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Does the plant mitochondrion integrate cellular stress and regulate programmed cell death?

Alan Jones

Research on programmed cell death in plants is providing insight into the primordial mechanism of programmed cell death in all eukaryotes. Much of the attention in studies on animal programmed cell death has focused on determining the importance of signal proteases termed caspases. It has recently been shown that cell death can still occur even when the caspase cascade is blocked, revealing that there is an underlying oncotic default pathway. Many programmed plant cell deaths also appear to be oncotic. The shared features of plant and animal programmed cell death can be used to deduce the primordial components of eukaryotic programmed cell death. From this perspective it is apparent that the mitochondrion is a common factor, which can serve in plant and animal cell death as a stress sensor and as a dispatcher of programmed cell death.

Until recently, research in programmed cell death (PCD) in animals focused more on the role of the proteolytic cascade of caspases than on the morphotype of death. Caspases play a pivotal role in animal cell death, serving both as the amplifiers of extracellular death signals and the means by which specific cellular components are disassembled by apoptosis. However, PCD can also occur independently of caspases; this finding led to a re-examination of the role played by these proteases in relation to cells death. In studies where the activity of caspases was blocked with general caspase inhibitors, apoptotic death was either prevented[1,2] or only partially executed[3]. Genetic knockouts of apoptotic factors of the caspase cascade [including Apaf-1 (Ref. 4) , caspase 3 (Ref. 5) and caspase 8 (Ref. 6)] did not prevent death even though some or all the apoptotic features were blocked. In spite of this apoptosis inhibition, the cells died – they exhibited a type of death termed oncosis[7], which is generally considered to be unprogrammed. The observation that cells can inducibly die, even when another programmed cell death is blocked, provokes the question, what is the basis of this oncotic-like death and can it be described at the molecular level? What is emerging from more critical analyses of cell death is that caspase-driven death might not be the central execution pathway[8], but instead operates on top of an underlying necrotic-like pathway. When caspase-driven death is blocked, the underlying death pathway is revealed as being oncotic.

PCD occurs in other organisms, including plants, but without the apoptotic morphotype where corpse morphology is the result of caspase action[9]. Thus, it is not altogether surprising that there is neither sequence similarity to caspase genes in yeast and plant sequence databases nor unequivocal evidence for caspase activity in these organisms. Is there a primordial death mechanism that is universal in all eukaryotes, but upon which the caspase proteolytic cascade operates in animal cells? Do caspases represent an evolved sophistication of cell death control and corpse management in animals? Analyses of death control and corpse management in organisms such as plants and yeast should answer these questions.

What features are shared among different cells?

Programmed cell death, which occurs independently of a central role for caspases, has focused

