA formation in the root¹².

In a series of recent studies, the cytology of embryogenesis-related cell deaths were characterized using *in vitro* cell cultures of fertilized ovules from Norway spruce (*Picea abies*). Chromatin fragmentation into clear nucleosomal fragments was observed without obvious chromatin condensation or nuclear BLEBBING, whereas vacuolation of the cytoplasm occurred earlier. Vacuole rupture occurs very late in the autolysis of the dying cells when most cellular compartments are already dismantled. Two separate waves of cell-death events were defined for the transition from pro-embryogenic mass to a somatic embryo¹³, with the latter wave mirroring those events observed during the developmental cell death that results in the selection of a single surviving embryo within the polyembryonic GYMNOSPERM ovule^{14,15}. Rapid cytoskeletal changes are observed in the early phase of embryogenesis and these could be important in orchestrating the relocation of essential cellular components that are involved in this developmental cell-death process¹⁶.

More-classic apoptosis-like features were observed for the HR, with nucleosomal laddering and apparent cellular fragmentation akin to apoptotic bodies reported in some cases of HR cell death¹⁷ but not in others^{5,18,19} (FIG. 2). Rapid reorganization of the cytoskeleton of plant cells has been observed at the infection site by fungal pathogens, and pharmacological studies have indicated that this might be important in disease resistance and HR cell death during an incompatible interaction²⁰. However, a recent study indicates that the dramatic changes in the cytoskeleton might be necessary but they are insufficient to account for HR cell-death activation²¹. New imaging tools²² should provide a more detailed and dynamic description of plant-cell-death morphologies under various scenarios.

Signalling for plant cell death

The ubiquitous nature of PCD highlights the idea that this might be a default pathway for a cell in the absence of the proper survival signal²³. In plants, this idea was supported by the finding that PCD of carrot cells cultured at low density could be reversed by putative intercellular factor(s) that are present in 'conditioned media' that was obtained from cultures at higher densities²⁴. So, numerous signals are probably constantly integrated by the cell to decide whether to activate the cell-death programme or not. In addition, the control of the boundaries and kinetics of the cell-death event would be crucial for the optimal deployment of this process. For example, in

senescence, PCD affects entire organs, occurs over a relatively long period and is linked with the recycling of fixed nitrogen. By contrast, for HR cell death, a rapid but restricted zone of cell death is used to effectively contain pathogens at their site of entry, and efficient recycling of the contents of the dead cell might be secondary.

The isolation and characterization of LESION-MIMIC mutants showed that cell death can be activated cell-autonomously and that survival signals are required to restrict lesion size²⁵. A relatively large set of genes that, when mutated, result in the lesion-mimic phenotype has now been isolated, predominantly from *A. thaliana*²⁶ (TABLE 1). In addition, some transgenes that perturb the physiology of the cell can also induce lesion-mimic phenotypes²⁷. Although these findings are aiding our appreciation of cell-death control in plants, separating primary signals for a cell-death pathway from those that might affect the physiology of the cell, and thereby potentiate the observed death phenomenon as a secondary consequence, is not trivial. This is especially problematic when core regulators for cell-death pathways are not defined for plants as yet, and so a mechanistic description of how a signal ultimately connects to a particular cell-death pathway would be difficult at best.

Phytohormones as PCD-signal regulators

Salicylic acid and nitric oxide. Different phytohormones are involved in modulating HR cell death under various conditions. Salicylic acid is a well-known mediator of SYSTEMIC ACQUIRED RESISTANCE (SAR) in plants²⁸. Aside from this key role, the involvement of salicylic acid in cell death has been tested by crossing *A. thaliana* plants that are engineered to suppress salicylic-acid accumulation with various lesion-mimic mutants. Interestingly, although the spontaneous cell-death phenotype could be abolished in some of these mutants, others showed only a quantitative reduction or no effect at all when salicylic acid is removed (reviewed in REF. 26). So, salicylic acid might promote cell death in some signalling pathways but have only a minor role in others. Studies of the lesion-mimic mutants *agd2* (REF. 29) and *acd6* (REF. 30) also showed a possible involvement of the salicylic-acid-dependent pathway(s) on HR-cell-death suppression as well as cell growth. One current view of salicylic-acid functions is that, at high concentrations, such as those that might be produced by plant cells at the site of pathogen entry, salicylic acid probably functions as a cell-death promoter in collaboration with other signals. As salicylic acid, and possibly other systemic signals, is transported outward from these initial sites of synthesis, salicylic acid at low concentrations becomes a survival signal instead and helps to establish the border of spreading lesions²⁸. Added to this complexity is the likely existence of feedback controls through the action of reactive oxygen species (ROS) on salicylic-acid synthesis and the salicylic-acid-dependent generation of ROS³¹.

Nitric oxide (NO) cooperates with salicylic acid to induce HR cell death and activate defence, which is analogous to its role in animal systems³². Increased NO production is sufficient to induce cell death in an *A. thaliana* cell culture³³. It has been proposed that the balance between intracellular NO and hydrogen peroxide (H₂O₂),

XYLEM

A tissue that comprises a large collection of specialized cells that are involved in the transport of water and solutes in vascular plants. Mature xylem vessels contain essentially only the cell wall.

MESOPHYLL

The photosynthetic tissue of a leaf that is internal to the epidermis, and contains both palisade and spongy cell types.

AERENCHYMA

Cortical cells that have undergone programmed cell death in the roots of grasses under low-oxygen conditions.

BLEBBING

Invagination of the membrane.

GYMNOSPERM

Non-flowering plants represented by pine and spruce.

LESION MIMICS

Also known as 'disease lesion mimics', this describes a class of phenotypes in which spontaneous cell death occurs as discrete 'zones' or 'lesions' that resemble the cell death that is observed during the hypersensitive response.

SYSTEMIC ACQUIRED RESISTANCE

(SAR). General, long-lasting plant defence that is activated at a distance from the initial site of attempted invasion by an avirulent pathogen of a host plant.

