OPINION PAPER

Classes of programmed cell death in plants, compared to those in animals

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Abstract

Relatively little is known about programmed cell death (PCD) in plants. It is nonetheless suggested here that tonoplast rupture and the subsequent rapid destruction of the cytoplasm can distinguish two large PCD classes. One class, which is here called 'autolytic', shows this feature, whilst the second class (called 'non-autolytic') can include tonoplast rupture but does not show the rapid cytoplasm clearance. Examples of the 'autolytic' PCD class mainly occur during normal plant development and after mild abiotic stress. The 'non-autolytic' PCD class is mainly found during PCD that is due to plant–pathogen interactions. Three categories of PCD are currently recognized in animals: apoptosis, autophagy, and necrosis. An attempt is made to reconcile the recognized plant PCD classes with these groups. Apoptosis is apparently absent in plants. Autophagic PCD in animals is defined as being accompanied by an increase in the number of autophagosomes, autolysosomes, and small lytic vacuoles produced by autolysosomes. When very strictly adhering to this definition, there is no (proof for) autophagic PCD in plants. Upon a slightly more lenient definition, however, the 'autolytic' class of plant PCD can be merged with the autophagic PCD type in animal cells. The 'non-autolytic' class of plant PCD, as defined here, can be merged with necrotic PCD in animals.

Introduction

In animals, three main types of programmed cell death (PCD) are currently distinguished: apoptosis, autophagy, and necrosis (Kroemer *et al.*, 2009). These PCD categories are based on cell morphology, not on biochemical features. In plants, even less is known about the biochemistry underlying PCD than in animals and it therefore seems that classes of plant PCD also have to be recognized mainly on the basis of cell morphology.

The purposes of this paper are (i) to argue what seems to be the best method for classification of plant PCD, (ii) to reassess which classes of PCD might be distinguished in plants, and (iii) to evaluate whether these classes of plant PCD can be merged with categories of PCD that are presently recognized in animals.

The present review is also meant as an update of van Doorn *et al.* (2011). Some of the PCD classes identified in

the paper will also be recognized here, but it will be argued that the intermediate PCD class is arbitrary and therefore ambiguous. In addition, the name of one of the main PCD classes ('vacuolar'), it will be argued here, is logically incorrect.

A criterion for dividing plant PCD classes

It can be contended that PCD classification requires at least the following: (i) it has to entail all known examples of *in situ* (i.e. *in planta*) PCD, (ii) it has to follow rather simple rules, (iii) there has to be as little doubt as possible on how to draw the lines between the classes, and (iv) it preferably results in groups that also have biological significance. The requirements of (ii) and (iii) imply that there should be no

arbitrariness, i.e. the classes should, if possible, not be based on weighing the relative importance of more than one feature. If weighing becomes involved it might introduce considerable bias and thus arbitrariness.

Any classification has to pinpoint a criterion (or a few criteria) that define a class. In animal PCD, for example, two simple criteria have been used that define three major PCD classes. These criteria are (i) the presence of apoptotic bodies or cell protrusions and engulfment of these by other cells and (ii) the presence of autophagosomes and derived vesicles. The first and second criteria define the apoptotic and autophagic class of animal PCD, respectively. The third group of PCD in animals is defined by not answering to either criterion (i) or (ii) (Table 1). In animal PCD, there is little overlap between the defining features of the first and the second class (Kroemer et al., 2009).

It will be asserted here that it is possible to distinguish, rather sharply, two major classes of plant PCD. The feature chosen to define these two major classes is the rupture of the tonoplast followed by rapid clearance of the whole cytoplasm and sometimes most of the cell walls. According to this criterion, the following classes of PCD in plants can be defined: one that shows this feature and another that does not (Table 1). The first class will be called 'autolytic' PCD here and the second class 'non-autolytic' PCD. An additional requirement for an example of PCD to be called 'autolytic' is that it is likely that tonoplast rupture followed by rapid cytoplasm clearance takes part in killing the cell. So if the rupture and clearance come only after the cell is already dead, for which some examples exist (to be discussed later on), an example is taken to be 'non-autolytic'.

An advantage of the chosen criterion is that it distinguishes between two large PCD classes that also represent a biological difference: 'autolytic' PCD occurs mainly during normal plant development and during mild abiotic stress, whilst 'non-autolytic' PCD takes place mainly during plant–pathogen interactions.

Numerous morphological and biochemical changes occur prior to tonoplast rupture and cytoplasm clearance. Morphological changes include chromatin condensation or condensation of the nucleus. However, these are quite variable among examples of PCD and therefore cannot be used to distinguish PCD classes that otherwise also make biological sense. Another early morphological change is the increase in autophagy-like structures in the cytoplasm: vacuole-like vesicles that are apparently involved in the initial cytoplasm degradation. This is found in many examples of PCD but is not observed in many other examples. Its presence or absence is quite well correlated with the presence or absence of the rapid clearance of the cytoplasm at the end of PCD. The presence of autophagic structures, therefore, might also have served as the criterion for distinguishing between the same two large groups of plant PCD that are now distinguished by cytoplasm clearing. Nevertheless, the presence of autophagic structures has not been chosen here to distinguish these two classes, as in most examples of PCD it is (i) not known if the autophagic structures actively participate in PCD, (ii) are irrelevant to PCD, or (iii) are a means to delay PCD. It has been shown in a few examples that autophagic-like structures, and the presence of autophagy genes, delay PCD rather than cause it (Doelling et al., 2002; Hanaoka et al., 2002; Yoshimoto et al., 2004). To choose a feature that can delay death as a criterion to distinguish large classes of PCD seems counter to logic. Although it is often not known precisely whether tonoplast rupture and clearance of the cytoplasm are a cause of death, it at least has not been shown to delay death, and it seems generally to participate in cell death.

