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COMMENTARY

Reactive Oxygen Intermediates as Mediators of Programmed Cell Death in Plants and Animals

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ABSTRACT. Programmed cell death (PCD) is a physiological process occurring during development and in pathological conditions of animals and plants. The cell death program can be subdivided into three functionally different phases: a stimulus-dependent induction phase, an effector phase during which the wide range of death-stimuli are translated to a central coordinator, and a degradation phase during which the alterations commonly considered to define PCD (apoptotic morphology of the nucleus and chromatin fragmentation) become apparent. Recent studies suggest that mitochondrial permeability transition is the central coordinator of PCD and deciding whether or not a cell will die. There is increasing evidence that reactive oxygen intermediates (ROI) serve as direct and indirect mediators of PCD in mammalian and plant cells. Overexpression of genes encoding pro- and antioxidant enzymes in transgenic animals and plants has been informative regarding the function of ROI. Recent data imply a dual role of ROI in the apoptotic process: first, as a facultative signal during the induction phase, and, second, as a common consequence of mitochondrial permeability transition leading to the final destruction of the cell. The present review discusses and compares new insights into the function of ROI during PCD in mammalian cells and in human and plant diseases. BIOCHEM PHARMACOL **57**;3:231–245, 1999.
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KEY WORDS. apoptosis; hypersensitive response; necrosis; programmed cell death; oxidative burst; reactive oxygen intermediates

PCD† is an integral part of many aspects of animal development [1] and selectively eliminates unwanted cells [2]. Cell suicide programs may also be activated in response to abiotic or biotic stimuli. In plants, PCD of single cells or small groups of cells occurs during reproductive and vegetative stages of development such as sex determination, gamete development, embryogenesis, formation of fluid-conducting channels called vessels and tracheids, leaf abscission, and the HR to pathogen infection [reviewed in Refs. 3–6]. The HR describes the rapid host cell death at and around the site of infection, a common feature of disease resistance in plants, with no connotations of immunological hypersensitivity. It can be triggered by a variety of pathogens and is thought to prevent systemic proliferation of the infection. In addition, plants have evolved genetic programs that trigger death of whole organs, such as leaves, flowers, and fruits, when they reach a certain developmental stage, and this is generally known as senescence [7].

THE "SHAPE" OF CELL DEATH: THE APOPTOSIS/NECROSIS PARADOX

PCD is often described by the use of morphological criteria. These features include chromatin aggregation, cytoplasmic and nuclear condensation, and partition of cytoplasm and nucleus into membrane-bound vesicles, so-called apoptotic bodies. These attributes are accompanied by cleavage of the chromatin at internucleosomal sites resulting in DNA fragments that are multimers of about 180 bp [8]. The term apoptosis was introduced by Kerr and colleagues [9] to distinguish this particular type of PCD from necrotic cell death in animals. Necrosis results from high doses of cytotoxic agents or severe injury, such as trauma and ischemia, and is characterized by cell and organelle swelling and membrane rupture. The leakage of cell contents may induce an inflammatory response in the surrounding tissue, while apoptotic cells are mostly sequestered by neighboring cells or by phagocytes before they can lyse. Therefore, apoptosis and necrosis have been regarded as morphologically and conceptually distinct modes of cell death.

Nevertheless, an increasing body of evidence is accumulating to suggest that apoptosis and necrosis represent just extreme ends of a wide range of possible morphological and biochemical deaths [10]. Many diverse triggers of cell death such as TNF, heat shock, viruses, protein synthesis inhibition, oxidative stress, hypoxia, or nitric oxide can induce

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† *Abbreviations:* AIF, apoptosis-inducing factor; BHA, butylated hydroxyanisole; DFF, DNA fragmentation factor; HR, hypersensitive response; ICE, interleukin-1β converting enzyme; $\Delta\Psi_m$, mitochondrial transmembrane potential; PCD, programmed cell death; PT, permeability transition; ROI, reactive oxygen intermediates; SA, salicylic acid; SOD, superoxide dismutase; and TNF, tumor necrosis factor.