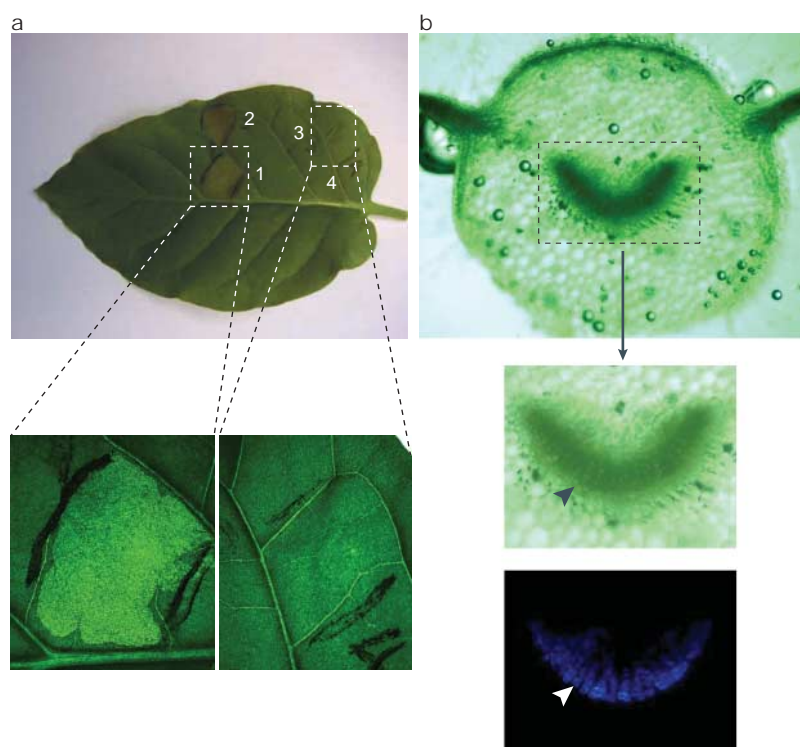


Figure 1 | Examples of programmed cell death in plants. **a** | An example of the hypersensitive response: a tobacco leaf is infiltrated with two different strains of *Pseudomonas syringae*. Zones 1 and 2 are infiltrated with strain NPS3121 and zones 3 and 4 with strain NPS4000. The latter strain is isogenic to NPS3121 except for a mutation in the *HRP* locus, which renders it incapable of inducing the hypersensitive response in tobacco. Visible cell death of the inoculated regions can be observed in zones 1 and 2 one day post-inoculation, whereas no significant morphological changes were observed in zones 3 and 4. Enlarged views with back lighting show cleared cells with little chlorophyll remaining in zones 1 and 2, in contrast to zones 3 and 4. **b** | An example of developmental death that has a critical role in the housekeeping function of mechanical support and long-distance transport is the formation of the xylem in vascular plants. The top panel shows a cross-section of a tobacco leaf at the mid-rib region, with the boxed region showing the central files of tracheary elements that are critical for transport of solutes and water. These cells have already undergone programmed cell death and the remaining corpses have reinforced secondary cell walls that are highly autofluorescent (shown by arrowheads in enlarged views). Top enlarged view: transmission light microscopy image. Bottom enlarged view: epifluorescence of the same viewing field with excitation filter at 436 nm and emission filter at 480 nm.

ENDOSPERM

Storage tissue surrounding the embryo in the seeds of flowering plants to provide nutrients for the developing seed as well as the germinating seedling.

ALEURONE

Specialized cell layer surrounding the starchy endosperm of cereal seeds that is responsible for the secretion of hydrolytic enzymes to degrade the endosperm upon seed germination.

ELICITOR

Pathogen-derived molecules, such as fungal-cell-wall fragments and secreted proteins, that can trigger defence responses of plant cells that resemble the hypersensitive response.

but not superoxide, concentrations is the key determinant for the HR cell-death response³⁴. The recent identification of two plant nitric-oxide-synthase genes revealed that they share little, if any, structural similarities with their animal counterparts^{35,36}. This indicates that there are new modes of NO synthesis and that possible convergent evolution occurred in plant and animal systems to generate similar signalling molecules using structurally distinct enzymes. Mutants of these genes should provide useful tools to facilitate our understanding of the involvement of NO in cell-death processes from HR to senescence in plants.

Jasmonic acid and ethylene. In addition to salicylic acid and NO, two other phytohormones, jasmonic acid and ethylene, regulate cell death under stress conditions and during development. Jasmonic acid, a phytohormone that is produced after wounding, negatively

regulates cell death in *A. thaliana* under oxidative stress by ozone treatment³⁷, but it might work as a positive factor to promote cell death that is induced by a fungal toxin fumonisin B1 (REF. 38). Ethylene is involved in promoting senescence³⁹ as well as other forms of developmental cell death, such as the formation of aerenchyma in hypoxic roots⁴⁰ and ENDOSPERM cell death in cereals⁴¹. Ethylene also promotes cell-death activation by ROS as well as lesion formation in the lesion-mimic mutant *acd5* (REF. 42) and in cell-death induction by the toxin fumonisin B1 (REF. 38).

Brassinosteroid, gibberellic acid and abscisic acid. Three phytohormones that are involved in plant growth and development are also known to control cell-death signalling in a context-dependent manner. In the tracheary-element differentiation system of *Z. elegans*, brassinosteroid is produced by *Z. elegans* cells to initiate the final stage of tracheary-element differentiation, which involves the autolytic death programme^{6,43}. Two other plant hormones have opposing effects on PCD in the ALEURONE cell layers of developing barley endosperm. Gibberellic acid promotes cell death in this context in cooperation with ROS, whereas abscisic acid counteracts this death-promoting effect of gibberellic acid. The mechanism of this interplay between the two hormones could relate to their opposing effects on the expression of ROS-scavenging enzymes⁴⁴. The role of abscisic acid in repressing cell death has also been reported for developing barley anthers⁴⁵, so this type of hormonal potentiation of cell death might not be restricted to aleurone cells.

Membrane channels and lipid metabolism

Rapid ionic fluxes are among the earliest documented responses to HR that are induced by avirulent pathogens. Plasma-membrane-localized receptors, as well as enzymes that are involved in ROS metabolism, are also important in the perception and propagation of signals for cell growth and death in both animals and plants. It is therefore no surprise that several plant-cell-death signalling proteins associate with membrane channels or have lipid-associated functions.