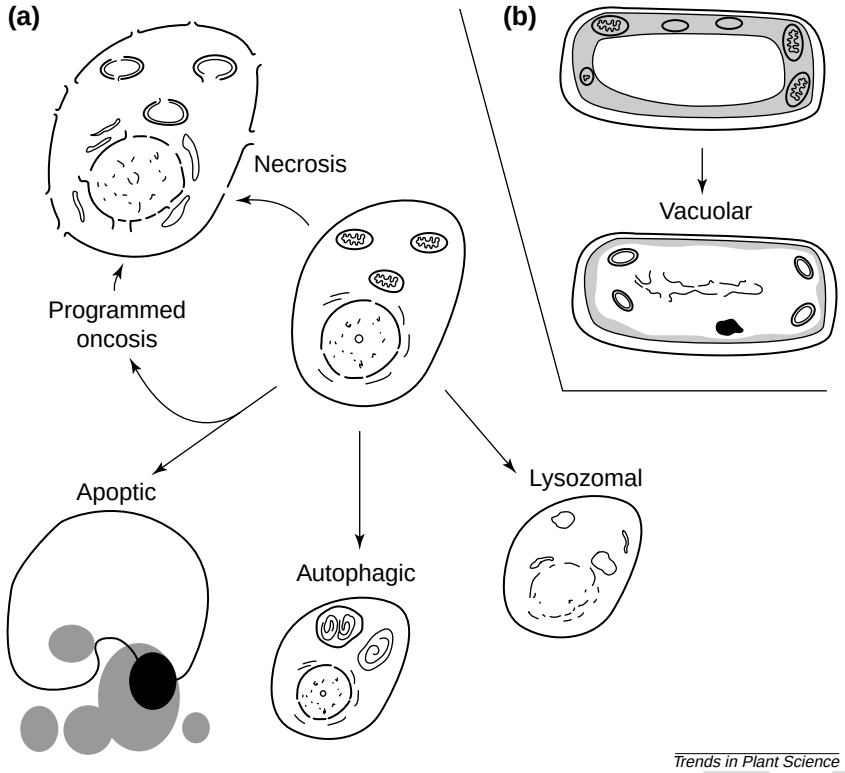

Fig. 1. Death morphotypes. (a) The morphology of the cell corpse depends on the way a cell dies. Apoptotic, autophagic and lysozomal are three types of programmed cell death. Apoptosis occurs in animal cells and has the hallmarks of nuclear condensation, cytoplasmic blebbing and the involvement of a macrophage to remove the corpse. Autophagic degenerative cell death involves autophagic organelles but often no nuclear condensation. Examples of this type are found in both plant and animal cells. Lysozomal degenerative cell death involves the loss of cytoplasm probably by the local secretion of hydrolases. Plant cells might use a form of this type, discussed in (b). In contrast with a programmed cell death, oncosis is viewed as accidental death where metabolic homeostasis fails. Oncotic cells and organelles are swollen and the plasma membrane is broken. One other possible outcome is for a cell to initiate apoptosis but fail to complete it. (b) Plant cell death is manifested as autophagic and oncotic, this is described as programmed oncosis. In addition, many plant cells trigger death by disrupting the tonoplast in a program called vacuolar degenerative cell death. These hydrolases, sequestered in the vacuole until the moment of triggering death, are synthesized *de novo* in response to signals that prepare the cell to die.

attention on the death morphotype: are the different molecular mechanisms for programmed cell death in animals[10] and plants[11] manifested as different death morphotypes, or are the death morphotypes points along a continuum?

The morphology of dying animal cells has been thoroughly documented[12]. The first major distinction among dying cell morphotypes defines death as an active program, whereby the cell is dismantled in an orchestrated and predictable manner compared with the unprogrammed, unorganized management of corpse removal (Fig. 1). Programmed cell death in animals has been categorized into three morphotypes:

Morphotype 1 (apoptosis), has three ºhallmark features:

- DNA appears marginalized on the nuclear envelope (pyknosis) and is fragmented to nucleosomal-sized lengths.
- The nucleus and cytoplasm are fragmented into vesicles.
- Macrophages remove the corpse *in vivo*.

This type of PCD is the best understood and is used to study animal PCD.

Morphotype 2 (autophagic or cytoplasmic degenerative PCD), might or might not display nuclear degradation and pyknosis, and usually does not involve the participation of macrophages, although its hallmark feature is the consumption of cytoplasm by autophagic organelles. This morphotype is often found where death occurs *en mass*, such as the in the degeneration of the tadpole tail[13], the intersegmental muscle of insect larvae[10], and in chick neurons[14]. It has also been documented in plant cells, such as in the embryonic suspensor[15].

Morphotype 3 (lysozomal degenerative PCD) is the least understood. It is classified by its general loss of cytoplasm without the clear involvement of an organelle or another cell. The proposed mechanism involves the secretion of hydrolases from lysozomes. The mechanism has been debated in the past, but was eventually neglected without directly testing its function. However, recent examples of the mechanism of plant cell death might resurrect interest in this type of death mechanism: PCD in plant cells often involves the conversion of the vacuole into a lytic compartment that discharges its hydrolases when triggered.

In contrast with apoptosis, oncotic death, which is induced by cellular insults (both chemical and mechanical) is manifested by swelling cytoplasm, mitochondria and other organelles, and by the leakage of cellular contents into the surroundings. Although the presence of the cellulosic wall precludes cytoplasmic swelling, plant PCD shares many of the animal oncosis features, such as disruption of membranes and swelling of organelles[16–19].

Can oncosis be programmed in plants?

