Petal Senescence: New Concepts for Ageing Cells

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Abstract

Senescence in flower petals can be regarded as a form of programmed cell death (PCD), being a process where cells or tissues are broken down in an orderly and predictable manner, whereby nutrients are re-used by other cells, tissues or plant parts. The process of petal senescence shows many similarities to autophagic PCD in animal cells including a massive breakdown of protein, DNA and RNA, the formation of autophagic vacuoles for the breakdown of cytoplasm and organelles therein and, the eventual rupture of these vacuoles that kills the cell. Chromatin condensation and DNA and nuclear fragmentation (traditionally considered being apoptotic-like features) are observed in both autophagic animal cells and in senescing petal cells.

We present a conceptional model underlying petal senescence that integrates elements that have been associated with both apoptotic and autophagic types of PCD.

FLOWER SENESCENCE RESEARCH IN A NUT SHELL

Rapid senescence of flower petals is highly undesirable from a postharvest perspective. However, from an ecological point of view senescence should be regarded a functional process. In many flowers, the petals rapidly senesce following pollination, presumably to direct pollinators to other flowers on the plant, to prevent wasting energy necessary to maintain the petals any longer than necessary, and to retrieve nutrients from the senescing tissue. In the absence of pollination, such flowers have a relatively long life span (e.g. carnation, petunia, orchids). In other types of flowers, petal senescence seems to follow a tightly controlled program independent of external stimuli such as pollination. Such flowers often have a relatively short life span, often less than one day (e.g. *Ipomoea*, *Iris*, *Hibiscus*), but several flowers with a longer life span also belong to this category (e.g. *Aster*, *Gerbera*).

The mechanism of petal senescence has been the focus of extensive research. Increased ethylene production is the signal mediating the pollination-induced senescence. Ethylene is also the signal in any species with ethylene sensitive petal senescence, even if the flowers do not become pollinated. Chemical and molecular genetic strategies have been developed to block ethylene production or perception to prolong the life span of such flowers (Woltering, 2003; Serek et al., 2006). For the other type of flowers, no clear master switch that regulates the senescence has been identified yet. It is, however, expected that the senescence process itself shows many similarities in these two types of flowers.

Major early work, using morning glory (*Ipomoea tricolor*) as a model, has identified massive breakdown of proteins and nucleic acids and showed that petal senescence is accompanied by autophagic processes. In senescing petal cells, numerous vesicles and structures reminiscent of mitochondria were found in the vacuole and this compartment was recognized as the main site of autophagic activity. The breakdown of the tonoplast was regarded as the last phase of the senescence process. These observations indicated that senescence proceeds in an ordered and predictable way culminating in tonoplast rupture and death of the cells (Winkenbach, 1970a, b; Matile and Winkenbach, 1971; Wiemken-Gehrig et al., 1974; Wiemken et al., 1976).

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In later years, research has been focused primarily on membrane properties. The general idea was that senescence was the result of ageing-related changes in the oxidative status of the cell, leading to increased membrane lipid peroxidation and subsequent loss of semi-permeability of the plasma and vacuole membranes, which would result in cell death. The latter process was regarded more or less passive and the result of accidental events (Eze et al., 1986; Fobel et al., 1987; Borochov et al., 1994). In addition, a change in the phospholipid/sterol ratio, presumably due to decreased synthesis and enhanced degradation of membrane phospholipids, was supposed to induce a gradual decrease in membrane integrity. The data obtained thus far have not substantiated either of these hypotheses. Nonetheless, it can not be excluded that the final rupture of the vacuolar membrane is related to phospholipid degradation or other mechanisms of inducing membrane instability.

In the early nineties, with the introduction of molecular biology in flower senescence research, it was recognized that senescence is a very active event which, apart from the massive breakdown of protein and nucleotides, needs the synthesis of new mRNAs and proteins (Borochov and Woodson, 1989). Currently we have entered the episode where research is focussed on the regulation of senescence at the gene expression and proteomic levels, with an effort to improve our understanding of the underlying mechanisms (Fig. 1). The availability of gene micro-arrays has allowed us to study the expression of many thousands of genes during the course of senescence (van Doorn et al., 2003; Breeze et al., 2004; Hoeberichts et al., 2007), and functional analysis of the most interesting genes will follow in the near future.