Membrane-associated signals. The observation that transgenic expression of a bacterial proton channel, bacterioopsin (bO), in tobacco (*Nicotiana tabacum*) resulted in a lesion-mimic phenotype indicated that perturbation of the pH homeostasis in plant cells might activate HR-like cell death and the associated defence response⁴⁶. Point mutants that abolish the proton-channelling activity of bO failed to induce lesion-mimic phenotypes. Both bO and the various point mutants accumulate to similar levels in transgenic tobacco plants and probably localize to the plasma-membrane fraction⁴⁷. Another recent study points to an anion transporter that is involved in nitrate efflux as an essential component of ELICITOR signalling for HR cell death in tobacco⁴⁸. In addition, two genes that encode members of the CYCLIC-NUCLEOTIDE-GATED CHANNEL (CNGC) family in *A. thaliana* have been identified as regulators of HR cell death. CNGC2, a cation channel that

conducts calcium, is encoded by the *DND1* (defence, no death-1) locus⁴⁹. The *dnd1* mutant suppresses HR cell death when challenged with certain avirulent pathogens without altering disease resistance. However, this mutant also shows micro-lesions, indicating a low level of constitutive activation of HR cell death. Recently, a lesion-mimic mutant, *hlm1*, which shows constitutive HR-like cell death and pathogen resistance, was shown to encode CNGC4, a channel protein that can mediate transport of K⁺ and Na⁺, and that is activated by both cyclic GMP and cyclic AMP⁵⁰. These results support the view that the alteration of ionic homeostasis is a key step in the early signalling process for HR cell death.

Lipid-related signals. Intracellular signals that are derived from lipid metabolism are also being recognized as mediators of HR induction and associated cell death. Two mutants that are defective in pathogen-response signalling, *pad4* and *eds1*, revealed genes that encode proteins that are related to triacylglycerol lipases^{51,52}. Interestingly, these genes are also required for the spontaneous phenotypes of some of the lesion-mimic mutants such as *lsd1*, *cpr1* and *cpr6* (reviewed in REF. 26; TABLE 1). This provided genetic evidence that phospholipid signalling is involved in the induction of HR cell death. Phosphatidic acid — which can be produced by the action of phospholipase D (PLD) on the precursor phosphatidylcholine, or from phosphorylation of diacylglycerol by a kinase — increases after induction of the plant-cell-defence response⁵³. One particular isoform of PLD in *A. thaliana*, PLD δ , has been genetically shown to attenuate H₂O₂-mediated activation of cell death that might mediate many biotic and abiotic responses⁵⁴. This indicates that the phosphatidic-acid production, which was mediated by PLD in plant tissues that were challenged with pathogens, might serve as a negative signal of PCD for the management of cell-death propagation during the development of the HR. PLD δ is apparently stimulated by oleic acid⁵⁵, and deficiency in oleic acid in the *ssi2/fab2* mutant (TABLE 1) leads to a lesion-mimic phenotype, possibly due to a decrease in phosphatidic-acid production from lower levels of PLD δ activity⁵⁶. These results support the importance of the phosphatidic-acid-lipid-derived signal as a negative regulator of certain cell-death pathways, in particular those that are mediated through oxidative stress.

Recent suppressor analyses of the *ssi2/fab2* mutant resulted in the identification of the *SFD4/FAB6* gene (which encodes a plastidic $\omega 6$ -desaturase) and other *SFD* loci, which function as modulators of the *ssi2* phenotypes⁵⁷. However, the differential effects of these suppressors on *ssi2* plants indicate a complex interplay between lipids containing polyunsaturated fatty acid and the various signalling pathways that mediate defence and wound responses. Finally, a loss-of-function mutation in the *A. thaliana* gene *ACD11*, which encodes a sphingosine-transfer protein, also results in a lesion-mimic phenotype that is salicylic acid and light dependent, in addition to a requirement for PAD4 and EDS1 (which are involved in salicylic-acid signalling; REF. 58). The facilitation of sphingosine

transfer between membranes might also potentiate cell-death activation and its perturbation could trigger cell death in plants.

NADPH oxidases and ROS are cell-death signals. The involvement of ROS in the activation of PCD is well known in animals, plants and yeast^{5,7}. The generation of an 'oxidative burst' during the early and late phases of plant-pathogen interaction has been well documented⁵⁹, and more-recent observations support a role for ROS in cell-death signalling^{60–62}. In animal systems, a membrane-associated NADPH oxidase in complex with the small G-protein RAC is responsible for ROS production⁶³. Recently, mutations in two *A. thaliana* genes, *rbohD* and *rbohF*, that encode orthologues of the mammalian *GP91^{phox}* NADPH oxidase catalytic subunit were analysed. Loss-of-function mutants in *A. thaliana rbohD* and *rbohF* resulted in decreased ROS production during the HR, with a corresponding decrease in cell death⁶⁴. These results provided the first direct genetic evidence for ROS generation by plant NADPH oxidases and their roles in a cell-death response. Paradoxically, although *A. thaliana rbohD* seems to be more prominent in the generation of total detected ROS, *A. thaliana rbohF* is more important for the activation of HR cell death. The intracellular location of ROS synthesis might be an important factor that determines the efficacy of cell-death induction by this signal. Consistent with the role of plant orthologues of *GP91^{phox}* in ROS generation and HR induction, studies with rice-derived RAC have provided evidence that this small G-protein functions in the control of HR cell-death induction and the associated resistance to fungal and bacterial pathogens⁶⁵. However, a recent study that reported gene suppression of *RACB* in barley indicated that this small G-protein is required for successful fungal infection in a genotype-specific manner⁶⁶. Perhaps, the differential accumulation of superoxide, instead of H₂O₂, could be an important determinant of resistance and possibly HR cell death?

Organelles and cell death in plants

As the energy status of the cell can potentiate cell death in animal and yeast systems, it is not surprising that the mitochondria and plastids in plants are also important signal generators for cell-death regulation. In addition to their abilities to generate ROS through electron-transfer intermediates that are intimately involved in functions associated with these organelles, a number of other cell-death signalling pathways also seem to depend on organelle components. Several well-characterized cell-death mediators exit the mitochondria to promote apoptotic cell death in animal systems. Cytochrome *c* (the release of which activates procaspase-9, REF. 67), apoptosis-inducing factor (AIF; which functions as a CASPASE-independent activator of nuclear DNA cleavage⁶⁸), and the recently described endonuclease G (REF. 69) are some of these animal proteins that might have counterparts in plants.

In plants, although cytochrome-*c* translocation to the cytoplasm has been reported (reviewed in REF. 5), its

CYCLIC-NUCLEOTIDE-GATED CHANNEL (CNGC). Conserved protein family with six predicted transmembrane helices that can form cation-conducting channels and is activated by the binding of lipophilic cyclic nucleotides such as cAMP and cGMP.

CASPASES
A family of cysteine proteases with specific target-site sequences that contain an aspartate residue at the P1 position. In addition to activating programmed cell death (PCD), caspases also function in non-PCD-related roles such as the processing of cytokines.