It should be noted that breakdown of the nucleus and the tonoplast are not always enough to kill a cell (Sjölund, 1997; Wang et al., 2008). Mature sieve tubes undergo what has been called partial PCD, which is characterized (apart from the disappearance of the nucleus) by the disappearance of the Golgi bodies and many ribosomes. All this does not result in killing the cell (Wang et al., 2008). The sieve

Table 1. Main defining morphological criteria, and features other than the defining criteria, for classes of programmed cell death (PCD) in animals (Kroemer et al., 2009; Ravichandran, 2010), compared with suggested defining morphological criteria, and features other than the defining criteria, for classes of PCD in plants

	Defining criteria	Other criteria
Animals		
Apoptosis	Apoptotic bodies, or blebs on cell surface	Chromatin condensation. Nuclear fragmentation
	Degradation in other cells, after phagocytosis	Find-me signal. Eat-me signal
Autophagy	Increased numbers of autophagosomes, Autolysosomes, and small lytic vacuoles	
Necrosis	None of the above defining criteria	Cell swelling. Organelle swelling. Plasma membrane rupture
Plants		
Autolytic	Rapid clearance of cytoplasm, after rupture of tonoplast	Chromatin condensation. Increase of vacuolar volume (decrease cytoplasma volume)
Non-autolytic	No rapid clearance of cytoplasm	Swelling of organelles. No increase in vacuolar volume

tube example also shows that the disappearance of the tonoplast is by itself not sufficient to kill a cell. Sieve tube vacuoles disappear because the tonoplast collapses and becomes degraded (Thorsch and Esau, 1981; Wang *et al.*, 2008). In sieve tube cells, there seems to be an insufficient release of hydrolases from the vacuole to destroy the cytoplasm fully and thus to kill the cell.

Two classes of plant PCD

The two main classes of plant PCD, as recognized here, will briefly be described, followed by a description of the three main PCD categories in animals. Thereafter an attempt will be made to describe the position of these plant PCD classes in relation to the PCD categories in animals.

Autolytic PCD: rapid clearance of the cytoplasm

The defining feature of this PCD is rapid cytoplasm clearance after tonoplast rupture (Table 1). It is still far from clear how tonoplast rupture comes about. It is clear, by contrast, that the disappearance of the cytoplasm is due to the release of hydrolases from the vacuole, which degrade the cytoplasm.

In most examples of this type of PCD, death is preceded by the appearance of small vacuoles in the cytoplasm, which merge, and the merging of intermediate size vacuoles into a big one. Single membrane-bound vesicles are often observed in the small and large vacuoles (van Doorn *et al.*, 2011). The cytoplasm thereby becomes replaced by vacuolar volume. Considerable numbers of cytoplasmic organelles, in particular, plastids, ribosomes, ER membranes, and peroxisomes, disappear during this process. These changes are very similar to autophagy in animal and yeast cells, although it is not yet clear how the plant process is regulated and what organelles are involved (van Doorn *et al.*, 2011).

The autophagy-like ultrastructure very likely relates to the remobilization of macromolecules that is often associated with 'autolytic' PCD. In senescent leaves and petals, for example, extensive degradation of DNA, RNA, lipids, complex carbohydrates, and protein has been observed. These become degraded to sucrose, amino acids, and amides, which are readily transported, through the phloem, out of the organ that is undergoing PCD and into other organs of the plant (van Doorn, 2004; Gregersen *et al.*, 2008). Similar processes seem to take place during PCD in germinating seeds (Young and Gallie, 2000).

Other processes that are associated with 'autolytic' PCD can include, in about the order of description, an increase in cytoplasmic calcium ion concentration (Hoeberichts and Woltering, 2003; Bosch *et al.*, 2008; Fagerstedt, 2010), induction of MAPK (mitogen activated protein kinase) signalling, acidification of the cytosol, as well as changes in the microtubule and actin cytoskeletons (Smertenko *et al.*, 2003; Bosch *et al.*, 2008), organelle swelling (Bosch and Franklin-Tong, 2008), degradation of the contents of the chloroplast (Inada *et al.*, 2000), DNA degradation (Yamada *et al.*, 2006a, b), chromatin aggregation and movement of

condensed chromatin to the periphery of the nucleus (Gunawardena *et al.*, 2001), condensation of the nucleus to a smaller diameter (Yamada *et al.*, 2003, 2006a, b) and breakup of the nucleus into smaller fragments (Yamada *et al.*, 2001, 2003, 2006a, b; Kladnik *et al.*, 2004). These changes generally occur before tonoplast rupture. In some examples of 'autolytic' PCD, however, the changes in the nucleus only become apparent after tonoplast rupture (Obara *et al.*, 2001). Another early event associated with at least one example (petal senescence) is closure of the plasmodesmata (van Doorn *et al.*, 2003). Such closure might prevent sugars from passing from cell to cell and thus might lead to a lack of ATP if sugars are also not loaded into the cell from the apoplast. Tulip petal PCD (senescence) has been shown to be related to early ATP depletion (Azad *et al.*, 2008).

'Autolytic' PCD often requires serine proteases and/or cysteine proteases (Pak and van Doorn, 2005), but the precise role of most of these proteases in PCD, other than degradation of bulk protein, is still largely unknown. KDEL-tailed cysteine proteinases reside in rough endoplasmic reticulum-derived organelles, called ricinosomes. It has been suggested that acidification of the cytoplasm after tonoplast rupture cause the ricinosomes to break open, releasing the proteases, which become activated and help degrade the cytoplasm (Senatore *et al.*, 2009).