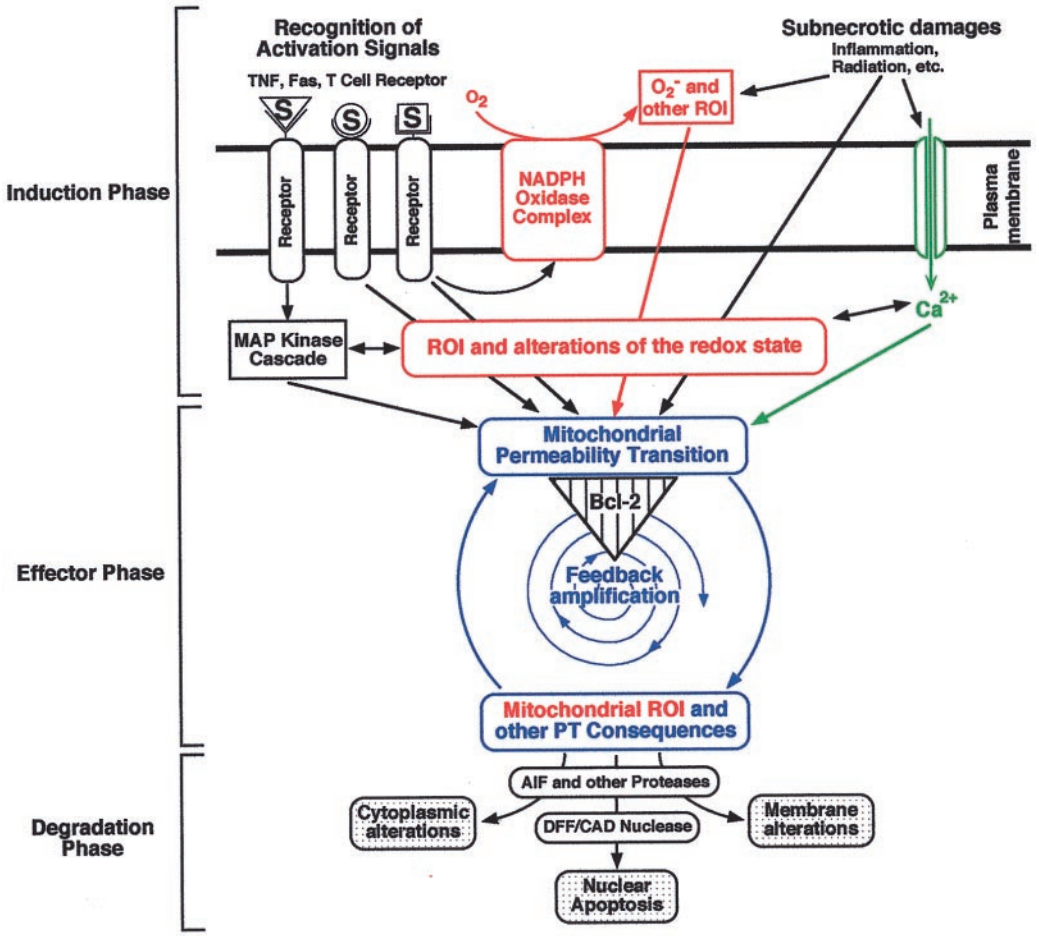

FIG. 1. Proposed role of ROI in apoptosis regulation in mammals. Apoptosis can be induced by receptor-mediated activation signals (S) and subnecrotic damages. The immediate downstream signaling events depend on the death stimulus. They involve MAP kinase cascades, ion fluxes (green), as well as ROI and alterations of the cellular redox state (red). Induction of mitochondrial PT (blue) serves as an integration and amplification step of these "private" pathways, which is controlled by the action of the antiapoptotic protein Bcl-2. Subsequent generation of mitochondrial ROI and other consequences of mitochondrial PT lead to the activation of the apoptotic degradation processes.

both apoptosis and necrosis [10, 11]. Moreover, similar signaling mechanisms involving ROI [12], Ca^{2+}, and stress-dependent transcription factors have been implicated for necrotic and apoptotic cell death [10]. Even proteins that were thought to be highly specific to apoptotic death programs, such as Bcl-2 and caspases, seem to participate in necrosis, too [12, 13].

Recent findings suggest that the "shape" of cell death (apoptotic or necrotic morphology) is determined by the intracellular ATP level [reviewed in Refs. 14 and 15]. The ATP requirement has been demonstrated in various apoptosis models. When ATP levels are reduced, triggers that usually induce apoptosis stimulate necrotic cell death [13]. This theory of apoptosis and necrosis as extremes of a continuum of multiple forms of PCD is supported by earlier studies that have shown that not all forms of PCD in animals and plants involve all of the morphological changes associated with apoptosis [16–18].

IS THERE A COMMON CELL DEATH MECHANISM THROUGHOUT THE PLANT AND ANIMAL KINGDOM?

The mechanisms leading to animal cell death have been studied extensively during the last 20 years, although they are far from being understood completely [19]. In plant science, this field of research is just emerging. The first mention of apoptosis in connection with plants was with the HR in 1986 [20], but little further characterization took place until cell death mutants from *Arabidopsis* were described in 1994 [21, 22].

Three functionally distinct phases (induction, effector, and degradation, see Figs. 1 and 2) are apparent in both plants and animals [3, 23]. During the induction phase, plant and animal cells receive the death-promoting stimulus, which can be as diverse as growth or survival factor withdrawal [24, 25], binding of TNF to its receptor [11],

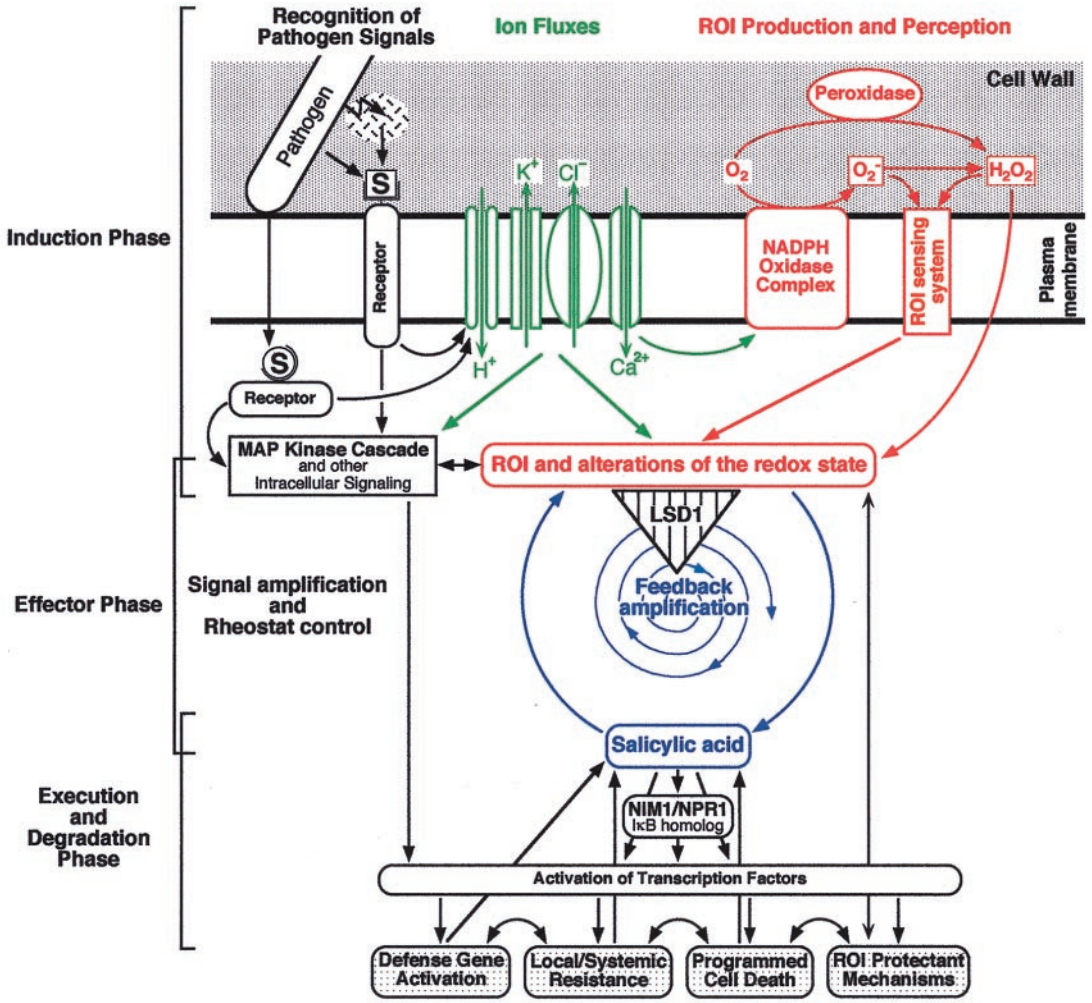

FIG. 2. Speculative model of programmed cell death regulation during the plant defense response. Plant receptor proteins interact with pathogen interaction-derived signal molecules (S). The subsequent cell death-inducing second messenger systems are not known and might depend on the plant–pathogen interaction studied. Participation of transmembrane ion fluxes (green), ROI and cellular redox state (red), MAP kinase cascades, and other intracellular signaling mechanisms has been shown. Strong biochemical and genetic evidence indicates an important role of the signaling molecule SA (blue), which might form a feedback amplification cycle in concert with alterations of ROI metabolism. The putative transcription factor LSD1 may function as a negative control element of this central coordination step. Downstream signaling of SA-dependent pathways involves an IκB homolog, NIM1/NPR1.