It is argued that apoptosis and oncosis in animal cells are at opposite ends of a continuum[20]. Apoptotic death is thought to involve an active program whereas oncosis is viewed as 'accidental' death and represents the cell's inability to either repair the damage or to initiate a death program that contains a corpse-processing subroutine manifest as one of the three morphotypes discussed[21]. Can death by either apoptosis or oncosis share the same early molecular mechanism? Can an oncotic corpse morphotype result from a programmed cell death in animals? From an evolutionary viewpoint, it is difficult to argue that animal cells would initiate oncosis because the undesirable leakage of cellular content would probably lead to tissue inflammation. However, this constraint does not apply to plant cells, and in several plants the developmental and pathogen-induced programmed cell death processes appear oncotic.

Recent results (showing that PCD can occur independently of caspase activity) indicate that underlying an apoptotic program is another program complete with hydrolases that manage the corpse up to the point of phagocytosis. This could represent a primordial part of PCD, shared by both plants and animals, which in animals evolved into the apoptotic programme.

Understanding how 'programmed oncosis' might operate requires a quick review of the role of the mitochondrion in PCD. When cell-free systems were developed to study PCD in animals, several apoptosis-activating factors (Apaf) were found[22,23]: one of these is cytochrome c. Cytochrome c acts in the cytoplasm by recruiting caspases via an adapter called Apaf1 (Ref. 24). In the current model, cytochrome c and ATP cause Apaf1, an

adaptor protein that contains caspase recruitment domains, to dimerize thus bringing procaspase 9 (another Apaf) into close proximity to transactivate each other and to activate downstream caspases.

How cytochrome c gets to the cytoplasm is a fascinating story that is still unfolding. It seems that there are at least two mechanisms[25] (Fig. 2):

(1) Via the formation of a transient pore into the mitochondrial matrix. This pore is called the permeability transition (PT) pore and is formed by the transient complex of the voltage-dependent anion channel (VDAC) on the outer membrane, the adenine nucleotide transporter (ANT) from the inner membrane and cyclophillin D in the matrix. The inner membrane is normally impermeable to anions, but when the PT pore forms, it is thought that the rapid movement of ions into the matrix followed by water causes this compartment to swell, rupturing the outer membrane to release cytochrome c.

(2) Via the VDAC (Ref. 26); this is not associated with mitochondrial swelling and rupture. The route for calcium-induced release of cytochrome c can differ depending on the tissue[27]. In rat liver, the PT pore forms to release cytochrome c, whereas in rat brain, cytochrome c is released by a PT-independent means, probably directly via the VDAC. In most, if not in all cases examined, calcium activates the release only at low ATP levels. This point is important as there are several cases where calcium induces cell death in plants when ATP pools are reduced.

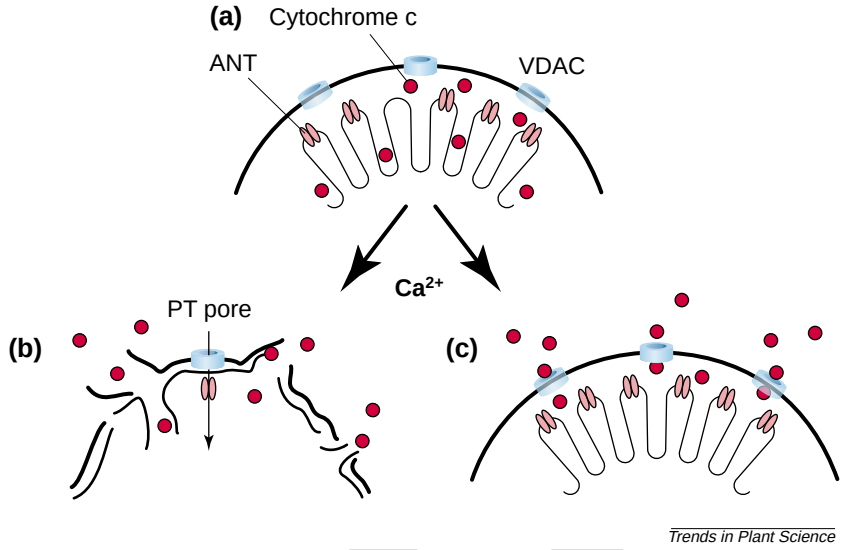

Fig. 2. (a) Cytochrome c release into the cytosol occurs in two ways (b and c), both require calcium flux at low cellular ATP levels. In the first (b), the permeability transition pore (PT pore) forms as a complex with the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), cyclophilin D (not shown) and the benzodiazepine receptor (not shown). The PT pore permits ions to move into the matrix; outer membrane rupturing occurs when the inner membrane swells. (c) Cytochrome c can also be released directly via the VDAC.