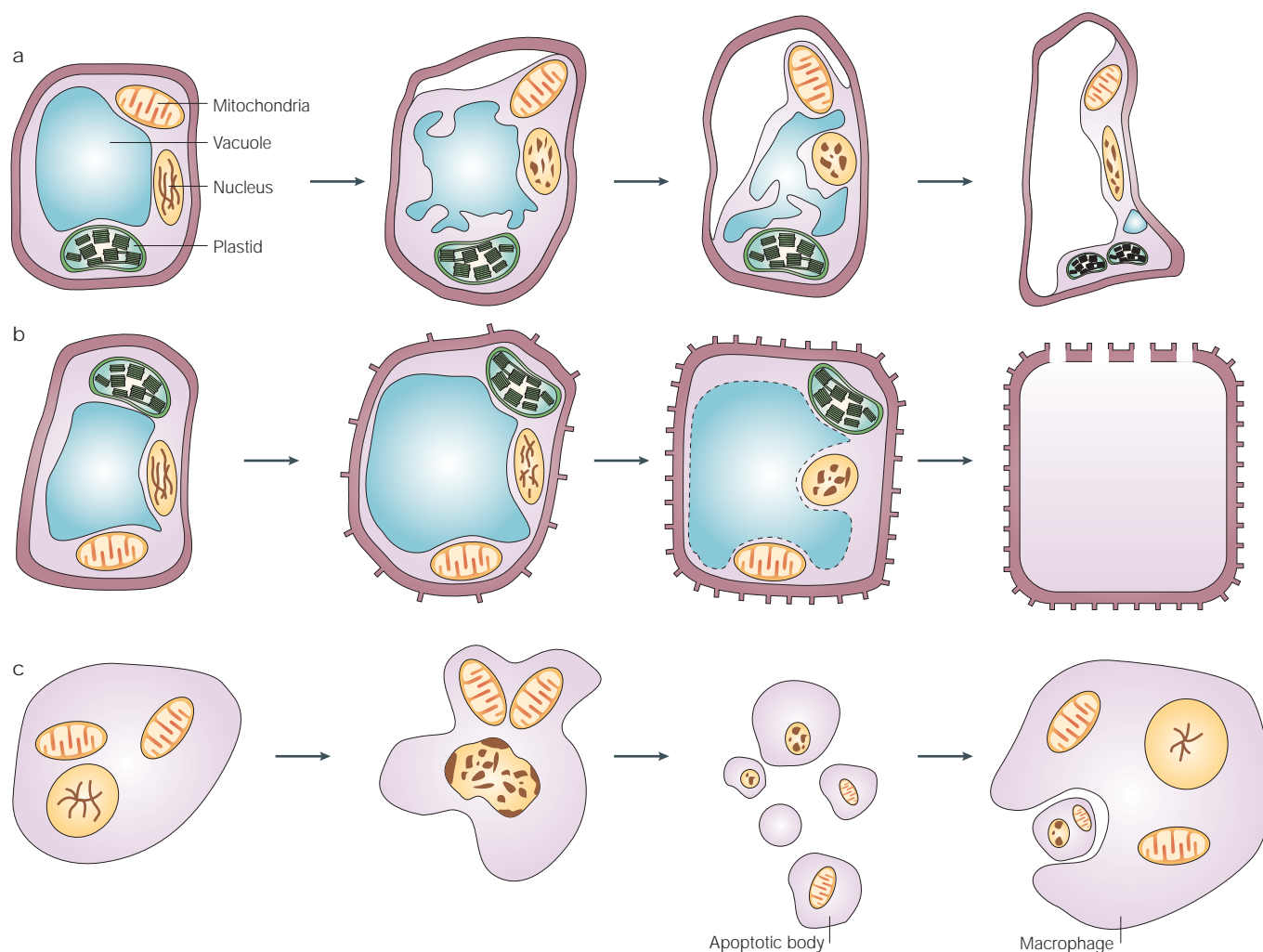


Figure 2 | Morphological comparison between programmed cell death in plants and animal apoptosis. a | In the hypersensitive response, chromatin condensation and DNA cleavage into 50-kb fragments were observed before the apparent disruption of the vacuole, which takes place during the late stages of cell death¹⁸. Blebbing of the vacuole and plasma membranes, and late destruction of organelles were also observed. At the final stage of this cell-death process, the plasma membrane collapses and separates from the cell wall^{18,19}, ending with the leakage of the dead cell's content into the apoplast. Fragmented nuclear DNA is shown throughout the figure as irregular, brown masses in the nuclei that are undergoing cell death. **b** | During the differentiation of tracheary elements, vacuole swelling and rupture is coordinated with the thickening and restructuring of the cell wall. The final collapse of the vacuole immediately precedes nuclear DNA fragmentation, which occurs at the late stages of the cell-death process before the final autolysis of the cell. Short stubbles on differentiating tracheary elements indicate reticulated secondary cell walls. Broken areas in the cell wall of terminally differentiated tracheary elements indicate spatially localized perforations. **c** | Apoptosis in animal cells initiates morphologically with chromatin condensation and fragmentation. Plasma-membrane ruffling is followed by the formation of apoptotic bodies from the repackaging of the cell content and their final engulfment by neighbouring cells or macrophages.

possible function as a cell-death signal — rather than it being a consequence of mitochondrial destruction — remains to be shown. Recent studies indicate that breakdown of the permeability of mitochondria might correlate with cell-death induction in some plant systems^{70–72}, but these pharmacological studies require more molecular and genetic studies to further elucidate the mechanisms involved. A Mg^{2+} -dependent nuclease was recently identified in the intermembrane space of the mitochondria using a cell-free nuclear system that recapitulates chromatin condensation and DNA fragmentation, and it might be biochemically similar to the recently described

mitochondrial endonuclease G (REF. 9). Increased phosphorylation of a mitochondrial chaperone, prohibitin, has also been reported in the rice lesion-mimic mutant *cdr1* (REF. 73). This correlates with the activation of NADPH oxidase in this mutant and might be part of the cellular responses that lead to the cell-death phenotype.