cross-linking of Fas/APO-1 by anti-Fas antibody [26], activation of the tumor suppressor gene p53 by genotoxic agents [27], recognition of a pathogen-derived avirulence (*avr*) gene product by the corresponding plant resistance (*R*) gene product [6, 28], heat shock [29], hypoxia [30, 31], or contradictory signal combinations [9, 32]. Furthermore, cytotoxic agents, such as treatments causing DNA damage, antitumor drugs [33], ceramides [34], mycotoxins [29, 35], SA [36, 37], UV [33, 38], and oxidative stress [39, 40], are able to kill animal and plant cells in a controlled manner, i.e. by apoptosis [reviewed in Ref. 32]. Many different signal transduction pathways participate in this induction phase of PCD, dependent on the cell context and on the lethal stimulus applied (Figs. 1 and 2) [41]. Beyond this stage, during the effector phase, universal regulatory events, including the anti- and proapoptotic function of the Bcl-2 family members, seem to take place and converge the initial diverse signaling processes into a few stereotyped pathways. Here, the cells pass a point of no return and are irreversibly committed to death. Thereafter, numerous degradation processes take place, which give rise to the characteristic morphology and biochemistry of apoptosis [9].

In plants, only a few similarities to the animal apoptotic signaling machinery have been elucidated. All attempts to identify plant homologues to the prototypic regulator of mammalian cell death, Bcl-2, or any other members of the Bcl-2 family, have until now been fruitless. This includes screening of cDNA and genomic libraries as well as searches of expressed sequence tag (EST) databases available for several plant species. Additionally, overexpression of the human antiapoptotic protein Bcl-X_L in tobacco did not block PCD in response to viral or bacterial pathogens [42]. Likewise, cysteine proteases similar to the ICE, also called caspases, which play a central role in the effector

phase of animal PCD [43], have yet to be described in plants.

Nevertheless, some molecular evidence that mechanisms underlying PCD may indeed be conserved in animals and plants has been unraveled recently. The gene encoding the *defender against apoptotic death* protein (DAD1) has been identified from *Arabidopsis* and rice EST databases [44, 45]. The *Arabidopsis* cDNA has been shown to be as efficient as human *dad1* in rescuing mutant hamster tsBN7 cells from apoptotic cell death [45]. Interestingly, the pea homologue of *dad1* is down-regulated during senescence of flower petals [46], pointing to a putative endogenous function as plant antiapoptotic regulator. In further analogy to animals, hydrolytic enzymes, such as nucleases and proteases, and the ubiquitin system are activated during the degradation phase of senescence and various other types of cell death in plants [4, 5]. Many of these proteinaceous components of the apoptosis jigsaw puzzle are continually being uncovered and put in place [for excellent reviews see Refs. 43, 47, and 48].

ROI AS COMMON MEDIATORS OF STRESS-INDUCED PCD

Although ROI are conventionally viewed as toxic by-products of cellular metabolism, a growing body of evidence suggests that they may regulate signal transduction in plant [49–51] and animal cells [recent reviews in Refs. 52–54]. As yet, there is no evidence in eukaryotes for proteins similar to the bacterial SoxR or OxyR capable of specifically sensing $O_2^{\cdot -}$ or H_2O_2 [55]. In contrast, there has been an enormous accumulation of experimental data to indicate that the functioning of many proteins is significantly dependent on their redox state. Such proteins include growth factor receptors, protein kinases [56] (some of which are MAP kinases [57]), protein phosphatases, G proteins [53], as well as a number of important transcription factors, including AP-1, NF-κB, and p53 [52], some of which are thought to be involved in the activation of PCD [27, 58].

During recent years, a large body of evidence has suggested that ROI can activate cell death programs (see Table 1) [59, 60]. Arguments for this come, in part, from studies showing oxidative stress in cells undergoing PCD. The most straightforward evidence is the induction of PCD by treatments with ROI themselves, such as low doses of H_2O_2 [61–63], $O_2^{\cdot -}$ [36], $^{\cdot}OH$ [62], or lipid peroxides [64]. Other indications are that oxidation-promoting agents, such as cytotoxic amyloid β protein [65], cyanide-induced hypoxia [30], and UV [38], are able to trigger cell death. In plant tissues, various conditions lead to ROI formation and subsequent PCD, such as senescence [66], cold [67, 68] and osmotic stress [69], ozone fumigation [70], UV radiation [71], SA [36], and inoculation with avirulent pathogens leading to hypersensitive cell death (HR) [28, 61]. Likewise, PCD can be triggered by reducing the ability of the cell to scavenge or otherwise detoxify ROI (Table 1). For example, treatment of fibroblasts with buthionine sulfoxamine causes efficient depletion of intracellular glutathione, which is followed by substantial cell death [72]. Similar results have been obtained with neuronal cells [12, 73].

INHIBITION OF APOPTOSIS BY ANTIOXIDANTS AND ANTIOXIDANT ENZYMES

Several studies assessed the inhibitory capacity of antioxidants on stress-induced apoptosis (Table 1). Antioxidants, such as the oxygen radical scavenger BHA, and metal chelators, which enhance the endogenous antioxidant defense systems, can inhibit or delay PCD to varying extents in UV-irradiated leukemic cells [33]. Similarly, BHA and other antioxidants protect cells against TNF-induced cytotoxicity [91] and p53-dependent apoptosis [83]. Moreover, cellular sensitivity or resistance to TNF- and growth factor deprivation-induced apoptosis is correlated with decreased or increased levels of antioxidant enzymes, respectively [76, 92].