How universal is this death signaling mechanism?

Under conditions where ATP pools are low, calcium is the fundamental pore-activator in all cells tested[28]. Oxidative stress also triggers PT pore-opening directly[29] and possibly also indirectly by elevating calcium levels[30]. Calcium-induced PT pore-opening can be blocked by scavengers of reactive oxygen species (ROS), therefore there appears to be dependency between ROS and calcium in pore opening[31,32]. Accordingly, inhibition of electron transport, such as with nitric oxide[33], reduces ATP pools[34], elevates ROS pools and, under conditions of elevated calcium, activates the pore. The mitochondrion is thought to integrate various stresses and, once triggered by a calcium increase, signals the cytoplasm to execute the PCD (Fig. 3).

Programmed oncosis might be the outcome of unsuccessful apoptotic corpse management in animal cells, but be a program in itself in plant cells. In animal cells, the death program is activated by cytochrome c release, but when there is insufficient ATP to carry out the

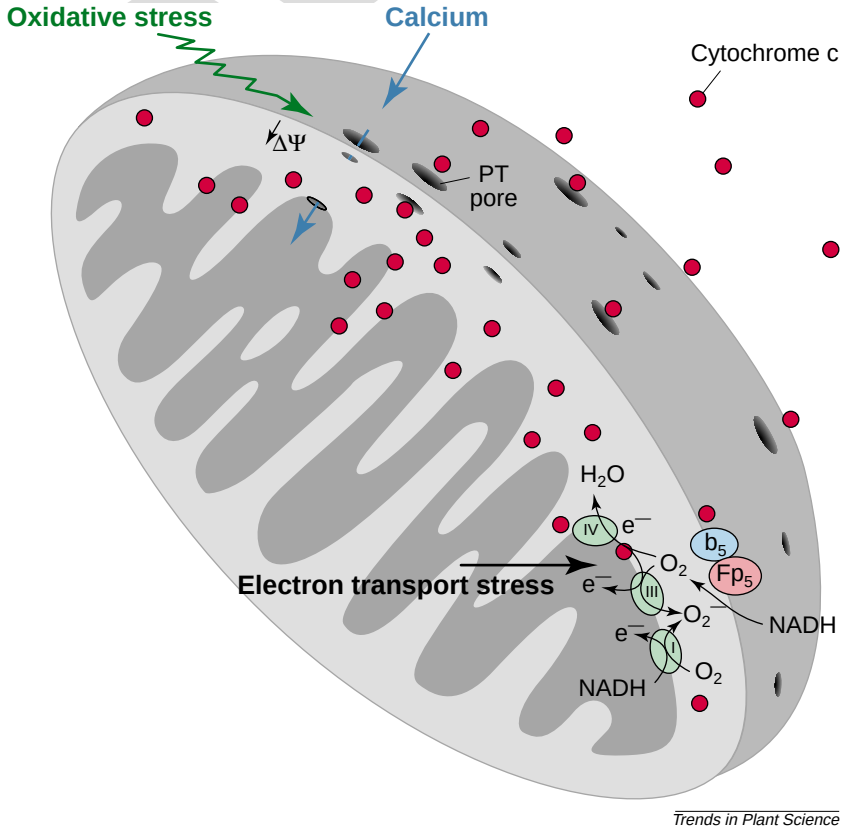

Fig. 3. Does the plant mitochondrion integrate stress signals for programmed cell death (PCD)? There are many different situations that lead to cytochrome c release. These include oxidative stresses that induce PT pore formation, stresses on electron transport and a rise in calcium levels. It is proposed that when cells are unable to maintain metabolic homeostasis and the stresses overwhelm the cell, that mitochondria release cytochrome c triggering death. These stresses are normal components of PCD in plant cells.

proteolytic caspase cascade the result might be oncosis-like death. Because this type of death is active and triggered, it can be thought of as programmed, but the morphotype is that of oncosis (Fig. 1).

