Dead cells do tell tales

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The most recent major advances in the study of programmed cell death (PCD) in plants include the observation that peptide inhibitors of caspases inhibit the hypersensitive response. Nitric oxide has been shown to be required for the induction of disease related PCD. Mutant analysis has led to the cloning of the first genes involved in PCD related disease resistance, *LSD1* and *MLO*.

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Abbreviations

APX	ascorbate peroxidase
HR	hypersensitive response
NO	nitric oxide
PCD	programmed cell death
PR	pathogen-related
R	resistance
ROI	reactive oxygen intermediates
SA	salicylic acid
TE	tracheary element

Introduction

Intrinsically programmed cell death is, by now, an accepted requisite for life in multicellular eukaryotes. Extra- and intracellular inputs dictate whether cells find themselves in the right place, at the right time in development, and surrounded by the right neighbors [1•]. If reinforcing microenvironmental signals are perceived, the cell lives and continues about its business. But when spatial and temporal signals clash, and the 'I'm OK, you're OK' monitoring system is perturbed, cells follow an intrinsic pre-set default pathway leading to their own death [2]. Cells which beat the system, through mutations disarming the default death pathway(s), can become malignant. Conversely, it is well established that some cells are destined to die as part of normal developmental processes, or can be triggered to die quickly as part of a response to infection.

This broad outline was established in animal systems. Recent data suggests that several fundamental aspects of this model can also operate in plants. Possible mechanistic overlap and molecular parallels between cell death control in plants and animals are under investigation. Here we summarize very recent publications which add to the unraveling of how plants program cell death; recent reviews provide detailed background material [3–5]. Our definition of a 'program' is one which is intrinsically encoded by the plant cell. Hence, mutants which interrupt or induce cell death might define steps in pathways which either respond to, or regulate, cellular homeostasis. This definition, at first glance, could preclude cell death initiated by pathogen-produced toxins. But if that toxin has a specific cellular target, and that target performs a cellular task which is monitored for fidelity, then lack of fidelity in the process could trigger an intrinsic death program [3–6]. Our definition would, however, probably preclude the death engendered by non-specific toxins like heavy metals. Key unanswered questions to bear in mind include: are some or all plant cell deaths driven by a default pathway? How many inputs do plant cells monitor which, when perturbed, lead to cell death? Is there more than one 'execution' pathway? Have plant pathogens learned to interdict the death process to suit their lifestyle needs?

Programmed cell death (PCD) occurs at many points during plant development, but the most progress to date has been made using the hypersensitive cell death response (HR) to pathogen infection as a model. Whether the HR, per se, stops pathogen growth or whether it is a consequence of the mechanism(s) which does is not clear. Yet, it is very often correlated with disease resistance. As well, common disease symptoms — the outcome of a successful infection — also very often include the death of host cells. It is important to consider that the deaths engendered in these two outcomes could be mechanistically different; these differences might reflect the fact that in the HR the plant is controlling its own cellular destiny, whereas during the onset of disease symptoms it is the pathogen which may use interdiction of a normal cellular signaling pathway to further its goal of growth and propagation. With these caveats in mind, we concentrate on the HR in this review.

If it looks dead...

Many, but not all, programmed cell deaths in animals lead to a stereotyped cellular dismantling known as apoptosis. Apoptosis is the outcome of the integration of a wide range of signals, all of which feed into a conserved cysteine proteinase (caspase) cascade [7]. This cascade is under constitutive negative regulation. If that regulation is removed, the default pathway is activation of caspases and orderly cellular dismemberment. Apoptosis, contrary to vernacular usage, is not synonymous with PCD, as there are other programs which can operate to initiate cell suicide in animal cells (e.g. [8]).

Some facets of apoptosis are shared between kingdoms. Broadly, signals are transduced, ion fluxes occur, and specific proteinases are activated during plant cell death. Nuclear condensation and DNA cleavage first into large fragments, and subsequently into fragmented 'ladders', is observed in some, but not all cases of pathogen-induced PCD. The lack of apoptotic bodies in plants is not surprising, if their

function is to facilitate phagocytosis of their contents by neighboring cells. There is no way for typical apoptotic bodies to pass through the cell wall. A recent study points out yet another difference between plant and animal versions of PCD processes. Tobacco cells pulsed with chemical inducers of PCD have a window within which they can reverse the program, even after chromatin condensation has begun [9•]. This is in contrast to the animal model where once initiated, there is no turning back from PCD.