Exogenous addition of catalase attenuates, but that of SOD exacerbates, peroxynitrite-induced DNA fragmentation in human leukemia HL-60 cells, suggesting that the H_2O_2 production predominantly contributes to this apoptotic process [80]. In a plant study using cultured soybean cells, catalase was demonstrated to reduce significantly H_2O_2-mediated hypersensitive cell death that was triggered by avirulent phytopathogenic bacteria [61]. In contrast, using a different system (the *Arabidopsis lsd1* mutant, which shows spontaneous cell death resembling HR symptoms), we revealed that this type of cell death can be inhibited by injection of SOD, whereas catalase treatment was inefficient, suggesting an important role for $O_2^{\cdot -}$ [36].

Transgenic plant and animal systems have been widely used to study the function of antioxidant enzymes during PCD induction. Plants expressing antisense RNA for cytosolic ascorbate peroxidase or catalase show spontaneous cell death and/or increased susceptibility for oxidative stress [86–88, 93]. On the other hand, increased oxidative stress tolerance can be gained by overproduction of antioxidant enzymes such as Fe-SOD and Mn-SOD in chloroplasts and/or mitochondria [89, 90]. In a human kidney cell line, overexpression of Mn-SOD confers increased resistance to cytotoxic TNF [94]. Conversely, expression of antisense Mn-SOD RNA renders these cells sensitive to TNF. Likewise, neuronal apoptosis can be delayed by overexpression of the cytosolic Cu/Zn-SOD, while expression of antisense SOD message exacerbates growth factor-deprivation-induced PCD [84]. Hence, the decision as to whether a cell is committed to death or to life may depend on the extent of ROI formation and on the ability of its antioxidative machinery to destroy these mediators of apoptotic cell death.

TABLE 1. Models of PCD: Induction, ROI generation and inhibition

Cell type	Inducer of PCD	Inhibitor of PCD	ROI*	Ref.
Mammals				
Thymocytes	Glucocorticoids	BA, trypsin inhibitor	+	[74]
	TNF	BHA	++	[75]
T and B cells	H_2O_2, nitric oxide	NAC	+	[63]
	Growth factor depletion	CAT	+	[76]
	Glucocorticoids	Bcl-2, NAC, rotenone	++	[40]
	HIV (Tat), TNF, NF-κB	GSH	+	[64, 77]
	anti-Fas, ceramide	NAC	+	[78]
	anti-Fas, ROI	Bcl-2, CsA, BA	++	[79]
T cell hybridomas	TNF, ceramide	Bcl-2, NAC	++	[40]
Fibroblasts	Staurosporine, anti-Fas		−	[62]
	Menadione	GPX, GSH, BHA	+	[62]
	ROI	CAT, GPX	+	[62]
	SA	Kinase inhibitor	ND	[37]
	GSH depletion	DMSO, catechol	+	[72]
Cancer cells (HL-60)	UV, cytotoxic drugs, H_2O_2	CAT, BHA, PDTC	+	[33, 38]
	UV	SOD	+	[38]
	Peroxynitrite	CAT, NAC	+	[80]
Cancer cells (U937)	Ceramide	NAC, PDTC, rotenone	++	[34]
Hepatocytes	TNF, ceramide	Rotenone	++	[81, 82]
Smooth muscle cells	p53	CAT, NAC, PDTC	+	[83]
Neurons	Glutamate	Cystine, NAC	+	[73]
	GSH depletion	Bcl-2, ascorbate	+	[12]
	Growth factor depletion	SOD	+	[84]
	Down's syndrome	CAT, BHA, NAC	+	[85]
	Amyloid β protein	CAT, DPI, neopterin	+	[65]
	Staurosporine	SOD, NAC	+	[35]
	Cyanide	CAT, ascorbate	+	[30]
Hamster tsBN7 cells	Temperature shift (*dad1*$^-$)	Human or plant *dad1*	ND	[45]
Plants				
Suspension-cultured cells	Host-selective mycotoxin		ND	[29]
	Arachidonic acid		ND	[29]
	Cyanide		ND	[29]
	Heat shock		ND	[29]
	Growth factor depletion		ND	[25]
	Cold stress, ABA		+	[68]
Progenitors of aerenchym	Hypoxia, ethylene, Ca^{2+}	Kinase inhibitor	ND	[31]
Leaf cells	Darkness, ethylene		+	[66]
	Pathogen infection	CAT, DPI	+	[28, 61]
	SA, pathogen, light	LSD1, DPI, SOD	+	[36]
	Spontaneous (*acd2*$^-$)	ACD2	ND	[21]
	Ozone	Ascorbate peroxidase	+	[70, 86]
	CAT deficiency		+	[87, 88]
	Paraquat	Fe-SOD, Fe-SOD	+	[89, 90]

Abbreviations not defined previously: ABA, abscisic acid; BA, bongkrekic acid; CAT, catalase; CsA, cyclosporin A; DPI, diphenylene iodonium; GPX, glutathione peroxidase; NAC, *N*-acetyl-L-cysteine; and PDTC, pyrrolidine dithiocarbamate.

* Key: (+) participation of ROI indicated; (++) dual role of ROI during PCD indicated by involvement of mitochondrial ROI generation as a consequence of PT; (−) ROI generation not involved in PCD; and ND, not determined.

MECHANISMS OF ROI-MEDIATED APOPTOSIS: DOES THE BCL-2 FAMILY COUNTERACT OXIDATIVE STRESS?

The prototypic regulator of mammalian cell death is the proto-oncogene *bcl-2* [reviewed in Ref. 39]. It was first identified as a gene overexpressed in human B cell tumors. Bcl-2 inhibits apoptotic cell death when overexpressed in various cell types irrespective of the inducing insult, with few exceptions [95, 96]. This general effectiveness of Bcl-2 confirms the theory that all forms of PCD may have common molecular mechanisms, as already proposed astutely by Kerr and colleagues in 1972 [9], inferred by common ultrastructural changes.

Several Bcl-2 family members have been identified in recent years. Some, such as Bcl-2, Bcl-X_L, and BAG-1, inhibit PCD, while others, such as Bax and Bak, promote PCD. Many of these proteins can interact with each other through a complex network of homo- and heterodimers [97], with one monomer antagonizing or enhancing the function of another. In this way, the ratio of pro- and antiapoptotic Bcl-2 family proteins in a cell may determine the likelihood of the cell undergoing PCD [98]. Phosphor-

ylation of these proteins may act as additional regulatory steps [reviewed in Ref. 99]. Very recently, it has been shown that Bcl-2 itself serves as a caspase substrate and is thereby converted to a Bax-like proapoptotic factor [100]. Thus, cleavage of Bcl-2 may ensure the inevitability of the cell death program.