Cytochrome c release might also be important in plant cell death. Recently, it has been shown that maize cells induced to die by the addition of D-mannose release cytochrome c to the cytosol[35]. Interestingly, D-mannose inhibits hexokinase and reduces ATP levels[36], a prerequisite for calcium-induced PT pore formation. A plausible explanation for mannose action is that it lowers ATP levels and raises ROS levels to a point where the high calcium levels found in the culture medium trigger the PT pore and cytochrome c release. Cytochrome c release also precedes heat-[37] and menadione-induced[38] death in plant cells. Furthermore, the addition of cytochrome c to carrot cytoplasmic extract induces apoptotic features of added mouse nuclei[39]. Admittedly, the evidence for cytochrome c release in plants is scarce, but the new data suggests that plants and animals share the mitochondrial component of PCD, and therefore this could represent the primordial component of the program.

Without prototypical caspases in the plant genome, one must wonder how cytochrome c activates plant PCD. It has been proposed that cytochrome c release generates lethal levels of ROS by blocking electron transport and therefore this might have been the primordial mechanism by which the endosymbiotic mitochondrion killed its host[40]. Equally plausible is the suggestion that caspase function is fulfilled by other divergent proteases in plants.

Are some plant cell deaths programmed oncosis?

Although an apoptotic morphotype has been described for plant cell death in association with the hypersensitive response (HR)[41], there is a general view that the typical HR morphotype is oncotic-like[18,19]. Dying cells leak, their organelles swell, and the corpse is unprocessed and left to be invaded by the surrounding cells. Although this response to pathogens might appear an almost accidental death, there is clear evidence that the death pathway is programmed. All known PCD in plants requires a calcium influx[42–45], but calcium alone is insufficient to trigger death, suggesting that calcium influx enables a cell to become competent to die. In addition, HR can be blocked pharmacologically and there are mutants that mimic the HR in the absence of a pathogen[46].

Cell death associated with the HR clearly involves ROS [Ref. 47; including multiple sources and types of ROS (Ref. 48)] and it is generally thought that these are for the purpose of defense[49]. In generating ROS, the cell places itself in a precarious position between mounting a defense and eliciting cytoplasmic oxidative stress. Indeed, the evolution of defensive ROS is accompanied by redox homeostatic mechanisms that presumably attenuate cytoplasmic oxidative stress[50,51]. It has been shown that a failure to control the cytoplasmic redox has dire consequences for the cell[52].

Are mitochondria integrators of stress and regulators of plant PCD?

It is clear that the mitochondrial PT pore complex is a sensor of cellular stress and that it releases apoptogenic factors that trigger death in animal cells. Is this the case in plant cells, and how might the role of calcium perturbation and ROS generation in plant PCD explain cell death regulation? Research on the signal transduction pathway leading to HR has led to the view that ROS is an inductive death signal[53]. However, ROS might not be a signal but rather a stress, one of many that are integrated by mitochondria. This alternative conclusion is more than mere semantics: for some responses, low levels of ROS might be sufficient to keep pathogens at bay whereas others require additional ROS and additional tactical maneuvers. It is known that plant cells have the means to attenuate ROS stress cytoplasmically and do so at high ROS levels during pathogen defense[54]. But is there some level of ROS that overwhelms the cell's ROS homeostasis machinery and favors the initiation of PCD? If so, the mitochondrion is the ideal organelle to integrate the level of stress and to coordinate PCD when warranted. ROS are generated by mitochondria, chloroplasts and other organelles during their normal activities, and at the same time are sensitive to reactive oxygen stresses. One mechanism for this is via the release of cytochrome c at high levels of calcium, and the converse is true: the release of cytochrome c leads to ROS generation.

The primary role for nitric oxide could be as an anti-bacterial agent – indirectly stressing cells to the point of triggering death by permeability transition pore formation

Much attention has been placed on the possible role of nitric oxide (NO) in plant cell death[55], with the conclusion that NO is the signal that triggers cell death in the HR. However, plants might use NO as an antibacterial agent (it forms peroxynitrite after reaction with oxygen) rather than a direct signal of cell death, making NO a stress rather than a signal. Furthermore, NO can release calcium by nitrosylation of the ryanodine channel[30] and therefore potentially can induce PT pore formation. Direct NO effects on mitochondria have also been noted, including PT pore formation[56]. Finally, NO has been shown to reduce ATP by the inhibition of electron transport[34], another requisite for PT pore formation. Thus, it is conceivable that the primary role for NO is as an anti-bacterial agent that might indirectly stress cells to the point of triggering death by PT pore formation.