Proteins in the BCL*2*-CED9 family can either prevent or trigger PCD in animals [10]. No genes encoding BCL*2* family members been unambiguously defined in public plant databases. Immunolocalization studies using antibodies to a human BCL*2* family member, however, detected a BCL*2*-like epitope in leaves and meristematic tissues of tobacco and maize. BCL*2* family members are mostly localized on the mitochondria in animals [11]. As might be expected, the BCL*2*-like plant protein localizes to mitochondria, nucleus and chloroplasts [12]. An additional tool to uncover genes operative in the *BCL2* control pathway, perhaps including plant genes, was recently described [13•] — yeast hybrid analysis was used to screen a human library for an inhibitor of BAX (a pro-apoptotic BCL*2* family member), and homologues of this inhibitor were found in *Arabidopsis*, mouse, and *C. elegans*, implying possible mechanistic conservation. Overexpression of the animal anti-apoptotic BCL-XL, however, in transgenic tobacco did not inhibit HR [14].

Animal viruses often encode proteins which inhibit caspase activation. When overexpressed in tomato and tobacco, the p35 protein from Baculovirus seems to inhibit both pathogen growth and disease symptoms caused by necrotrophic pathogens. Interestingly, growth of obligate biotrophs was not slowed although the eventual disease symptoms these pathogens produce was diminished (D Gilchrist, personal communication). This result strongly suggests that induction of conserved mechanisms for apoptosis are used by virulent, necrotrophic pathogens to kill cells upon which they can then feed. Another preliminary study concludes that TMV induced HR was inhibited by p35 overexpression in tobacco and that mutations in p35 which abolish caspase inhibition also abolish this phenotype (O del Pozo and E Lam, personal communication). A recent demonstration that plants induce a caspase-related proteinase activity preceding HR suggests that, among the many cysteine proteases in plants, at least one has the substrate requirements of an animal caspase [15••]. In animals, the CED-4/Apaf1 proteins activate caspases and these are tethered by BCL-*2* proteins until required [16]. Enticing homologies between these proteins and a domain in plant disease resistance proteins has been noted [17•], further suggesting structural similarities between pant and animal cell death control.

Signal traffic to control HR cell death

The signal cascade leading to HR is triggered through recognition of a pathogen avirulence gene product by the appropriate disease resistance (*R*) gene [18], or by an elicitor of plant defense responses recognized by a specific receptor (e.g. [19]). Recognition of either type of signal initiates the overall resistance response, in association with an influx of Ca^{2+} ions from the extracellular space, anion fluxes leading to alkalinization of the extracellular milieu, an oxidative burst producing reactive oxygen intermediates (ROIs), defense gene activation, development of local and systemic disease resistance (reviewed in [*20*,*21*••,*22*]). Measurements of increase in cytosolic calcium support the concept that it plays a very early role in this transduction chain [*23*,*24*•]. The overall order of these events seems to reflect the activation of multiple pathways, as inhibition of Ca^{2+} flux or oxidative burst prevents cell death but not defense gene activation [25••]. An exciting recent development is the identification of a MAP kinase which mediates a variety of input signals, is salicylic acid (SA) inducible, and leads to defense gene activation [*26*••,*27*••,*28*••]. Interestingly, this kinase acts either upstream to or independently of the oxidative burst (T Romeis *et al.*, unpublished data). This, or another, kinase cascade may end in the phosphorylation of a putative transcription factor for defense response genes [*29*•].

ROI are ordinarily generated during both metabolic and photoactivated processes, and damage the cell via uncontrolled oxidation of cellular components. Different ROI are produced within the cell, and, in combination with different sites of production, could drive cell death in a variety of cellular contexts. The oxidative burst initiated by pathogen attack results in superoxide synthesis, which can occur within regions of the cell wall adjacent to the pathogen [30]. Superoxide can spontaneously dismutate to the more stable product hydrogen peroxide (H_2O_2) or it can act as a localized signal molecule. H_2O_2 can serve multiple roles in plant disease resistance: as a direct antimicrobial activity; a component of structural defense through oxidative cross-linking of the cell wall; and as both an intra- and inter-cellular signaling molecule (reviewed in [31]).