'Autolytic' PCD in barley seed aleurone cells was associated with the induction of a gene encoding cathepsin B, a cysteine protease (Martinez *et al.*, 2003). A gene encoding a cathepsin B was also up-regulated during *Populus* xylem fibre PCD (Courtois-Moreau *et al.*, 2009). The knockout of three cathepsin B genes resulted in a delay in leaf PCD in *Arabidopsis*, along with a 7-fold reduction of the decrease in the transcript level of the senescence marker gene *SAG12* (McClellan *et al.*, 2009). Cathepsins have also been implicated in some types of animal PCD (Kroemer and Jäättelä, 2005). Animal cathepsins are localized in the lysosome (an organelle equivalent to plant vacuoles) and include, according to their active site amino acid, at least two serine proteases, two aspartate proteases, and 11 cysteine proteases (Groth-Pedersen and Jäättelä, 2010). Permeabilization of lysosomes can release cathepsins B and D into the cytoplasm. These cause proteolytic activation of the pro-apoptotic protein Bid, which then induces mitochondrial outer membrane permeabilization and PCD through caspase activation. In other examples, such as lung cancer cells, the release of cathepsins from the lysosome induced a caspase-independent PCD (Guicciardi *et al.*, 2004; Kroemer and Jäättelä, 2005; Boya and Kroemer, 2008; Turk and Turk, 2009).

'Autolytic' PCD is also often accompanied by an increase in caspase-like activities. Caspases are cysteine proteases that are required in many examples of animal PCD. They activate other caspases and activate degradative enzymes or proteins involved in producing the cell morphology that is typical of PCD.

In PCD associated with seed coat formation, a requirement of VPEδ (vacuolar processing enzyme δ, a cysteine protease with caspase-1 activity) was found (Nakaune *et al.*, 2005).

A 100-fold increase in VPEc expression was observed during petal senescence (Müller *et al.*, 2010).The processing of animal cathepsins B, H, and L was blocked after knockout of an animal VPE-analogue (Shirahama-Noda *et al.*, 2003), showing a potential relationship between the VPE requirement and the cathepsin requirement for plant PCD.

'Autolytic' PCD in xylem fibres was associated with increased expression of the autophagy genes *atg8c, 8d,* and *8f,* an orthologue of a mammalian BAG (Bcl-2-associated athanogene) gene, and a gene encoding a sphingosine-1-phosphate phosphatase (Courtois-Moreau *et al.*, 2009). The autophagy genes might relate to remobilization processes. BAG proteins are chaperone regulators that modulate diverse processes, including PCD. Plant BAG proteins are remarkably similar to their animal counterparts, and they also regulate PCD during development and during pathogen attack (Doukhanina *et al.*, 2006). Sphingosine might hypothetically be involved in tonoplast permeabilization. In animal cells, PCD stimuli can lead to increased levels of sphingosine, which accumulates in lysosomes and acquires detergent-like properties, leading to lysosomal membrane permeabilization (Johansson *et al.*, 2010).

In addition, PCD induced by water stress in roots tips was accompanied by increased active oxygen species (AOS), such as hydrogen peroxide (Duan *et al.*, 2010). Hydrogen peroxide was even required for the PCD in epidermal cells that disappear because of adventitious root formation (Steffens and Sauter, 2009). The PCD in aleurone cells in wheat seeds (Wu *et al.*, 2011) and the PCD associated with senescence in wheat leaves (Huang *et al.*, 2011) was modulated by changing the activity of haem oxygenase (HO). This enzyme confers protection against oxidant-induced cell injury, both in animals and plants. It regulates the conversion of haem into biliverdin. Biliverdin is subsequently reduced to form the potent antioxidant bilirubin. PCD in aleurone cells was associated with an increase in hydrogen peroxide, whilst the activity of catalase and ascorbate peroxidase decreased significantly. Gibberellic acid (GA) induces aleurone PCD. Treatment with the HO specific inhibitor, zinc protoporphyrin IX, before exposure to GA, decreased HO activity and accelerated GA-induced PCD. By contrast, the HO inducer, haematin, induced HO expression, and inhibited GA-induced PCD (Wu *et al.*, 2011). Very similar effects were found in the reversal of dark-induced senescence of isolated leaves, by treatment with a cytokinin. HO activity in this system was correlated with the activities of catalase, peroxidase, superoxide dismutase, and ascorbate peroxidase (Huang *et al.*, 2011). These results suggest the importance of oxidative reactions, probably involving hydrogen peroxide and superoxide, in at least some examples of 'autolytic' PCD.

'Autolytic' PCD during normal plant development includes the senescence of various organs (such as cotyledons, leaves, petals, and roots), the formation of pollen and of the ovary, the formation of dead xylem conduits and bark, that of laticifers and other ducts, and the death in several parts of developing and germinating seeds (several other examples are given in van Doorn and Woltering, 2005). Some examples have been included, in general terms, in Table 2. Examples of PCD during mild abiotic stress are the induction of aerenchyma in waterlogged roots, and the precocious yellowing of leaves because of adverse conditions, such as drought (Table 2).

It should be noted that 'autolytic' PCD is not limited to plants. In animals, the lysosomes can contain more than 50 acid hydrolases, including phosphatases, nucleases, glycosidases, proteases, and lipases. These are capable of digesting most or all of the macromolecules in the cell. Rupture of the lysosomal membrane can result in a rapid PCD due to the action of these lysosomal hydrolases on the cytoplasm (Boya and Kroemer, 2008).