Evolving from the subcellular localization of Bcl-2 in the nuclear, endoplasmic reticulum, and outer mitochondrial membranes [101], a model of Bcl-2 function opposing oxidative stress has been suggested, as these intracellular sites are commonly accepted as major sources of ROI production [9, 39]. In support of this hypothesis, various triggers of PCD have been found to stimulate intracellular ROI production [39, 102]. In particular, TNF-induced apoptosis is associated with mitochondrial ROI production [103]. Moreover, Bcl-2 seems to inhibit cell death via protection from oxidative stress [12, 39, 104]. Treatment of cells with pro-oxidants leads to apoptosis that can be blocked by *bcl-2* overexpression [12, 39, 104]. In addition, necrotic cell death triggered by glutathione depletion can also be inhibited by *bcl-2* overexpression [12], verifying that apoptosis and necrosis share common mechanisms. In a neural cell system, this inhibition is accompanied by a reduction of the net cellular generation of ROI and lipid peroxides [12]. In contrast, another study has shown that stress-induced death of neural PC12 cells is inhibited by Bcl-2 without reduction of ROI formation [105].

Thus, it is still a matter of debate whether production of ROI is required for the execution of the cell death program, as it has been shown that PCD also occurs under near-anaerobic conditions [62]. Other evidence suggesting that ROI may not be essential for PCD is the lack of protection against PCD by several antioxidants in FAS/APO-1- and staurosporine-induced PCD [62]. However, conflicting data have been obtained recently, indicating that PCD induced by these agents does involve and also requires transient synthesis of ROI, namely $O_2^{\cdot -}$ [26, 35].

MITOCHONDRIAL PERMEABILITY TRANSITION AS THE CENTRAL COORDINATOR OF DIVERSE ROI-DEPENDENT SIGNALING PATHWAYS

There is striking new evidence for the importance of signals from mitochondria during the effector phase of apoptosis [reviewed in Refs. 47 and 106]. Mitochondrial structure and function are altered significantly early during the apoptotic process [reviewed in Refs. 23, 41, and 107]. For example, several studies indicate that apoptosis-inducing agents can trigger the uncoupling of electron transport from ATP production, leading to a decrease of $\Delta\Psi_m$ and subsequent ROI production [40]. These biochemical parameters can be attributed to a well known phenomenon, mitochondrial PT, which is believed to facilitate the diffusion of low molecular mass compounds (<1500 Da) between the intermembrane space and the cytosol [81]. PT consists in opening of mitochondrial pores or megachannels that can be formed at the inner-outer membrane contact sites. These pore complexes consist of the inner membrane adenine nucleotide transporter and different outer membrane proteins, such as the peripheral benzodiazepine receptor and porin, a voltage-dependent anion channel. In addition, several other membrane and soluble proteins support the formation of the dynamic multiprotein pore complex [41].

Functional and genetic experiments indicate that loss of $\Delta\Psi_m$ by PT and subsequent nuclear apoptosis cannot be dissociated [106]. Consequently, cyclosporin A, which is a reversible inhibitor of PT, prevents $\Delta\Psi_m$ disruption as well as nuclear DNA fragmentation [108]. In addition, a further specific inhibitor of PT, bongkrekate, a ligand of the adenine nucleotide translocator, can inhibit the pre-apoptotic $\Delta\Psi_m$ disruption and all post-mitochondrial apoptotic changes in several different cell systems [74, 81, 108]. Similar results have been obtained with other inhibitors of PT such as the thiol reagent monobromobimane [109]. Overall, these results suggest that mitochondrial PT is the central coordinator of PCD, as outlined in Fig. 1.

The opening state of the PT pore complex is regulated by several physiological conditions, some of which are well-known signaling events of the early apoptotic process. Alkalinization of the mitochondrial matrix, sustained increases of cytosolic Ca^{2+} or other divalent cations, oxidation of the pyridine nucleotide pool, and primary activation of caspases favor the open state of the PT pore. Most interestingly, ROI, e.g. H_2O_2, are able to induce PT, too [74, 110]. Likewise, complete oxidation of glutathione triggered by cross-linking of mitochondrial dithiols with arsenite or phenylarsine oxide increases the opening probability of the PT pore [111]. This redox sensor capacity of the central coordinator of PCD reinforces the hypothesis that ROI and, thereby, alterations of the cellular redox state are important signaling components of the induction phase of apoptosis (see Fig. 1).

Several pieces of evidence suggest that Bcl-2 inhibits apoptosis (and necrosis) by direct regulation of the mitochondrial PT pore [reviewed in Refs. 47 and 107]. First, to exert its antiapoptotic function, Bcl-2 must be anchored to the outer mitochondrial membrane [39, 112]. Second, overexpression of *bcl-2* in the outer mitochondrial membrane directly abolishes PT induced by various agents such as the pro-oxidant *t*-butylhydroperoxide [81]. Third, Bcl-2 colocalizes with the PT pore complex to the contact sites between the outer and inner mitochondrial membrane [113]. Fourth, Bcl-2 family members form ion-conducting channels [114], which is compatible with their α-helical structure, reminiscent of bacterial ion-pore forming toxins. Taken together, these findings suggest that Bcl-2 and its homologues directly interact with the PT pore complex and thereby modulate their opening probability, an hypothesis that, although not yet proven, would be in accord with the antioxidative activity of Bcl-2. However, the precise biochemical mechanisms by which these proteins exert their influence on PT and thereby on cell destiny remain to be elucidated.

THE DEATH FINALE: ROI AND OTHER APOPTOGENIC CONSEQUENCES OF MITOCHONDRIAL PT

PT not only results in $\Delta\Psi_m$ disruption but also causes depletion of reduced glutathione and NAD(P)H [82], matrix Ca^{2+} outflow, and excessive generation of ROI [34, 40, 82]. Obviously, several of these consequences of PT themselves favor PT pore opening, thereby leading to a self-amplifying process. This positive feedback loop may act as an all-or-nothing switch, which is in good accord with the hypothesis that PT constitutes the central coordinator and executioner of apoptotic cell death. In fact, cells that undergo massive PT pass the point of no return and are irreversibly condemned to death.

Recent data suggest that the PT-dependent excessive generation of mitochondrial ROI is responsible for at least some of the post-mitochondrial apoptotic changes [75], thereby reinforcing the hypothesis of previous studies that mitochondria-derived ROI mediate TNF-induced cytotoxicity and gene expression [91]. Taken together, this points to a dual role of ROI in the apoptotic signaling cascade. Besides their function as inducers of PT as part of diverse signal-specific pathways during the induction phase of PCD, they also participate during the effector phase in signaling events downstream of PT, leading to the structural changes of the degradation phase of PCD.