The plastid-localized enzymes that are involved in porphyrin metabolism, which is important for haem and chlorophyll biosynthesis, are known to be involved in spontaneous cell-death activation in plant lesion mimics. These include the *lin2* and *acd2* mutants in *A. thaliana* and several lesion-mimic mutants in *Zea mays*^{26,74}. The

Box 1 | PCD mechanisms in eukaryotes: plants versus animals

Despite the widespread interest, the study of programmed cell death (PCD) mechanisms in plants remains hampered by the lack of a clear set of 'core regulators' that function as the key executioners of various death signals. In metazoans, from humans to *Caenorhabditis elegans*, the three known classes of conserved core regulators for apoptosis are the BCL-2/CED-9, APAF1/CED-4 and caspase/CED-3 families of proteins^{1,2,7}. The absence of structural orthologues for these proteins in the complete genome sequences of *Arabidopsis thaliana* and yeast species indicates that alternative, structurally distinct, regulators must have evolved to control cell-death activation in plants and fungi. However, recent experimental evidence has reinforced the idea that caspase-like proteases (CLPs) exist in plants to mediate some forms of developmental and induced cell death. Also, the effects of pro-apoptotic and pro-survival BCL-2-related proteins on plant PCD have been reported using transgenic approaches. These results indicate that, despite differences in the inherent morphology (see FIG. 2 for comparison), physiological requirements and regulators at the sequence level, similar strategies with functionally related molecules might have been conserved between animals and plants to orchestrate controlled cell death. One candidate for such a conserved cell-death regulator is the BAX inhibitor-1 (BI-1) class of proteins, which has been found to function as a cell-death suppressor in different eukaryotes such as animals, plants and fungi. Interestingly, although BI-1 can function in *Saccharomyces cerevisiae*, it is apparently absent from its genome. Whether it could be found in the genome of other fungi might help determine the evolutionary flux of this highly conserved gene within the eukaryote lineages.

effects of these mutations are not well defined, although it is likely that photo-oxidative damage mediated by accumulated toxic intermediates might be a common cause. The level of a plastid homologue of the bacterial protease FtsH, which is involved in clearing damaged proteins, negatively correlates with the induction of HR cell death. This tobacco gene, *DS9*, seems to positively affect photosynthetic-electron flow and its overexpression leads to an apparent delay in HR-cell-death activation⁷⁵. How these plastid-derived signals are all integrated together for cell-death signalling remains to be determined.

New plant-specific PCD regulators

Two plant genes that are emerging as interesting signalling mediators for HR-cell-death activation are *A. thaliana* *LSD1* and barley *MLO*. Both are conserved across monocots and dicots but seem to be specific to plants.

LSD1. *lsd1* was originally identified as a lesion mimic of the propagation class and it exhibits a so-called 'runaway cell death' (rcd) phenotype — that is, *lsd1* plants cannot restrict cell-death progression after an initial stimulus⁷⁶. It specifically responds to O₂⁻, but not to H₂O₂, to activate HR cell death, and recent genetic studies show that it is probably an important negative regulator downstream of the triacylglycerol lipases EDS1 and PAD4, as mutants of these loci suppressed the *lsd1* cell-death phenotype²⁶. In addition, salicylic acid is also required for the rcd phenotype, which provides strong genetic evidence for LSD1 as a key repressor of cell-death progression during plant defence⁷⁷. A highly conserved relative of LSD1, known as **LOL1** (LSD1-like-1), was recently characterized in *A. thaliana*. Both proteins contain a set of conserved zinc-finger motifs and are putative transcription factors or

scaffold proteins⁷⁸. Unlike LSD1, which is predicted to negatively regulate cell death, LOL1 is thought to be a positive regulator of cell death. Suppression of LOL1 expression in an *lsd1* background suppressed rcd, whereas increased expression of LOL1 accelerated HR cell death in a wild-type background. It has been suggested that LSD1 and LOL1 might represent plant-cell-death potentiators that function in an antagonistic fashion to establish a threshold for cell-death activation. Although the roles of these proteins in HR cell death is clearly established, an interesting question is whether they are specific for pathogen signalling or whether they could be involved in controlling other forms of PCD in plants. It would also be interesting to further clarify the possible *in vivo* association between these two proteins, their biochemical activities and target(s), as well as the role of the third member, **LOL2**, of this emerging family of plant-cell-death regulators.

MLO. The *MLO* gene family was originally identified in barley, with a loss-of-function mutant showing a spontaneous lesion-mimic phenotype of the initiation class — that is, it spontaneously activates PCD in the absence of any obvious inductive signals. MLO, as well as its related proteins, is predicted to contain multiple transmembrane helices and is located in the plasma membrane. Loss of MLO leads to enhanced disease resistance in barley, and it has also been shown that the spontaneous cell death of the mesophyll cells in *mlo* mutants might be the result of partly accelerated senescence⁷⁹. Furthermore, *MLO* seems to be transcriptionally activated under a variety of biotic and abiotic stresses and this might correlate with its role as a negative potentiator of cell-death activation. A clue to the function of MLO came from recent studies showing that CALMODULIN interacts specifically with the carboxy-terminal portion of MLO proteins⁸⁰. Point mutations that abolish this interaction result in a quantitative decrease in the ability of MLO to suppress resistance and this can be phenocopied by suppression of a barley calmodulin isoform by RNA interference⁸¹. These interesting observations provide evidence that MLO function could be modulated by Ca²⁺ concentrations and that it mediates signalling downstream of calcium channels to suppress cell death and resistance.

The elusive executioners or core regulators

Determinants of the 'point-of-no-return.' The first irreversible step in the activation of the cell-death programme would require high precision by the cell. Proteases such as metazoan caspases that function as regulatory switches can provide such a key function. They are usually expressed in a dormant form and subsequently activated, or they are sequestered in a subcellular compartment to protect the cell from misfiring the essential death trigger.

The characterization of a putative protease switch with a clearly defined set of subsequent targets and effectors in plant PCD has been relatively slow. For tracheary-element differentiation, multiple types of protease are thought to be important at different stages. Proteasome-inhibitor studies indicate that ubiquitin-mediated protein

CALMODULIN

A Ca²⁺ protein that is found in all eukaryotes, calmodulin has a high degree of structural conservation and can bind to target enzymes and modulate their activity as a function of the cytosolic Ca²⁺ concentration.