The type of PCD here discussed has been called 'autolytic' because of the quick clearing of the cell due to hydrolases released from the vacuole. Previously, it has been called autophagic (van Doorn and Woltering, 2005, 2010). The term was used to indicate what was called mega-autophagy, the lysis of the remaining cytoplasmic content. However, autophagy is now mainly defined by the activation of autophagy (*atg*) genes, which places 'mega-autophagy' outside of autophagy. Alternatively, if 'autophagic' PCD in plants is defined as being associated with the presence of autophagosomes, autolysosomes and vesicles produced by autolysosomes (the definition of autophagic PCD in animals), the term 'autophagy' for this type of PCD cannot be used, for reasons that will be discussed below. Nonetheless, if, by contrast, 'autophagic' PCD only means a PCD that is accompanied by autophagic structures, 'autophagic' PCD might be an acceptable term. Nonetheless, 'autolysis' is a better criterion to distinguish this type of PCD from the other type than 'autophagy', if 'autophagy' is defined as

Table 2. Examples of *in situ* plant PCD, as categorized to the two suggested classes of plant PCD ('autolytic' and 'non-autolytic' PCD). *In situ* refers to processes that occur in intact plants, not in cell cultures. For several more examples of developmental PCD see van Doorn and Woltering (2005).

PCD class	Examples
Autolytic	Developmental PCD, for example, PCD that occurs during the formation of the male and female zygotes, in seeds (except endosperm in cereals), in embryonic structures, and during development of roots and shoots. Mild abiotic stress, such as lack of oxygen (induces aerenchyma in roots), and drought (advances leaf yellowing and other senescence processes).
Non-autolytic	Hypersensitive response (HR)-related PCD. Necrotrophic PCD. Other examples of PCD where death is shown to occur prior to tonoplast rupture, where tonoplast rupture does not occur, or where tonoplast rupture is not followed by complete clearance of the cytoplasm. Endosperm in cereal seeds is an example of the second group (no tonoplast rupture).

being accompanied by autophagic structures, because in some examples of plant PCD autophagy delays death rather than induces PCD.

PCD that is not accompanied by rapid clearance of the cytoplasm (non-autolytic PCD)

The defining feature of this type of PCD is the absence of a rapid clearance of the cytoplasm (Table 1). There can, however, be increased permeability or even rupture of the tonoplast. Such changes apparently do not lead to the release of a massive amount of hydrolases that quickly clear the remaining cytoplasm.

'Non-autolytic' PCD is mainly found in three settings (Table 2). One is related to the hypersensitive response (HR), the second is the PCD that is due to necrotrophic plant pathogens, and the third is the PCD in the endosperm of cereal seeds. The PCD related to the HR can again be divided into two groups: one that shows the release of physiologically active proteins from the vacuoles and another that does not show this.

Vacuole-requiring plant PCD due to infection with plant pathogens, related to the HR

During the HR, the advancement of a pathogen is blocked by the plant through a series of defence reactions. The HR is usually associated with the death of a ring of cells around the intruding pathogen. This type of cell death can therefore be called HR-related PCD.

When a plant part becomes infected by a pathogen (viruses, bacteria, fungi, and oomycetes) the typical defence reaction involves two tiers. After penetrating a plant, the presence of conserved regions in pathogen molecules is detected through plant pattern recognition receptors. Examples of the detected pathogen molecules are bacterial flagellin and fungal chitin. The perception of these molecules induces a basal immune response by the plant, which inhibits the further growth of the pathogen. However, pathogens have developed so-called effector molecules (including polypeptides, proteins, and oligosaccharides) that suppress the basal plant immune response. These bacterial molecules block either signal transduction (from the pattern recognition receptors to the genome) or the output of plant defence-related genes. Here the second tier of plant defence comes in. To counteract the effect of the pathogen effector molecules, plants have evolved resistance (R) genes. The R genes encode receptors which recognize pathogen effector molecules. The R gene-encoded receptors initiate a signalling cascade that results in gene expression. Among the effects of the R genes are the induction of systemic resistance in the host, stomatal closure, and the death of a layer of plant cells surrounding the point of entry of the pathogen (Mur et al., 2008; Hayward et al., 2009).

The PCD that is related to the HR is often shown to be preceded by calcium ion influx into the cytoplasm, activation of a MAPK signal transduction cascade, and the production of salicylic acid, active oxygen species, and nitrogen oxide. It is accompanied by an oxidative burst (Hong et al., 2008; Hayward et al., 2009). The constitutive over-expression of the cystatin AtCYS1, a natural inhibitor of cysteine proteases, suppressed an HR-related PCD in tobacco plants and in cultured Arabidopsis cells. This suggested that this type of PCD may be regulated by cysteine proteinases (Belenghi et al., 2003).

Disruption of the tonoplast has even been proposed to be the critical event for cell death in at least some examples of 'non-autolytic' PCD (Greenberg and Yao, 2004; Hofius et al., 2009). Hatsugai et al. (2004) showed that vacuolar collapse was apparently required for a virus-induced HR-related PCD in tobacco plants. Using gene silencing, vacuolar processing enzyme (VPE), a protease, was found to be essential for the vacuolar collapse. VPEs function in activating other hydrolases and account for a large part (or all) caspase-1 activity in plants (Zhang et al., 2010). Other data from Hatsugai et al. (2004) indicated that cell death was preceded by the collapse of the tonoplast. However, the disruption of the tonoplast apparently did not kill the cell by a massive release of hydrolases, as in 'autolytic' plant PCD: numerous organelles and ample cytoplasm remained after the collapse had taken place (Hatsugai et al., 2004). This indicates that the cell was killed by other means than the massive release of hydrolases from the vacuole. One candidate for killing the cell is a vacuolar cathepsin.