However, ROI generation may not be the only and also not the most important consequence of PT. Mitochondria undergoing PT release at least two apoptogenic proteins, one of which is cytochrome *c* [115, 116]. Once released into the cytosol, cytochrome *c* induces the activation of a protease(s) resembling caspase-3, thereby leading to downstream apoptotic events [117]. Cytochrome *c* by itself is unable to process the precursor form of caspase-3; additional cytosolic factors are required. However, recent results indicate that mitochondrial cytochrome *c* release occurs upstream of caspase activation and independently of mitochondrial PT [118].

The other apoptogenic factor is a novel ~50 kDa protein called AIF. AIF is sufficient to cause apoptotic chromatin condensation and DNA fragmentation even in the absence of additional factors [79]. It is likely to be a protease, as it is directly inhibited by *N*-benzoyloxycarbonyl-Val-Ala-Asp.fluoromethylketone (z-VAD.fmk), an inhibitor of ICE-like proteases. In addition, AIF itself is an inducer of PT, thereby also participating in the positive feedback loop described above [119]. This apoptogenic protease may also determine the mode of death: cells treated with PT inducers alone undergo apoptosis, whereas cells kept in identical conditions in the presence of z-VAD.fmk die from necrosis [120]. These observations are in accord with the hypothesis that PT as the central coordinator of PCD is the rate-limiting step. But it might be the activity of AIF and other apoptogenic proteases that determines the choice between the two death modalities.

Chromatin degradation is thought to be one of the prominent processes during the degradation phase of apoptosis. Very recently, one of the executioners of this final step of apoptotic cell death has been identified: DFF [121]. This endonuclease was found to be activated by caspase-3, thereby earning its synonym, caspase-activated DNase (CAD) [122]. Caspase-3 exerts its activity by digestion of an inhibitory partner protein of CAD, called ICAD (inhibitor of CAD). In summary, these data draw a direct signal transduction chain for the final steps of apoptosis: caspase-3 to CAD/DFF to DNA fragmentation.

DISEASES ASSOCIATED WITH ROI-MEDIATED PCD

A deregulation or aberrant induction of apoptosis can lead to cancer, autoimmune diseases, or the destruction of the immune system after HIV infection. Furthermore, PCD in specific brain neuron cells can give rise to neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease, familial amyotrophic lateral sclerosis (FALS), and Parkinson's disease.

While evidence is largely correlative thus far, oxygen free radicals have been implicated in various animal disorders (atherosclerosis, diabetes, Alzheimer's disease, arthritis, muscular dystrophy, cancer, Down's syndrome, multiple sclerosis, and other inflammatory diseases) and plant disorders (fungal and bacterial infection, drought, and osmotic and UV stress), most of which involve PCD [6, 123]. Furthermore, studies using a variety of *in vitro* experimental models indicate that cell death in these diseases is mediated, in part, by an increase in oxidative damage.

In the following section, four examples are presented that illustrate the importance of ROI in the pathology of human diseases:

First, Down's syndrome has been correlated with overexpression of the human Cu/Zn-SOD gene (*sod1*), which resides on chromosome 21. Overexpression of the *sod1* gene, due to gene dosage, may disturb the steady-state equilibrium of ROI within the cells. In accord with this theory, Down's syndrome neurons generate increased levels of intracellular ROI, leading to neuronal apoptosis [85]. Conclusive evidence has been obtained by modeling Down's syndrome in transgenic mice, which overexpress *sod1*. These mice have abnormal thymi resembling those of Down's syndrome patients, and their thymocytes show enhanced apoptotic cell death [124]. This higher susceptibility to apoptosis is associated with an increased ROI production and a higher degree of lipid peroxidation, confirming the role of oxidative stress for the onset of PCD during the pathology of Down's syndrome. However, not all clinical studies could confirm the pathological role of ROI or SOD1 [125].

Second, several other disorders have also been tracked down to mutations in SOD genes, such as FALS, a neurodegenerative disease. FALS patients and their relatives share point mutations in a single gene, namely *sod1* [126]. The crystal structure of human SOD1 established

that all 12 observed FALS mutant sites alter conserved amino acid residues critical to SOD1 dimerization [127]. Red cells from heterozygotes have less than 50% normal SOD activity, consistent with a structurally defective SOD1 dimer. Thus, defective SOD1 is linked to motor neuron death and may carry implications for understanding and possible treatment of FALS.

Third, ROI have been implicated in Alzheimer's disease. Neural cell death triggered by amyloid β protein results from increased levels of ROI and lipid peroxides [65]. Catalase and other antioxidants protect cells from amyloid β toxicity. In addition, amyloid β protein induces the activity of NF-κB, a transcription factor thought to be activated by oxidative stress.

Fourth, progression of HIV infection to AIDS is characterized by increased susceptibility of peripheral lymphocytes to apoptotic T-cell death. The HIV protein Tat stimulates TNF-induced activation of the redox-dependent transcription factor NF-κB and TNF-mediated cytotoxicity [77]. Recently, it has been reported that HIV-infected T-cells are extremely susceptible to oxidative stress-induced apoptosis, due to an HIV-associated catalase and glutathione peroxidase deficiency [64]. Moreover, even virus-free T lymphocytes are condemned to apoptosis, giving rise to the immune deficiency in AIDS patients [128]. These findings suggest that efficient drug therapy in HIV infection should include agents targeted at preventing apoptosis of uninfected T cells.

ROI AS MEDIATORS OF PCD IN PLANT DEFENSE RESPONSES

Analogous to apoptosis in animals, plant cell death may be triggered in response to pathogens [reviewed in Refs. 6, 129, and 130]. Among the disease-related circumstances in which PCD has been suggested to play an important role is the HR that is characteristic of several incompatible, i.e. resistant, plant–microbe interactions. Several lines of evidence suggest that the HR results from the activation of an intrinsic PCD pathway. First, the HR requires active plant metabolism, including transcription and translation machinery [131, 132]. Second, certain fungal elicitors can induce many aspects of the multicomponent defense response during disease resistance [133] and lesions that match the HR in the absence of pathogens [134]. Third, expression of various foreign genes sometimes activates HR-like PCD [135, 136]. Fourth, a large class of plant mutants, so-called lesion mimics, display spontaneous or conditional cell death, which, in some cases, resembles the HR [summarized in Ref. 135]. Thus, HR cell death is not caused directly by the destructive potential of the pathogen but rather results from the activation of an intrinsic plant genetic program [137].