Table 1 | *Arabidopsis thaliana* lesion-mimic and related mutants that affect PCD signalling

Mutant or gene name	Phenotype	Predicted function
<i>acd1</i>	Propagative, necrosis	Chloroplast targeted, Rieske (2Fe-2S) domain protein
<i>acd2</i>	Propagative, HR-like	Red chlorophyll catabolite reductase
<i>acd11</i>	Propagative, chlorosis	Sphingosine-transfer protein
<i>svn1</i>	Propagative, vascular necrosis	Membrane protein
<i>lsd1</i>	Propagative, necrosis, rcd	Scaffold protein or transcription factor
<i>lol1</i>	Suppressor of <i>lsd1</i> ; enhances HR when increased	Homologue of <i>LSD1</i>
<i>acd5</i>	Initiation class, chlorosis	Lipid kinase
<i>acd6</i>	Initiation class, few lesions	Ankyrin-domain-containing transmembrane protein
<i>cpn1</i>	Initiation class, necrosis	Copine protein
<i>cpr5</i>	Initiation class, chlorosis	Transmembrane protein
<i>dnd1</i>	Initiation class, few lesions	<i>A. thaliana</i> CNGC2
<i>hlm1</i>	Initiation class, HR-like	<i>A. thaliana</i> CNGC4
<i>lin2</i>	Initiation class, necrosis	Coproporphyrinogen-III oxidase
<i>ssi2/fab2</i>	Initiation class, HR-like	Stearyl-ACP desaturase
<i>ssi4</i>	Initiation class, chlorosis	TIR-NR-LRR protein related to resistance genes
<i>sfd4/fab6</i>	Suppressor of <i>ssi2/fab2</i>	Plastidic ω 6-desaturase

Selected lesion-mimic mutants that have been identified in *Arabidopsis thaliana* and their related genes are listed along with their known phenotypes and predicted functions on the basis of their deduced amino-acid sequence or biochemical evidence. Most genes described in this table are discussed in the main text and their characterization has been reviewed elsewhere²⁶. Propagative mutants are defective in the control of lesion size and, therefore, once activated by endogenous or exogenous signals, have the phenotype of spreading large cell-death sectors. Initiation-class mutants show spontaneous cell-death activation in the absence of obvious death-inducing signals. Chlorosis is the relatively slow death of tissues that is typified by the loss of chlorophyll, yellowing of tissues and minor collapse of cells. ACP, acyl carrier protein; HR, hypersensitive response; rcd, run-away cell death; CNGC, cyclic-nucleotide-gated channel; PCD, programmed cell death; TIR-NB-LRR, *Toll*-interleukin-receptor-nucleotide-binding-leucine-rich-repeat domains.

turnover is essential during the commitment phase, but not the autolytic phase, of this process^{6,82}. A potential role for extracellular serine proteases has also been postulated⁸³. Early work with ROS-induced cell death has indicated the importance of serine- and cysteine-protease activities¹⁹. Subsequent studies identified cystatin, a cysteine-protease inhibitor, as a possible inhibitor of ROS- and bacteria-induced HR cell death⁸⁴. On the other hand, caspase-specific peptide inhibitors specifically abolished HR cell death in tobacco leaves⁸⁵. Caspase-like protease (CLP) activity was also detected in extracts that were prepared from tissues undergoing HR cell death. Notably, cell death was uncoupled from the defence-gene response by peptide inhibitors, which indicated that the loss of cell-death induction was not due to inhibition at the early step of host-pathogen signalling. Although the use of pharmacological reagents has the usual caveat of specificity and the identity of the targets is still uncertain, these early results, nevertheless, might signify that a protease or proteolytic cascade involving multiple proteases and their regulation by endogenous inhibitors could function as a critical switch in plant cell death.

Since the publication of the complete *A. thaliana* genome, it has become clear that canonical caspases with high structural similarities to the well-studied metazoan homologues are unlikely to be found in plants. Nevertheless, proteases with activities akin to caspases have been implicated in plant-cell-death activation using a variety of experimental conditions and model systems^{4,5,85}. In addition to peptide inhibitors, biological inhibitors for metazoan caspases have also been reported

to suppress plant cell death. The baculovirus proteins Op-IAP (inhibitor of apoptosis protein) and p35 have been shown to interact with caspases *in vitro* and to suppress apoptosis activation in animal systems⁸⁶. Transgenic expression of Op-IAP in tobacco suppressed cell death that was induced by necrotrophic fungal pathogens, and expression of the p35 protein in tobacco delayed HR cell death in several different systems^{85,87-90}. Mutants of p35 that cannot inhibit animal caspases are also unable to suppress cell death in transgenic plants⁸⁸⁻⁹⁰ — providing important support for the specificity of the phenomena that are observed as the result of CLP inhibition.

Identifying elusive CLPs. What might be the identity of plant CLPs? Iterative searches based on the structural features of caspases have identified two related families of proteases, known as PARACASPASES and METACASPASES⁹¹. These families of cysteine proteases do not contain any obvious variants of the QXCRG active-site motif that is found in all canonical caspases, but their predicted structures are likely to have strong homologies to the caspase-haemoglobin fold. Metacaspases are found in fungi, protozoa and plants, including nine predicted metacaspase-encoding genes in the *A. thaliana* genome. Three of these *A. thaliana* genes belong to the type-I class, containing a prodomain that has interesting homologies to the LSD1 protein, whereas the other six type-II metacaspases contain a conserved insertion in the carboxy-terminal half of the predicted protein sequence.

Recently, the single yeast type-I metacaspase, *Yca1*, has been shown to mediate oxidative stress and ageing-

PARACASPASES

A class of cysteine protease that is found in animals and slime mould and was identified as being structurally related to caspases by iterative sequence comparison.

METACASPASES

Predicted cysteine proteases that are found in plants, fungi and protozoa and which contain homologous sequences and domains that are structurally related to metazoan caspases.

Box 2 | Type-III effectors as regulators of host programmed cell death

More than 30 proteins could be injected from Gram-negative bacterial pathogens into cells of their host plants by a type-III secretion system¹¹². A type-III effector that has been reported to repress cell death is the *Pseudomonas syringae* HopPtoD2 protein. This protein was recently shown to have protein tyrosine phosphatase (PTP) activity and its expression in the tobacco *Nicotiana benthamiana* suppressed the activation of HR cell death by an incompatible strain of *Pseudomonas syringae*¹¹³. HopPtoD2 also attenuates hypersensitive response (HR) cell death that is induced by ectopic expression of a constitutively active mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK), MEK2^{DD}, perhaps by counteracting this phosphorylation-dependent signalling step in disease resistance. Correspondingly, the loss of HopPtoD2 results in enhanced HR cell death as well as a reduction in the proliferation of the *Pseudomonas syringae* in the host plant.