VPEs were also found to contribute to PCD in other HR-related systems. In a mycotoxin-induced PCD, the knock-out of VPEγ resulted in less PCD (Rojo et al., 2004; Yamada et al., 2004). Single-silenced (NbVPE1a) or dual-silenced (NbVPE1a/b) N. benthamiana plants also failed to show HR-related PCD after treatment with the bacterial toxin harpin (Zhang et al., 2010). These data also point to the importance of VPE and thus of caspase-1 activity.

Exogenous cathepsins induced a plant PCD which was very similar to the one related to the HR (Hofius et al., 2009). Moreover, cathepsin B was required for the HR-related PCD induced in N. benthamiana by the fungus Phytophthora (Gilroy et al., 2007). McLellan et al. (2009) showed that cathepsin B genes were required for the HR-related PCD triggered by the protein AvrB in Arabidopsis, while it was not required for the HR-PCD triggered by AvrRps4.

Pathogen-induced PCD that does not require the vacuole, during the HR

Tonoplast rupture was apparently not involved in cell collapse during the HR of cowpea epidermal cells to the cowpea rust fungus (Heath et al., 1997). Another fungus, Cochliobolus victoriae, the causal agent of victoria blight in oats, can induce HR-related PCD via secretion of its host-selective toxin, victorin. Victorin-induced PCD was characterized by shrinkage of the cytoplasm. It required Ca^{2+} import, and involved both caspase-like proteases and collapse of the mitochondrial transmembrane potential. PCD occurred in the absence of plasmodesmatal closure.

This PCD also occurred without tonoplast rupture and even without permeabilization of the plasma membrane (Curtis and Wolpert, 2004; Williams and Dickman, 2008).

PCD during interaction with a necrotrophic pathogen

Necrotrophic interactions, whereby a pathogen consumes dead plant cells, are accompanied by the induction of PCD in the plant cell. An important example is the fungus *Botrytis cinerea* (van Kan, 2006; Tudzynski and Kokkelink, 2009). This fungus kills host cells by means of toxic molecules such as botrydial and oxalic acid. Infection with *B. cinerea* induces a significant oxidative burst, leading to the accumulation of active oxygen species. This is probably the reason for lipid peroxidation and the depletion of antioxidants, and might contribute to death (Gechev *et al.*, 2006). A mutation in plant VPEγ, but not a mutation in the other plant VPEs (α, β, δ), reduced the PCD induced by *B. cinerea* in *Arabidopsis* (Rojo *et al.*, 2004).

Sclerotinia sclerotiorum, another necrotrophic fungal pathogen, mainly secretes oxalic acid. Mutants defective in oxalic acid synthesis did not induce cell death, while exogenous application of physiological concentrations of oxalic acid induced cell death in this mutant. Treatment with exogenous oxalate produced an increase in H_2O_2 in the plant cells, and led to PCD. When this oxalate-induced H_2O_2 production was inhibited, PCD did not occur (Williams and Dickman, 2008). These data show that increased H_2O_2 production was required for PCD.

The necrotrophic fungus *Fusarium moliniforme* secretes fumonisin B1 (FB1), which is sufficient to induce PCD. Its effect was enhanced after knockout of the gene encoding the ER-located Bax-inhibitor 1, suggesting a role of ER stress (Watanabe and Lam, 2006). Knockout of all four VPE genes in *Arabidopsis* prevented the effect of FB1, and also prevented the disappearance of the tonoplast. The main VPE involved was VPEγ, but the other VPEs were required for the full effect. TEM data indicated that the disappearance of the tonoplast did not result in the destruction of the remaining organelles (Kuroyanagi *et al.*, 2005). This might suggest the absence of release of massive amounts of hydrolases from the vacuole.

Death of inner endosperm cells in cereal seeds; death of cells during somatic embryogenesis

There are currently two examples of PCD during plant development in which the cells are shown to be dead before tonoplast rupture occurs or die without apparent tonoplast rupture. One occurs in cereal seeds, the other during somatic embryogenesis.

Cells of the inner endosperm of cereals (Poaceae) die relatively early, i.e. during seed formation. The mechanism of cell death is not known, but no evidence has yet been given for permeabilization of the tonoplast. Features of this PCD include the increased expression of cysteine proteases, including caspase-like protease, induction of RNase and DNase, a decline in RNA and DNA content

as well as in the content of soluble proteins, and loss of plasma membrane integrity (Young and Gallie, 2000; Borén *et al.*, 2006; Nguyen *et al.*, 2007). The dead endosperm cells stay intact until seed germination. The material inside the dead endosperm cells then becomes degraded by hydrolases secreted by the surrounding aleurone tissue, which is also part of the endosperm. The hydrolysed material is then utilized by the growing embryo (Young and Gallie, 2000; Sreenivasulu *et al.*, 2004). This type of cell death is different from that in the seeds of many other species, whereby the endosperm cells die only by the time of germination. In these seeds, endosperm cell death is apparently due to hydrolases that are released from the vacuole of the dying cells themselves. (Greenwood *et al.*, 2005; DeBono and Greenwood, 2006). PCD in cereal endosperm seems the only example, thus far, of an *in situ* PCD during normal plant development that is not 'autolytic', and thus must be placed under 'non-autolytic' cell death (Table 2).