Several recent reviews provide an excellent summary of induction and signal transduction during the HR [138, 139]. Therefore, this review will mainly summarize the role of ROI and recent findings unraveling new components of the HR cell death program, some of which appear to be similar to those in mammalian cells. The production of ROI plays a key role in plant defense and HR in a way that is probably functionally and mechanistically analogous to ROI generation by mammalian macrophages and neutrophils in response to infection by pathogens (Fig. 2). In plants, ROI are generated rapidly and transiently after challenge at the site of infection, commonly referred to as the oxidative burst [50]. ROI can act as antimicrobial agents [140], as substrates for cell wall strengthening (a common mechanism to stop the microbial invader before it enters the plant cell) [141, 142], as well as second messengers of the plant multicomponent defense response [51] and the HR-associated cell death [36, 61].

Although there is still some debate concerning the primary source of the oxidative burst [142], recent findings indicate that plants contain a mechanism for $O_2^{\cdot -}$ production that is highly homologous to the macrophage NADPH oxidase complex. ROI generation and defense responses can be blocked by diphenylene iodonium, an inhibitor of mammalian NADPH oxidase at micromolar concentrations [51, 61]. Antibodies against some components of the human neutrophil NADPH oxidase complex, e.g. $p47^{phox}$, $p67^{phox}$, and the small G protein Rac2, cross-react with plant proteins of a similar size [143, 144]. Furthermore, the plant homologue of the main catalytic subunit, $gp91^{phox}$, has been identified from the rice EST database [145].

Recent evidence suggests that the first event in triggering the HR is determined by the direct interaction of a pathogen-derived avirulence (*avr*) gene product (ligand) with the corresponding plant resistance (*R*) gene product (receptor) either at the plant plasma membrane or in the cytosol (Fig. 2) [138]. The oxidative burst together with rapid ion fluxes are the earliest changes observed following pathogen recognition [51, 61, 133, 134]. In a study using elicitor-stimulated cultured parsley cells, we demonstrated that ion fluxes (Ca^{2+} and H^+ influx, K^+ and Cl^- efflux) across the plant plasma membrane causally precede $O_2^{\cdot -}$ production [51]. Moreover, ion fluxes and ROI production are equally necessary and sufficient for activation of the plant defense machinery, as indicated by detailed loss- and gain-of-function experiments [51]. In contrast, using a soybean cell culture system, the reverse causal relationship was established, i.e. that ROI production induces the rapid influx of Ca^{2+}, then leading to cell death.

There is increasing evidence that ROI are essential mediators of the cell death program during the HR [61]. One of the questions still unsolved is which ROI are the most important during the HR cell death program? While some studies suggest that H_2O_2 is sufficient to cause HR-like cell death [61], compelling evidence indicates that $O_2^{\cdot -}$ is the key ROI triggering cell death in the *Arabidopsis* lesion mimic mutant *lsd1* [36]. Furthermore, infiltration of SOD into tobacco leaves infected with tobacco mosaic virus compromised the development of the HR, confirming a significant role of $O_2^{\cdot -}$ [146]. Similar results were obtained when *Arabidopsis* leaves were inoculated with avirulent

strains of phytopathogenic bacteria in the presence of a 0.5 μM concentration of the NADPH oxidase inhibitor diphenylene iodonium.*

Only a few studies of the molecular events following these initial steps of the HR cell death program have been published until now [reviewed in Ref. 139]. Very recently, a MAP kinase was identified that acts downstream of ion fluxes but independently or upstream of the oxidative burst (Fig. 2) [147]. Upon receptor-mediated activation, this MAP kinase is translocated to the nucleus, where it might interact with transcription factors that induce defense gene activation. However, a direct participation of this or other components of MAP kinase cascades in triggering the HR remains to be demonstrated.

Much emphasis has been placed on the search for mutants in loci essential for normal HR development [148–151]. In addition, genetic approaches have been useful to identify "private" (stimulus-specific) signal transduction pathways during the induction phase of the HR. In barley, the *rar1* mutation abolishes *Mla*-based hypersensitive cell death and resistance against powdery mildew, while it does not suppress the HR of the *Mlg*-mediated resistance [152]. Likewise, the *Arabidopsis* mutant *eds-1* suppresses resistance to various isolates of the fungal pathogen *Peronospora parasitica* but not to an avirulent race of the bacterial pathogen *Pseudomonas syringae* [151]. In contrast, the *ndr1* mutant suppresses resistance to bacteria and *P. parasitica* [153]. In sum, these mutations provide compelling evidence for the convergence of signals downstream of distinct recognition scenarios into a single pathway, triggering the HR very much like the mammalian cell death program (compare Figs. 1 and 2) [6].

The nature of the executioner(s) of the HR cell death program remains unclear. Several compounds of the multicomponent plant defense response have been suggested as potentially toxic for the plant cell (e.g. ROI, lipid peroxides, SA, and enzyme activities, such as lipoxygenases) and therefore may directly participate in the HR cell death. However, little direct evidence has been presented until now [154].

As already mentioned, the HR is under genetic control, some of which may be contributed by antioxidative enzymes, such as SOD, catalase, peroxidase, glutathione-*S*-transferase, and glutathione peroxidase. Expression of these genes correlates with the induction of the HR [61] and may protect neighboring cells from uncontrolled diffusion of death signals, such as ROI, throughout the entire leaf. Likewise, transgenic plants with lowered antioxidative capacity often develop necrotic lesions and induce defense gene expression in the absence of pathogens.

Nevertheless, the following problems remain to be solved concerning the genetic control of the HR: Do other anti-cell death pathways exist in plants apart from the antioxidant enzymes mentioned, which, for example, might inhibit the induction of PCD in the leaf tissue surrounding the area of an HR lesion? What determines the size of the leaf area undergoing PCD after pathogen recognition, as there is no cell differentiation-dependent determination? And even more intriguing, if it exists, what constitutes the central executioner making this decision to die or not to die?

As already mentioned, Bcl-2-like functions have not been described yet in plants. The function of plant homologues of the anti-apoptotic protein DAD1 during the HR remains to be demonstrated. Nevertheless, anti-cell death pathways in plants do exist, as indicated by the existence of cell death control mutants, such as *acd2* [21], *lsd1* [22], and *lls1* [155]. These mutants exhibit impaired control of cell death in the absence of a pathogen and cannot control the spread of cell death once it is initiated. Very recently, the *Lls1* gene was found to encode an aromatic ring-hydroxylating dioxygenase [155], suggesting that its target, most likely a mediator of cell death, might be a phenol. One candidate that may fit well in this role is SA, which exhibits a 10- to 50-fold increase during the HR and some types of oxidative stress-induced PCD [139]. In addition, SA is known to cause an increase of intracellular H_2O_2 [49], to potentially form a cell-damaging free-radical [156], and to promote cell death during the HR [154] and in *Arabidopsis* cell death mutants, such as *lsd1*. In sum, this suggests that LLS1 acts as a cell death suppressor by scavenging SA or a related death-promoting phenolic compound.