A more general PCD repressor could be the *Pseudomonas syringae* AvrPtoB effector protein¹¹⁴. Unlike many of the type-III effector proteins, AvrPtoB is apparently conserved in the genomes of diverse plant pathogenic bacteria including *Xanthomonas* sp., *Erwinia* sp. and some strains of *Pseudomonas* sp. It can trigger HR cell death as well as resistance in tomato plants expressing the resistance gene *PTO* but, surprisingly, it functions as a cell-death suppressor when expressed in the tobacco *N. benthamiana*. The carboxy-terminal region of AvrPtoB contains the death-repressor domain, and its chromosomal deletion from the AvrPtoB locus resulted in a strain that can elicit immunity in a previously susceptible *L. esculentum* background. Expression of wild-type AvrPtoB in *trans* restored disease, thereby providing strong evidence that this cell-death-repressor domain of AvrPtoB is an important determinant for pathogenicity. In addition to suppressing the induction of cell death that is elicited by many plant-resistance genes, AvrPtoB also can suppress BAX-induced PCD in plants as well as yeast. These results provide strong indications that AvrPtoB, more specifically, its carboxy-terminal domain, functions as a general PCD inhibitor for several signalling pathways, and its target(s) should be a good candidate for a core regulator of cell-death control in plants.

related cell death. A *yca1* strain shows dramatically longer life in culture in addition to high tolerance to H₂O₂ (REF. 92). The wild-type Yca1 protein — but not one with a point mutation at the cysteine residue predicted to be the active site — was shown to be proteolytically activated upon oxidative stress, which correlated with the appearance of new CLP activities. These results therefore establish the first genetic and biochemical evidence that metacaspases could be candidates for the role of executioner protease in plants, fungi and protozoa. Indeed, expression of a *Trypanosoma brucei* metacaspase, but not a variant that was mutated at its predicted active-site cysteine, caused cell death in yeast⁹³. Recent findings showed the increased expression of a type-II metacaspase during necrotroph-induced, but not chemical-induced, cell death of tomato (*Lycopersicon esculentum*) leaves, perhaps linking this class of proteases to the induction of plant cell death⁹⁴. Comprehensive analysis of the metacaspase gene family, using reverse-genetic and molecular approaches, should allow us to determine their biochemical characteristics and *in vivo* relevance to plant cell-death control in the near future.

BI-1-related cell-death regulators. A large class of conserved core regulators that are related to the BCL-2 protein are important in potentiating death-inducing signals in animals^{7,95}. Although plant and yeast genomes lack any obvious orthologues of BCL-2-related proteins at the primary sequence level, expression of metazoan pro-apoptotic BAX activated the cell-death programme, whereas

expression of the pro-survival BCL-2 or BCL-X_L provided protection in these organisms^{4,5,7}. An induced-lethality suppression screen was carried out in yeast to identify new mammalian proteins that could counter the pro-death activity of BAX⁹⁶. Two proteins — BAX inhibitor-1 (BI-1) and bifunctional apoptosis regulator (BAR) — were identified, and BI-1 homologues were found as conserved genes in plants. Plant BI-1 homologues, as well as a number of proteins that are involved in ROS homeostasis such as iron-superoxide dismutase, were also found using this yeast lethality-suppression screen with *A. thaliana* and tomato cDNA expression libraries⁹⁷. In *A. thaliana*, the expression of BI-1 was induced during plant-pathogen interaction, and overexpression of *A. thaliana* BI-1 — as well as the rice orthologue of BI-1 in animal cells, transgenic tobacco and *A. thaliana* — has been shown to suppress BAX-induced cell death^{97–100}. Suppression of tobacco BI-1 expression correlated with increased AUTOPHAGY, nucleosomal DNA fragmentation and cell death after carbon starvation and hypo-osmotic shock¹⁰¹. Another study found that decreased BI-1 expression correlated with chemical-induced resistance of barley to powdery mildew fungal infection. Furthermore, overexpression of barley BI-1 at a single-cell level induced hyper-susceptibility and could reverse the fungal resistance that is conferred by the loss of *MLO*, a negative regulator of resistance and HR-like cell death¹⁰². BI-1 could therefore function as a cellular survival factor that promotes fungal infection, and its downregulation would correlate with heightened fungal resistance, as is the case for *mlo* mutants of barley^{79,81}. Consistent with this conclusion, transgenic rice that overexpressed rice BI-1 showed improved cell survival upon challenge with a fungal elicitor from *Magnaporthe grisea*¹⁰³.

Although *A. thaliana* BI-1 was reported to localize to the endoplasmic reticulum of plant cells⁹⁹, the mode of action for this protein remains enigmatic in animal, plant and yeast systems. All known BI-1-related proteins contain 5–7 predicted transmembrane α -helices in their deduced sequence, which are conserved in the two other *A. thaliana* BI-1-related genes, BI-2 and BI-3. Database searches using the amino-terminal region of *A. thaliana* BI-2 uncovered 13 genes, which are known as *A. thaliana* BI-2-related (ABR) genes⁹. No obvious homologues to ABRs were found in animal or yeast genomes, which indicates that these genes might have evolved specifically in plants. This new family of proteins has been speculated to function in an analogous fashion to Bcl-2-related proteins — perhaps pro-survival and pro-death members could regulate cell death under different contexts. Consistent with this is the presence of six predicted transmembrane helices in all of the ABR members, similar to BI-1. Interestingly, expression of *A. thaliana* BI-1 in a human fibrosarcoma HT1080 cell line induced apoptosis that could be repressed by co-expression of the caspase inhibitor XIAP¹⁰⁴. This finding showed that *A. thaliana* BI-1, and perhaps its related proteins, could function as either pro-survival or pro-death factors in a context-dependent manner. Further detailed studies on the activity and function of BI-1 and its related proteins in plants

AUTOPHAGY

A pathway for the recycling of cellular contents, in which materials inside the cell are packaged into vesicles and are then targeted to the vacuole or lysosome for bulk turnover.

Box 3 | The plant vacuole: key player or supporting role?