The second example has been found during somatic embryogenesis, *in vitro*, in *Picea abies*. Somatic embryogenesis starts with the selection of some cells of the mother plant and growing these to an amorphous mass under the influence of auxin and cytokinin. Withholding these growth regulators triggers embryo formation. Most cells in the amorphous mass then show PCD. Addition of abscisic acid (ABA) is required for the differentiation of the early embryonic cell mass into suspensor cells (connecting the embryo with the amorphous cell mass) and the embryo. ABA treatment also leads to PCD in the suspensor cells. Many cells in the cell mass were permeable to Evans Blue. They can therefore be considered to be dead, as their plasma membrane is no longer semipermeable. But these cells did not show loss of turgor. This indicates that these cells were dead while their tonoplast had not yet ruptured. Evans Blue staining confirmed this: the stain was not found inside the large vacuoles. The same was found in the suspensor cells. Tonoplast rupture occurred later on, apparently as a mechanism to remove the corpse (Filonova *et al.*, 2000; Bozhkov *et al.*, 2002, Smertenko *et al.*, 2003).

During somatic embryogenesis the dying cells exhibited progressive disappearance of the cytoplasm and an increase in vacuolar size. The nucleus showed lobing and the nuclear DNA underwent fragmentation (Filanova *et al.*, 2000). The microtubule network was partially disorganized in the dying embryonal tube cells, and was completely degraded in the dying suspensor cells. Actin in cells not undergoing PCD was thin. By contrast, it showed thick cables in the dying suspensor cells. F-actin depolymerization drugs abolished PCD in the suspensor, which suggested that changes in the actin network are quite important (Smertenko *et al.*, 2003). Upon tonoplast rupture the dead cytoplasm disappeared, leaving only the cell wall (Filanova *et al.*, 2000). This example of PCD is not included in Table 2, as it refers to cell culture and not *in situ* PCD. Whether or not the suspensor cells in embryos in seeds are dead before tonoplast rupture remains to be established.

Comparison with an earlier paper suggesting categories of cell death in plants

In a previous paper, the two main categories of plant cell death, as here identified, were also recognized, in addition to an intermediary group (van Doorn et al., 2011). The two large groups represent what is here called 'autolytic' and 'non-autolytic' PCD. However, the delineation between the three groups was not sharply defined. Such lack of definition creates problems of arbitrariness because it is not immediately obvious what example of PCD has to go where. The lack of definitions becomes especially problematic with the delineation of the intermediary class. No clear reasons were given for placing several examples of PCD in this class. For other (also arbitrary) reasons these examples might therefore have been placed into the main classes. In the present classification, the delineation between the two major classes should be immediately clear, and there is no (fuzzy) intermediary group.

One PCD class identified in van Doorn et al. (2011) was given a name that is logically incorrect. The PCD class that is here called 'autolytic PCD' was called 'vacuolar cell death'. The term 'vacuolar cell death' for this category is wrong as the vacuole is also involved in the PCD of examples that were ascribed to other categories than the one called 'vacuolar cell death', such as the HR-related PCD that requires cathepsins, which are vacuolar proteins (Gilroy et al., 2007; McLellan et al. 2009).

Categories of programmed cell death in animals

'Apoptosis' (Table 1) is the type of cell death that is usually accompanied by rounding-up of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, little or no ultrastructural modifications of cytoplasmic organelles, the production of large cell protrusions on the surface or the fragmentation of the cell (thereby producing apoptotic bodies), and engulfment of these protrusions or apoptotic bodies by mobile phagocytes or by neighbouring cells. The degradation of the cell thus occurs in another cell. The key defining features (distinguishing apoptosis from the other types of PCD in animals) are the production of cell protrusions or apoptotic bodies and the destruction of these by other cells, after phagocytosis (Elmore, 2007; Taylor et al., 2008; Wang and Youle, 2009).

'Autophagic PCD' (Table 1) is in several ways opposite to apoptosis. Autophagy is a normal process in animal cells, which increases upon starvation. The degradation of less important compounds then provides the necessary energy as well as materials for synthesis. Autophagic PCD occurs in cells that show no apoptosis but exhibit ultrastructural features that are typical of autophagy. This type of PCD, therefore, is accompanied by degradration of cellular material within the dying cell itself. Autophagic PCD in animals, to be more precise, has been defined by a death that is accompanied by an increase in the number of autophagosomes, autolysosomes and small lytic vacuoles produced by autolysosomes. These changes have together been termed 'autophagic vacuolization'. Remarkably, 'autophagic PCD' in animals is defined independent of autophagic vacuolization being a cause of death. Thus the expression 'autophagic PCD', although sounding like an invitation to believe that the death is executed by autophagy, only describes cell death with autophagic morphological features and does not imply that autophagy is the cause of death. (Kroemer et al., 2009). Rather, there are many examples of animal cells where autophagy delays PCD (Tsujimoto and Shimizu, 2005; Kroemer and Levine, 2008; Kourtis and Tavernarakis, 2009). The autophagy category of PCD in animals therefore stands on quite a weak footing: the defining morphological feature (autophagosomes and derived vesicles) is very often not the cause of death. Prominent researchers of animal PCD have therefore even called autophagic PCD a 'misnomer' (Kroemer and Levine, 2008). Due to these problems the category of autophagic PCD in animals might well become subject to revision.

Thus far, involuting Drosophila melanogaster salivary glands are one of the two in vivo example in animals that show a requirement of autophagy genes (atg) for PCD (Berry and Baehrecke, 2007). The other example is found in the nematode Caenorhabditis elegans (Samara et al., 2008). In many other animal systems studied, the knockout of atg genes advanced PCD (Kroemer et al., 2009).

'Necrosis' is the last major type of PCD in animals (Table 1). For a long time, necrosis has been considered merely to be an uncontrolled form of cell death, but there is now much evidence that the execution of most examples of necrotic cell death is finely regulated, and under the control of gene expression. It thus is a PCD (Kroemer et al., 2009).

This programmed necrotic cell death in animals is mainly identified morphologically in negative terms, by the absence of apoptotic or autophagic morphological features. Nonetheless, it is also often accompanied by a gain in cell volume, swelling of organelles such as mitochondria, plasma membrane rupture, and the subsequent loss of the intracellular contents.