Another negative regulator of plant PCD is the LSD1 protein from *Arabidopsis* [157]. *lsd1* mutant plants show a lowered threshold for triggering HR and enhanced resistance to pathogens. However, they cannot control the lesion size, a phenomenon termed runaway cell death. The *LSD1* gene encodes a novel zinc finger protein that may regulate transcription of cell death-effectors [157]. Interestingly, $O_2^{\cdot -}$ is necessary and sufficient to initiate lesion formation in *lsd1* mutants. It accumulates before the onset of cell death and, subsequently, in live cells adjacent to the spreading *lsd1* lesions. Thus, $O_2^{\cdot -}$ is the critical ROI species whose perception and clearance are perturbed by the *lsd1* mutation. Interestingly, recent experiments indicate that the activity of several antioxidant enzymes, such as Cu/Zn-SOD and catalase, is altered in *lsd1* mutant plants.† In sum, these data suggest that the putative LSD1 transcription factor monitors a self-amplifying signal normally leading to cell death in plants (Fig. 2). Furthermore, LSD1 may act as a rheostat, sensing signals that activate the HR cell death program. LSD1 could trigger the initiation or spread of cell death at high signal levels and slow down or stop the cell death program as signal levels fall. This rheostat sensor might respond to alterations of the cellular redox state or increases of SA or ROI levels, as found in the close vicinity of pathogen infection sites. Alternatively, LSD1 could inhibit constitutive low level signals feeding into the

* Kiefer I, Daims H, Slusarenko AJ and Jabs T, unpublished results.

† Jabs T, Hermanns H, Kiefer I, Koch M and Slusarenko AJ, unpublished results.

ROI-generating system of the oxidative burst. This demonstration that $O_2^{\cdot -}$ is a critical signal in a feedback amplifying process monitored by a putative transcription factor to regulate the spread of cell death reinforces the hypothesis that similar strategies are used to control PCD in plants and in animals.

In addition to local resistance, often manifested by the HR, plants have developed a secondary, systemic, broad-range resistance referred to as Systemic Acquired Resistance (SAR). SAR is biologically triggered by pathogens that cause local cell death or can be induced by chemicals such as SA or synthetic SA analogs, such as 2,6-dichloroisonicotinic acid or benzothiadiazole. However, the causal relationship between cell death-stimuli and the establishment of SAR is not yet clear. Nevertheless, localized inoculation of avirulent pathogens has been shown recently to trigger low-frequency cell-death in uninoculated (systemic) leaves in addition to the HR symptoms in the infected leaf [158].

Many questions remain open about the induction, regulation, and execution of the HR. Are ROI really obligatory signals of the HR? Do they exert a dual function as in some mammalian systems? Do other essential signals exist, e.g. lipid peroxides resulting from either lipoxygenase activity [159] or membrane damage by the interaction with ROI or SA? Do mitochondria act as central coordinators, as in animal cells? Or could chloroplasts, also organelles of bacterial origin, take charge of this function in plant cells, as indicated by the common observation that HR development is compromised in the dark? In addition, disruption of chloroplast membranes is only observed during the final stages of the degradation phase [160]. Recent reviews raise the question about a putative social role of cell death [6], indicated, in part, by the findings that cell death may be required for triggering defense response in neighboring cells in uninoculated leaves [158]. Answering these questions may help to understand the regulation and function of PCD during plant disease resistance.

CONCLUSION

ROI have long been known to be potent mediators of PCD in various animal systems and for about 4 years now in plant systems, too. It is clear that antioxidants and antioxidative enzymes can protect cells under various death-promoting conditions. Whether ROI production is the primary trigger for apoptosis or a contributing factor is not yet clear. Another question still unsolved is which ROI are most important during PCD?

Based on recent findings that various distinct stimuli-specific PCD pathways converge to a common signaling pathway at the level of mitochondrial PT, it is hypothesized that PT is the central coordinating event of the apoptotic effector phase (Figs. 1 and 2). This central role of PT predicts a dual role of ROI in the apoptotic process. First, ROI participate in diverse signal-specific pathways during the induction phase of PCD and serve as inducers of PT. Second, ROI are one of several apoptogenic consequences of PT translating to the structural changes of the degradation phase of PCD. Recent findings indicate that the prototypic regulators of mammalian cell death, Bcl-2 and its homologues, exert their anti- or proapoptotic function by direct regulation of the PT pore opening probability or by modulation of the ion permeability of the mitochondrial membranes.

Numerous studies have documented increases in the markers of oxidative damage in a variety of diseases. However, data showing a causal and functional relationship between ROI, PCD, and pathogenesis are still sketchy. An unsolved problem is: If ROI and mitochondrial PT are implicated in all the diseases mentioned above, then how does one explain the specificity of cell death in each case? In this regard, the ability to effectively model human diseases in transgenic mice may provide a powerful tool with which to examine the *in vivo* relationship between ROI, PCD, and pathogenesis.

Recently, several striking homologies have become apparent between animal and plant PCD, although only few of the death regulators identified in animal systems, such as DAD1, have been hitherto identified in plants. Plants and animals share many typical attributes of the PCD pathway, such as ROI-dependent signaling mechanisms, convergence of signals from diverse death-stimuli, the regulation by antiapoptotic pathways limiting the amount of cell death, and the biochemical and morphological changes during the degradation phase of PCD. In fact, even the enormous diversity of the PCD-inducing stimuli is shared, in part, by animals and plants. For example, oxidative stress, several ROI species, SA, cyanide, staurosporine, heat shock, hypoxia, ceramides and pathogens are found to activate PCD in both higher eukaryote kingdoms. Moreover, recent studies examining death-specifying or -regulating transcripts in plants and animals independently identified similar genes, some of them regulating the availability of intracellular ROI [36, 61, 155, 161]. Future studies using genetic approaches and inducible animal or plant models will contribute to our understanding of the molecular basis of PCD. However, the only way to thoroughly scan the biological landscape for the relevant modifiers will be a large throughput mutant screen, and that is something feasible, at present, only in the model plant *Arabidopsis*.

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