The prominent presence of the vacuole in most mature plant cells and its function as a store for proteases and nucleases makes it a probable partner in most, if not all, forms of programmed cell death (PCD) in plants^{12,115}. However, whether it functions as a key executioner that defines the 'point-of-no-return', or, whether it has a supporting role as a provider of the necessary enzymes for recycling of the contents of the dying cell, is not clear at present. Among the various types of proteases that are known to be present in the vacuole, several members of the cysteine protease VPE (vacuolar processing enzyme) family are known to be activated in their expression and localization in the lytic vacuole upon stress and senescence¹¹⁶. Interestingly, it has been found that VPE can cleave substrate target sites with an aspartic-acid residue at the P1 position instead of asparagine¹¹⁷. They might potentially account for some of the recently documented caspase-like-protease activities that were observed in plant cell extracts upon PCD induction. A compilation of the annotated *Arabidopsis thaliana* genome revealed 12 orthologues to the 15 known yeast autophagy genes¹⁰⁷. Interestingly, 3 of these 12 orthologues exist as multigene families with *A. thaliana* APG8 having 9 copies, whereas a single gene encodes Apg8 and its orthologues in *Saccharomyces cerevisiae* and metazoans, respectively⁹⁵. APG8 is a starvation-induced gene in *S. cerevisiae* and functions to regulate AUTOPHAGOSOME formation and its delivery to the vacuole. Plants might have elaborated this type of protein for more diverse functions of the vacuole under various stress conditions. Recently, mutants of *A. thaliana* APG7 and APG9 have been described^{107,108}, which both showed an early senescence phenotype in addition to hypersensitivity to nutrient starvation.

should help establish whether they are true components that modulate cell-death triggers of PCD.

Cell-death regulators from plant pathogens. The genomes of viral pathogens provide a good hunting ground for potential regulators that could repress cell-death switches in their host because of their obligate BIOTROPHIC lifestyle. The baculovirus is an excellent example in which two main classes of potent cell-death regulator — IAPs and p35 — were discovered, and cellular-IAP orthologues were subsequently identified in animals as important regulators of cell death⁸⁶. It is therefore interesting that the study of plant viruses has yet to uncover cell-death repressors that would shed light on the central control machinery of the host. By contrast, recent studies of TYPE-III EFFECTORS from Gram-negative bacterial pathogens have provided the first candidates that could enrich our understanding of cell-death control in plants (BOX 2).

The vacuole as a source of executioners. Alan Jones recently hypothesized¹² that the loading of distinct sets of hydrolytic enzymes into the vacuole, as induced by endogenous or extracellular cell-death-triggering signals, could define the various morphotypes of plant PCD. In this scenario, calcium flux was envisioned to have a critical role in mediating the trigger of the eventual vacuole collapse by the direct or indirect action of a common executioner downstream from the various death signals. It is interesting to compare the mechanism of plant PCD and that of autophagic cell death in animal systems. Developmental PCD in animal systems is now known to occur commonly through non-apoptotic pathways⁹⁵. One recently well-characterized system is that of hormone-induced PCD of the salivary gland in *Drosophila melanogaster* where typical cellular morphologies of

autophagic cell death, instead of apoptosis, were observed. Nevertheless, regulatory genes that were related to the core executioners, such as a *D. melanogaster* caspase, were found to mediate this type of cell death¹⁰⁵. So, overlapping sets of regulators could be used to activate diverse cell-death morphotypes with the choice of cell-death modes being defined by the particular cellular context as well as the identity of the trigger. The finding that Beclin-1, a mammalian orthologue of a yeast protein (Apg6) that is involved in autophagy, binds BCL-2 and inhibits virus-induced apoptosis as well as tumorigenesis¹⁰⁶ also indicates a possible connection between autophagy, the vacuole/lysosome and apoptosis. It would be interesting to determine whether the autophagy protein Beclin-1/Apg6 could be involved in modulating apoptosis via interaction with BCL2-related proteins, or conversely, whether BCL2-related proteins might also function to modulate autophagic cell death.

In the analysis of cell-death morphologies during somatic embryogenesis in gymnosperms, Filonova *et al.*¹³ made the interesting observation that they resemble a combination of apoptotic and autophagic modes of animal PCD. It is therefore intriguing to raise the question as to whether key components that are involved in autophagy, which is normally used for recycling of cellular components via the vacuole/lysosome during nutrient starvation, could be involved in plant PCD (BOX 3). Further studies of cell-death-related processes with mutants in the autophagy pathway^{107,108}, as well as mutants in vacuole biogenesis such as *vacuoleless-1* (REF. 109), should shed light on the possible connections between these phenomena that are related to the controlled turnover of the content within plant cells.

Conclusions

The distinct purposes of controlled cell death, coupled with the diverse cell types in which cell death must be carried out, probably contribute to the diverse PCD morphologies that are observed in plants. During senescence, which is a slower process that coordinates cell death at the organ and tissue level, efficient recycling of nutrients from the dying cells and tissues is the primary purpose, whereas a more rapid cell death is required during the HR to efficiently prevent viral pathogens from systemically infecting the host plant^{5,6,12,88}. A specialized cell-death pathway might therefore have evolved in plants for each distinct purpose and different sets of executioners might be activated. Alternatively, varied contributions from a relatively small set of distinct cell-death pathways could provide the means for generating the spectrum of cell-death morphotypes that have been reported. The latter 'combinatorial' model is consistent with the observation that when apoptotic cell death is inhibited during digit formation in *Xenopus laevis*, a slower necrotic cell death is observed¹¹⁰. The partial redundancy that is inherent to this type of combinatorial model might also explain the paucity of 'plant cell executioners' that have been genetically identified so far, compared with the many components of various signalling pathways that function to modulate the death decision^{25,26}. This is in contrast to *C. elegans* and *D. melanogaster*, in which a set

AUTOPHAGOSOME

A double-membraned structure containing content for turnover that fuses with the vacuole, or the animal lysosome, during autophagy.

BIOTROPHIC

A lifestyle of pathogens that is dependent on a living host.

TYPE-III EFFECTORS

Proteins that are injected by gram-negative bacterial pathogens into their host cells via a conserved type-III secretory system.

of core regulators for various death morphotypes has been described genetically^{2,7,95}.

In conclusion, elucidation of the pathway for controlling vacuole rupture, the identification of the different CLPs that have been suggested to be important for plant-cell-death pathways, and the functional characterization of BI-1-related genes should help to shed light on these possible models. To this end, the completed *A. thaliana* genome coupled with a large collection of sequenced insertions are invaluable resources that

should help mine the genome for components in the cell-death pathways of plant cells. Efficient, inducible gene-suppressing systems¹¹¹ should further enhance our ability to test for gene functions under different developmental conditions. Ultimately, the knowledge that is gained should be relevant to applications such as those related to plant-disease resistance and wood production. Moreover, comparisons with animal cell-death pathways also shed light on the evolution of this process in eukaryotes.

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The author declares that he has no competing financial interests.

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