PCD TYPES

According to the general definition, programmed cell death (PCD) applies to cell death that is part of the normal life of multicellular organisms. If so defined, PCD is found throughout the animal and plant kingdoms. It is an active process in which a cell suicide pathway is activated resulting in controlled disassembly of the cell. Besides cell death as a result of normal development (developmental PCD), cell death resulting from environmental stress (e.g. infection by pathogenic organisms, wounding and low concentrations of toxins) also often occurs through controlled disassembly of the cell and can therefore also be termed PCD (stress-induced PCD, including pathogen-induced PCD).

The autophagic processes in senescing petals described by the early workers on plant cells (see above) were also found, although many years later, in dying animal cells. In animal science the cell death showing large scale autophagy, and no uptake of the cell contents by other cells, was termed Autophagic PCD (Type II PCD). It shows features such as the disappearance of cytoplasm and organelles, formation of double membrane autophagosomes containing cytoplasm and organelles, and the formation of auto-lysosomes.

Apart from Autophagic PCD, other types of PCD have been identified in animal cells (Table 1). Most prominent is apoptosis (Type I PCD). Other types of programmed cell death have also been assigned based on cell death morphology but these have not been studied intensely and are presumably less important (Table 1).

Apoptosis is the main type of PCD in animal cells. For a considerable period of time the terms PCD and apoptosis have been used as synonyms by scientists working with animal cells. Apoptosis can easily be distinguished from autophagy, as the apoptotic cell is finally degraded inside another cell, whereas the autophagic cell is degraded by itself. Other features that have often been associated with apoptosis are cell shrinkage, blebbing of the plasma membrane, condensation and fragmentation of the nucleus and internucleosomal cleavage of DNA. The final stage of apoptosis is the fragmentation of the cell into cellular debris-containing vesicles called “apoptotic bodies” that are being phagocytosed by other cells (Hengartner, 2000). At the biochemical level, the apoptosis signal is transduced by a specific class of cystein proteases called caspases, and by transport of proteins out of mitochondria (Hail et al., 2006).

Thus, an important difference between autophagy and apoptosis is that during autophagy the cell content is degraded by the cell itself, i.e. by the lysosome of the same cell, whereas during apoptosis the cell often show protuberances or the cell is split into apoptotic bodies. These protuberances or apoptotic bodies are taken up (phagocytosed) by other cells, and their content is degraded in the lysosome of that cell.

Striking and, initially confusing, is that both type I and type II cell death may be accompanied by chromatin condensation, DNA degradation, and nuclear fragmentation. Such features were first observed in apoptotic cells and have therefore become presumed to be "apoptotic features", and they sometimes are still erroneously called as such. In addition, it has been found that caspases, first thought to be specific for apoptosis, may also be necessary for autophagic PCD in certain systems (Baehrecke, 2003; Lockshin and Zakeri, 2004). It has now been recognized that the same cell may use different ways to die, in particular by using other cells for final degradation or no such cells, depending on the triggers and the cellular context (Baehrecke, 2002; Lockshin and Zakeri, 2004).

PCD IN CULTURED PLANT CELLS AND PROTOPLASTS

Cultured plant cells and protoplasts have been widely used as model systems to study PCD. Synchronized cell death can reproducibly be induced in such cultures by treatment with mycotoxins, heavy metals and other chemicals or by physical treatments (heat shock, UV light). In the majority of cases, cell death in such systems is accompanied by a characteristic cell morphology (chromatin condensation, cytoplasm shrinkage, nuclear fragmentation), by DNA degradation (sometimes DNA laddering) and sometimes by cytochrome-c release from mitochondria (Reape et al., 2008). Given the similarity to the observed changes in apoptotic animal cells, this type of plant cell death is often called *apoptotic-like PCD*. As the characteristic feature of apoptosis (degradation of cell contents in lysosome of other cells) is lacking in these plant systems, this type of cell death may in fact better be called *non-lysosomal PCD* (van Doorn and Woltering, 2005).