During necrosis an increase in the cytosolic concentration of Ca^{2+} has often been described. This can result in mitochondrial membrane permeabilization and in the activation of non-caspase proteases. Mitochondria uncoupling is found as well as the production of active oxygen species. Lysosomes can be involved through an increase in lysosomal membrane permeabilization, often resulting in the release of PCD-inducing proteases such as cathepsins. Other processes are ATP depletion and lipid degradation following the activation of phospholipases, lipoxygenases, and sphingomyelinases (Zong and Thompson, 2006; Galluzzi and Kroemer, 2008; Vandenabeele et al., 2008; Vanlangenakker et al., 2008).

Comparison between categories of PCD in animals and in plants

The presently recognized three main categories of animal PCD will here be taken as a start, and it will be discussed to what extent the two plant PCD classes that have been recognized here might relate to these animal categories.

Apoptosis

To date, there are apparently no examples of apoptosis in plant cells. Plants do not seem to have cells that devour other cells. Plant cells have also been shown not to show outgrowths at the cell surface or fragmentation of the whole cell into apoptotic bodies. Apoptosis, therefore, can be excluded as a category of cell death in plants (Table 3).

Autophagic cell death

As indicated, autophagic PCD in animals is defined morphologically by the increase in the number of autophagosomes, autolysosomes, and small lytic vacuoles produced by autolysosomes (Kroemer et al., 2009).

The formation of various autophagic structures in plants will be described here in some detail, as it is important in the decision about whether to merge the PCD in plants that requires the massive release of vacuolar hydrolases with 'autophagic PCD' in animals.

In young plant cells, small lytic vacuoles are formed (Mesquita, 1969; Marty, 1997) and, during cell differentiation, these small vacuoles merge with other vacuoles until a few large ones are, or a single one is, produced. Plant vacuoles often contain circular membraneous structures. These must be due, apparently, to microautophagy or macroautophagy (Marty, 1997).

Microautophagy is the uptake of cellular constituents by an invagination at the lysosomal membrane or the tonoplast. The invaginated space contains a portion of the cytoplasm, usually excluding large organelles. The invagination becomes restricted at the lysosomal membrane or the tonoplast, resulting in a vesicle that moves into the lysosome/vacuole. The vesicle membrane and the vesicle contents can then become destroyed (Thompson and Vierstra, 2005).

In animal and yeast cells, macroautophagy is the development of a double-membrane-bound structure, the initiation membrane, in the cytoplasm. This structure becomes folded around a portion of the cytoplasm, thereby sequestering it. By then the structure is called an autophagosome. In animals, the autophagosome subsequently merges with a vesicle containing hydrolases (called a lysosome). The autophagosome thereby becomes an autolysosome. The hydrolases destroy the inner membrane of the autophagosome as well as the cytoplasmic contents that were enclosed by the inner membrane. In yeasts, the autophagosome joins a large vacuole, where degradation of the autophagosome contents takes place (Yang and Klionsky, 2010).

In plants, a sequence of macroautophagic events has been described that is, in some ways, different from the formation of autophagosomes and autolysosomes in animal cells (Buvat and Robert, 1979; Marty, 1999). Small tube-like organelles protrude from the interface between Golgi bodies and ER. These tubules contain hydrolases. They form a network around a portion of the cytoplasm. The tubules then merge laterally. This results in a double membrane-bound organelle around a portion of the cytoplasm, which is similar to an autophagosome with the exception that there are hydrolases between the two membranes at the outset of the formation of the organelle. These hydrolases subsequently degrade the inner membrane and also the cytoplasmic contents inside the inner membrane (Marty, 1978, 1999; Buvat and Robert, 1979). Because of the presence of hydrolases, the double-membrane-bound structures in plants, once they have sequestered a portion of the cytoplasm, are similar to autolysosomes in animal cells. They may therefore be called autolysosome-like organelles. Plant macroautophagy has been described, mainly, in very young meristematic root cells (Coulomb and Coulomb, 1973; Marty, 1973; Buvat, 1977). Very little evidence has been given for a possible role of plant-type macroautophagy during PCD. One exception is the presence of macroautophagic structures prior to cell death in laticifers (Marty, 1970, 1971; Wilson and Mahlberg, 1980). Another is the presence of macro-autophagic structures prior to death in nucellus cells (Hiratsuka and Terasaka, 2010, Fig. 5h-l). Plastids that are apparently autophagic, as shown prior to suspensor cell PCD (Gärtner

Table 3. Suggested unification of PCD classes in plants and animals

PCD class	Examples in animals and plants
Apoptotic	Widespread in animals, no examples in plants
Autophagic	
Only associated with autophagic morphology	Many animal cells.[a] 'Autolytic' PCD in plants.[b] A few examples of 'non-autolytic' PCD in plants[c]
Requires *atg* genes for death	In animals, only two examples known to date: Drosophila[d] and Caenorhabditis[e]
	In plants, only two examples known to date: tracheary elements[f] and some HR-related PCD[g]
Necrotic	Ubiquitous in animal cells. Most examples of 'non-autolytic' PCD in plant cells

[a] In many examples of animal cells there is no proof that autophagy is causal in PCD. In several other examples it has been shown that autophagy even delays PCD (see text).

[b] In many examples of plant cells there is no proof that autophagy is causal in PCD. In other examples it has been shown that autophagy even delays PCD.

[c] Increasing vacuolation, indicating increasing autophagy, has been described in some examples (Greenberg and Yao, 2004; Hara-Nishimura et al., 2005).

[d] Berry and Baehrecke, 2007.

[e] Samara et al., 2008.