Oxidative burst, in isolation, does not lead to cell death, but high levels of hydrogen peroxide (much greater than those produced during a typical oxidative burst) can kill cultured soybean cells [32]. In addition, mutant bacteria which cannot trigger HR still generate a normal oxidative burst [33] and the oxidative burst is apparently not a sufficient signal for HR in the cowpea–cowpea rust fungus pathosystem [34]. Nitric oxide (NO) can act to potentiate PCD in animals [35] and important recent evidence supports a similar role for NO in plants [36••,37••]. If NO production is inhibited in cultured soybean cells or tobacco leaves, the HR is blocked and resistance to avirulent bacteria is moderately attenuated. Furthermore, exogenous generation of NO and ROI synergistically promote cell death and induce gene expression of both PR1 (SA-dependent) and PAL (SA-independent) defense genes. How do these local events relate to the onset of systemic signals? Recent evidence

suggests that ROI may also be rapidly induced at distal uninfected leaves [38•] following a local oxidative burst in infected leaves. The notion here is that rapid systemic signaling can be used as an early warning system which primes the SA-dependent systemic responses.

The cell has numerous compounds and enzymes which serve to scavenge ROI before untoward damage can occur. These antioxidants are typically upregulated in times of oxidative stress (e.g. high light, ozone exposure). Ascorbate peroxidase (APX) uses ascorbate to detoxify hydrogen peroxide. APX is found throughout the cell, and is believed to be one of the main scavengers of peroxides. Like other antioxidative enzymes, the levels of APX declines in cells undergoing HR [39•,40•,41]. Overproduction of APX in the chloroplast, however, does not protect tobacco against ozone induced damage [42].

Are there relationships between the set of signal events leading to HR and any case of developmentally controlled cell death? One highly developed system for analyses of PCD during development is tracheary element (TE) formation in transdifferentiating zinnia mesophyll cells [43]. Cell death during TE formation is not apoptotic and does not generate an observable oxidative burst. A calcium influx and perception of extracellular signals, mediated by a serine protease, however, are required for TE cell death [44,45•]. Thus, most features of this developmental cell death differ from signaling in HR as outlined above.

Salicylic acid and the potentiation of cell death

In tobacco and *Arabidopsis*, endogenous SA levels rise following pathogen attack and correlating with expression of pathogenesis-related (*PR*) genes as well as the onset of SAR. Exogenous addition of SA induces *PR* gene expression as well as heightening disease resistance (reviewed in [21••,46]). Yet, SA acts downstream of the oxidative burst and SA addition without pathogen triggers neither substantial increases in ROI nor cell death. A variety of experiments strongly support a model whereby SA and ROI (and probably NO) potentiate the overall HR and defense response [47–49,50••]. Transgenic expression of salicylate hydroxylase (*nahG*) under the control of temporally different promoters demonstrates that SA accumulates during pre-necrotic phases of TMV infection in tobacco and that this accumulation is required to curtail viral spread. [51]. SA pretreatment of parsley cell suspensions also potentiates subsequent induction of various defense genes by both elicitor-dependent and -independent modes. SA-potentiated, pathogen-induced cell death is also supported by observations of reduced lag time to cell death from eight to four hours [52]. The most likely scenario is that ROI production triggers both NO and SA synthesis. Superoxide and NO can combine to form the very dangerous peroxynitrite radical, and high levels of NOS activity could lead to more superoxide production [53], resulting in an amplification which produces more SA and NO. It is critical to note that amplification mechanisms containing an extracellular component (ROI production or signals emanating from dying cells), must be negatively controlled by desensitization once a sufficient response has been reached. This desensitization could respond to gradients of ROI or SA around HR sites. For example, recent work suggests that although the levels of ROI and SA in live cells around HR sites are sufficient to induce defense gene transcription, they are not enough to trigger HR [51,54•].