Functional plant equivalents of animal cell caspases are presumably involved in the process. Among these are the vacuolar processing enzymes (VPEs) and some other cysteine and serine proteases (de Jong et al., 2000). The production of reactive oxygen species (ROS; for example hydrogen peroxide; de Jong et al., 2002) and reactive nitrogen species (RNS; for example NO) by mitochondria and by the plasma membrane-associated NADPH-oxidase is instrumental in this type of cell death (Fig. 2). Although ethylene alone does not induce cell death in these systems, it seems an important stimulator of the process as blocking ethylene production or perception usually greatly diminishes the effect of the chemical and physical cell death inducers.

PCD IN SENESCING PETALS

As discussed, the early work in morning glory by Philippe Matile and co-workers, showed the possible involvement of autophagic processes in petal senescence. Others found similar evidence in other species. In senescing carnation (Smith et al., 1992), *Hemerocallis* (Stead and van Doorn, 1994) and *Iris* (van Doorn et al., 2003) petals, for example, an increase in vacuolar size was observed that was accompanied by a decrease in the amount of cytoplasm and with the disappearance of most organelles, including most of the endoplasmic reticulum and attached ribosomes.

In addition to these autophagic features, a vast amount of evidence for the occurrence of what have often been called "apoptotic-like" features in senescing petals is currently available (Table 2).

DNA degradation is a general feature of senescing petals (e.g. van der Kop et al., 2003; Xu and Hanson, 2000; Jones et al., 2005). DNA in animal cells is often first degraded into large parts (about 50 kbp), then into smaller parts consisting of about 180 bp or multiples of 180 bp. In senescing petals DNA degradation into fragments containing multiples of about 180 bp, showing a ladder pattern on gel, has so far only been observed in a limited number of species e.g. in pea, petunia, *Freesia* and *Alstroemeria* petals (Orzaez and Granell, 1997; Xu and Hanson, 2000; Yamada et al., 2001; Wagstaff et al.,

2003). It is not clear if the absence of visible laddering on gels represents lack of laddering. Judged from the DNA gels presented by several authors in plant senescence/PCD it seems that only a small portion (<5%?) of the DNA actually shows laddering. If no laddering is reported, it may therefore have escaped observation.

Using methods to visualize nuclear features (electron microscopy, TdT mediated dUTP-biotin nick end labelling [TUNEL], DNA staining and flow cytometry) several nuclear ultrastructural changes were observed in senescing petal cells (Table 2). The nuclei may show blebbing and chromatin often clumps into patches that are found throughout the nucleus or at the nuclear periphery (Serafini-Facassini et al., 2002; Wagstaff et al., 2003) or, nuclei may show a decrease in size due to chromatin condensation, fragment into separate masses and may contain degraded DNA (Hoeberichts et al., 2005; Yamada et al., 2006a, b, 2007).

Generally, nuclear morphological changes and in situ DNA degradation are observed in early stages of senescence. In *Gypsophila*, for example, TUNEL-positive nuclei were observed in cells throughout the petal well before the rise in ethylene production, at a time the flower had fully opened but did not yet show a visible sign of senescence (Hoeberichts et al., 2005). This indicates that, at least in *Gypsophila*, the majority of petal cells show nuclear morphological changes and DNA breakdown very early in the senescence process. A similar result was obtained in *Iris* and *Alstroemeria* flowers. However, in these species a clear distinction with respect to the timing of cell death was observed between the mesophyll and the epidermis cells. Most of the mesophyll cells in Iris petals were already dead and gone by the time the petal showed visible signs of senescence (van Doorn et al., 2003). In *Alstroemeria* some degenerative changes were found in the nuclei of petal cells even before the flower had fully opened (Wagstaff et al., 2003). Together these observations support the idea that cell death can begin very early, can be regulated by ethylene, and is an integral part of the developmental program of the petal.