[f] Kwon et al., 2010.

[g] Hofius et al., 2009.

and Nagl, 1980), might constitute a different mechanism of autophagy related to plant PCD, but such plastids have not yet been reported in other types of PCD.

There is now a growing consensus that autophagy must be defined as an event that depends on autophagy (*atg*) genes. Although ATG proteins are increasingly being studied in plants, it has not yet been firmly elucidated which ultrastructural features in plant cells relate to ATG-proteins.

Kwon *et al.* (2010) found that both PCD and autophagy (the decrease of the cytoplasm and concomitant increase in vacuolar volume) in *Arabidopsis* tracheary elements were inhibited in a plant in which the autophagy gene *atg5-1* was knocked out by T-DNA insertion. They showed many 'autophagosome/autolysosome-like structures' in the wild-type, although these might well also be termed small vacuoles, and there were fewer of these structures in the knockout plants. The location of the ATG5-1 protein in the cell was not investigated, hence the relationship between ATG5-1 and the autophagic processes remained unclear.

Similarly, Hofius *et al.* (2009) showed examples of a HR-related PCD that required *atg7* and *atg9* genes. Some triggers of death required specific receptors and these *atg* genes, but another PCD trigger did not require the *atg* genes. They observed the absence of 'autophagosome-like structures' in the *atg7* and *atg9* knockout mutants. These structures resembled small vacuoles merging with the large one. It was not elucidated, however, to what ultrastructural features the ATG proteins were related.

The work of Kwon *et al.* (2010) and Hofius *et al.* (2009) showed an *atg* gene requirement and thus the requirement of autophagy for plant PCD. These examples can therefore be added to the few examples in animal cells where *atg* genes were found to be required for PCD (Table 3).

Very strictly speaking, then, it must be concluded that we do not know if there is any PCD in plants that is related to autophagosomes, autolysosomes, and vesicles produced by autolysosomes. Some data suggest that plant autophagy is different from that in animals and does not involve the same type of organelles exactly. This might lead to the conclusion that no plant PCD can be merged with autophagic PCD in animals. However, when this is taken a bit more leniently, autophagic PCD might be defined as being related to a decrease of cytoplasm and an increase in organelles involved in gradual cell degradation. On such an interpretation, all 'autolytic' plant PCD falls into the category of autophagic PCD in animals (Table 3).

If such an interpretation would be considered to be incorrect, 'autolytic' plant PCD might be taken to be a class of PCD in addition to apoptotic, autophagic, and necrotic PCD. This might also be an option when the autophagic class in animal PCD would become removed because of the problems involved in autophagy not being a cause of death, but a mechanism whereby death is delayed, as described above. In another, but rather far-fetched, possibility, 'autolytic' plant PCD might then even be considered to be part of necrotic PCD.

In some examples of the hypersensitive response (HR)-related PCD, a cell death related to infection with pathogens, a progressive vacuolization of the cytoplasm has been described (Greenberg and Yao, 2004; Hara-Nishimura *et al.*, 2005). Although no rapid clearance of the cytoplasm has been found, and thus these examples are 'non-autolytic', the increasing vacuolization indicates the presence of autophagic processes. They therefore have to be placed in the autophagic category of animal PCD (Table 3).

Necrotic cell death

Programmed necrotic cell death in animals has been defined mainly in negative terms, by the absence of morphological markers of apoptosis or autophagic PCD. By these defining criteria most examples of 'non-autolytic' plant PCD would agree with the category of necrotic cell death in animals (Table 3).

The necrotic type of PCD in animals has often been characterized, morphologically, by an increase in cell volume and by the swelling of organelles. Swelling of the cell volume is apparently not reported in plant PCD. Swelling of mitochondria has been found in at least one example of plant PCD (germinating pollen; Bosch and Franklin-Tong, 2008), but as it is not a defining criterion, it need not occur for a plant PCD to be grouped under necrotic PCD or, conversely, when it occurs, it does not automatically mean that the example is to be classified as a necrotic PCD.

Conclusions

Two major classes of PCD can be distinguished in plants. The first ('autolytic' PCD) is associated with the release of hydrolases from the vacuole, after vacuolar collapse, resulting in rapid clearance of the cytoplasm. The second ('non-autolytic' PCD) is, in some cases, not related to vacuolar collapse, but in other examples it is also due to vacuolar collapse. In neither case, however, is it associated with the rapid clearance of the cytoplasm.

These two classes of plant PCD do not fit with the key morphological features of apoptosis: the formation of cytoplasmic outgrowths or apoptotic bodies and the consumption of these by other cells.

'Autolytic' plant PCD can be subsumed under 'autophagic PCD' in animals, because it is always associated with the presence of autophagy-like structures in the cytoplasm. This classification is not based on a very strict interpretation of 'autophagic' PCD in animals (PCD associated with autophagosomes, autolysosomes, and vesicles produced by autolysosomes), as our knowledge about autophagic structures in plants is rather rudimentary and the autophagy-like structures in plants do not seem to be completely the same as in animals.

The 'non-autolytic' plant PCD does not fit the description of apoptotic or autophagic PCD in animals and, therefore, can generally be subsumed under necrotic PCD in animal cells, as it is defined by the absence of apoptotic and autophagic morphological features. However, some examples of non-autolytic PCD that relate to the HR have been

shown to require *atg* genes (Hofius *et al.*, 2009; Kwon *et al.*, 2010). These are therefore placed under autophagic PCD in animals. Insofar as progressive vacuolization has been observed in examples of 'non-autolytic' PCD (Greenberg and Yao, 2004; Hara-Nishimura *et al.*, 2005) these must also be placed under autophagic PCD in animals.

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