Genetic screens to identify mimics of cell death have been successful[46]. Some of these genetic lesions might define genes in a signal transduction pathway in PCD. However, it remains possible that many lesions lead to death by altering plant cell redox or inappropriately causing PT pore formation, such as the *les22* lesion mimic in maize[57], which has been cloned and found to encode uroporphyrinogen decarboxylase, an enzyme in the porphyrin biosynthesis pathway. The *les22* mutants have elevated uroporphyrin, a compound with a similar structure to protoporphyrin IX. Interestingly, protoporphyrin IX, induces PT pore formation in animal cells[58], suggesting that a similar mechanism triggering death could occur in these plant cells. Alternatively, because catalase and peroxidase are heme-containing enzymes they might be less active in the *les22* mutants and therefore more susceptible to internally-generated ROS (Ref. 57). In either case, the explanation leads ultimately to PT pore formation as a possible cause of death.

Do plant cells regulate permeability transition pore formation and cytochrome c release with Bcl-like proteins?

In animal cells, apoptotic and anti-apoptotic proteins operate by interacting with mitochondria. Although it is not fully understood how this occurs, anti-apoptotic proteins, such as Bcl-2, might bind to the VDAC and prevent cytochrome c release[59,60]. Bcl-2 also has other downstream effects on caspases[61,62], but if its fundamental role is to block cytochrome c release from mitochondria, it might block cell death even in organisms lacking Bcl-2 homologs and downstream caspases. Apoptotic proteins, such as Bax, counter the action of Bcl-2, probably by promoting cytochrome c release. Recent evidence indicates that Bcl-2 and Bax fulfill similar roles in plant cells and yeast. Mitochondria-targeted Bax triggered cell death when expressed via the tobacco mosaic virus (TMV) in TMV resistant plants: leaves developed lesions with a tissue and kinetic pattern similar to the HR (Ref. 63). Conversely, when the anti-apoptotic gene Bcl-$_{XL}$ was expressed in tobacco, death induced by UV, paraquat or TMV (known inducers of cellular ROS) was suppressed[64]. What do these results tell us about the role of

BCL-like proteins (BLPs) in plant PCD? One possible interpretation is that divergent proteins might act in plants, suggesting conservation of the pathway. However, an alternative view is that only the PT pore complex is structurally conserved between plants and animals. Thus, BLPs can operate in plant cells, but they do not necessarily do so.

Even if plants lack prototypical caspases and BLPs (as appears likely given the failure to identify candidates in the public databases), the activity of heterologous BLPs in plant cells suggest a similar role for mitochondria in both plant and animal PCD, namely, regulating a cell death program.

Is corpse processing in plant programmed cell death plastic?

One clear difference between plant and animal PCD is that animal PCD appears to be instantly ready for activation and is a rapid process. Consequently, animal cells have PCD dampers and complex feedback controls. By contrast, plant cells must first acquire the competency to trigger death and they process the corpse cell autonomously, although the processing differs between plant PCDs. For example, during tracheary element differentiation PCD results in complete autolysis of the cytoplasm, while leaving behind the extracellular matrix. During lysigenous aerynchyma formation PCD results in the complete removal of the corpse, whereas HR-induced PCD shows no evidence of corpse removal. In each case, different internal and external signals direct the cell on how to prepare for its death and how the corpse will be processed. Several genes encoding hydrolases that are up-regulated are common to all plant PCDs, whereas some genes are unique to each type of 'preparation' for death. Thus, differences in the profiles of these nascent hydrolases might account for the underlying differences in corpse removal.

The plant cell is unique in that its central large vacuole can sequester hydrolases. The disruption of the tonoplast and the subsequent release of the sequestered hydrolases appears to be a common feature of plant PCD (Fig. 1). Perhaps it is time to define a new type of PCD based on the role of the vacuole, thus avoiding a term based on morphology, such as 'programmed oncosis'.

Finally, we need to rethink the role of ROS in controlling death. It is unlikely that these molecules simply serve as cellular signals for cell death, at least not by the traditional definition of a signal. We need to test the hypothesis that ROS, including NO, might act as stress inducers at the level of the mitochondrion or the chloroplast. Oxidative stress, not ROS, could be the cell death signal and the target could be the mitochondrion as it is in animal cells.

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