Misregulation of HR cell death in mutants

A number of mutants misregulate cell death, suggesting that the wild-type function of the genes they define may be in PCD control. They are collectively termed 'lesion mimics' because their phenotypes are reminiscent of either HR or disease symptoms (reviewed in [3,55]). Some lesion mimics map to disease resistance genes, suggesting that misregulation of R function can lead to inappropriate cell death. For example, the *Rp1 R*-gene locus in maize is prone to unequal crossing over that generates new fungal resistance specificities. Derivative alleles exist that cause lesioning in the absence of pathogen [56]. Some genes whose mutant phenotypes are increased disease resistance and propensity for cell death have been cloned. The *Arabidopsis LSD1* gene is a negative regulator of cell death which responds to a superoxide dependent signal. It encodes a zinc finger protein which may function as a transcriptional regulator [57,58••]. In barley, the *MLO* gene encodes a novel transmembrane protein [59••]. In *mlo* mutants, defense pathways are primed; this leads to both a low level lesion phenotype and resistance against downy mildew. In contrast, *lsd1* mutants are generally resistant to virulent bacterial and oomycete pathogens. Thus, *MLO* negatively regulates a subset defense response components active against one species of pathogen, while *LSD1* negatively regulates a potentially broader set. These two mutants are instructive because they suggest that defense systems are in part pre-existing and active in the absence of negative control. Mutations in negative regulators which interpret activation signal thresholds can lead to default cell death.

In addition to negative regulators controlling the extent of HR, there are a number of lesion mimic mutants, mostly dominant, that induce the SA-dependent disease resistance pathway in *Arabidopsis*. These mutations can be sometimes genetically upstream of requirements for SA accumulation in defense gene induction and disease resistance [60]. Alternatively, they can function in the amplification loop of SA responses such that SA is both required for lesion formation and able to potentiate lesion formation ([61], DH Aviv *et al.* unpublished data). There are also a number of constitutive disease resistant mutants which do not make lesions. In particular the *dnd* mutation is resistant to virulent pathogens in the absence of HR and makes a smaller and less obvious HR when challenged with avirulent pathogen [62]. This phenotype is in keeping with classic examples of systemic tobacco mosaic virus resistance in tobacco and suggests that the potential

normal requirement for HR in either local or systemic resistance can be bypassed.

Do all mutants that trigger PCD define genes whose wild-type function is either in pathogen recognition or response pathways? Evidently not. *Les22* in maize was recently cloned and found to encode an enzyme in the porphyrin pathway [63•]. Mutation in this enzyme causes a build up of a photoactivatable intermediate, which generates ROI in response to high light. Additionally, this pathway seems to impinge on heme formation, which is required for catalase and APX function, resulting in an inability to detoxify the ROI produced. If leaves are protected from high light until they are fully developed, thereby lowering flux through this pathway, lesioning disappears. Thus, ROI resulting from metabolic perturbation of pathways can trigger PCD and yet not be involved in defense. The maize *LLS1* gene is a negative regulator of cell death, and encodes an enzyme believed normally to degrade a phenolic signal. The absence of this enzyme leads to cell death [64]. Neither *Les22* nor *lls1* mutants induce disease resistance pathways. Mechanistically, this class of mutants suggests that cells sense metabolic perturbations and can follow a default pathway, normally under negative control, to cell death. Alternatively, these mutants result in accumulation of toxic compounds which then kill the cell.

Lesion mimics can also result from transgene overexpression. Several recent papers illustrate how these may trigger the disease resistance pathway much as the lesion mimics upstream of SA accumulation described above. Transgenic tobacco plants with reduced levels of catalase form lesions and express PR genes in response to high light, and this death increases protection against TMV and Pseudomonas [65–67] in an SA-dependent manner [68]. Expression of the light driven proton pump bacterio-opsin in tobacco results in increased systemic SA levels, PR gene expression, and resistance to several pathogens [69]. Expression of the same gene in potato imparted resistance to certain fungal isolates, but unexpectedly increased susceptibility to Potato Virus X [70]. Hence, inducible artificial lesion mimics resulting from transgene expression may not be a panacea for disease resistance in agriculture, but may prove useful in certain cases.

Conclusions

We hope to have conveyed the spirit of recent advances in understanding the control of HR and responses to pathogens where cell death occurs as an overall model of PCD in plants. It is apparent that we are at the early stages of this understanding, that some parallels exist with much more detailed examples from animal cell biology, and that genetic, biochemical and pharmacological tools are available to more clearly dissect these processes.

Note added in proof

The paper referred to in the text as T Romeis *et al.* has now been accepted for publication [71].

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