A CONCEPTIONAL FRAMEWORK FOR PETAL SENESCENCE

Piecing all the evidence together it may be suggested that cell death during petal senescence should be classified as an autophagic type of PCD (Type II). In the few systems that have been thoroughly studied with respect to morphological and ultra-structural changes during senescence (e.g. *Ipomoea*, carnation, *Iris*) several autophagic features were observed well before visible senescence. These features include the disappearance of cytoplasm, ER, and various organelles, an increase in size of the vacuole and the presence of “autophagic vesicles” outside and inside the vacuole. This has been called macro-autophagy. Most importantly, the rupture of the tonoplast is an autophagic phenomenon. This results in the release of numerous hydrolytic enzymes into the cell, resulting in the destruction of anything that has remained. These events have been termed mega-autophagy (van Doorn and Woltering, 2005). Autophagic cell death in senescent petal cells (or even in plants in general) is therefore not only associated with autophagic features, such as autophagic vesicles in the vacuole, it is due to the autophagy that is the result of tonoplast rupture. These results, however, need verification in other systems to determine how universal these phenomena really are.

Macro-autophagy is a normal physiological process involved in organelle turnover. Macro-autophagy is highly up-regulated during senescence, and synthesis is decreased, thus the rate of destruction highly exceeds the rate of synthesis. Whether the upregulation of macro-autophagy is in any way related to the onset of mega-autophagy is as yet not known. It is at present not at all clear what events precede the rupture of the tonoplast, thus it is not clear what finally kills the cell.

The measured increase in the leakage of ions from ageing petals, which has earlier been ascribed to a gradual decrease in overall integrity of cell membranes, may in this scenario be due to an increase in the amount of dead cells that release their contents after tonoplast rupture. A progressive dying of individual cells will be reflected in a gradual increase in ion leakage.

In ethylene-sensitive petal senescence in flowers such as carnation and *Gypsophila*, the increased production of this gaseous hormone during senescence may play a role in synchronizing the death of cells throughout most of the petal. This follows from the absence of a clear difference in the onset of senescence in mesophyll and epidermis cells, in *Gypsophila* (Hoeberichts et al., 2005). In contrast, in ethylene-insensitive flowers the mesophyll and epidermins cells started to die at different points in time, and thus do not seem to die in a synchronized way. In such flowers (e.g. *Iris*, *Hemerocallis*) it is important not only to remember that cells in the different types of tissues (epidermis, mesophyll) may be in different stages of senescence, but also that differences exist over the length of the petal, whereby the distal parts die earlier than the basal ones.

The occurrence of "apoptotic features" during petal senescence is not contradictory to the idea that petal senescence is a form of autophagic PCD. Also in animal cells, "apoptotic features" are often observed in autophagic cell death. The term "apoptotic features" actually seems mistaken, as the same features are found both in apoptotic and in autophagic PCD in animals. A cell can die in at least two ways: by mega-autophagy or by being taken up by another cell (apoptosis). The co-existence of autophagic and "apoptotic" features in the same tissue or the same cell of a petal is quite understandable, as the "apoptotic" features are not leading to the cell being taken up by another cell. The "apoptotic features" mainly refer to changes in the nucleus (nuclear condensation, chromatin condensation, DNA degradation including laddering, and nuclear fragmentation). These features are part of the degradation machinery of a cell that also shows macro-autophagy and will die by mega-autophagy.

In one scenario, then, macro-autophagy is only a remobilisation process, which also yields energy. Independently, some process leads to tonoplast rupture (macro-autophagy). As an alternative scenario, the cell death process in plants may be viewed solely as a result of increased macro-autophagy, which would somehow be the cause of mega-autophagy.

In both cases, the processes leading to the eventual rupture of the tonoplast are essential. As yet, we do not know how this comes about. Perhaps the caspase-like vacuolar processing enzymes (VPEs) play a important role (Hara-Nishimura et al., 2005). Tobacco plants in which VPE activity was blocked showed delayed tonoplast rupture and cell death following TMV infection (Hatsugai et al., 2004). VPEs show YVADase acivity and can be blocked by some human caspase inhibitors indicating that a "caspase-like" protease may be responsible for rupture of the tonoplast (mega-autophagy).

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Tables

Table 1. Types of PCD recognized in animal cells.

Type	Name	Features
I	Apoptosis	Shrinkage of nucleus and cytoplasm, membrane blebbing, nuclear condensation, DNA degradation and nuclear fragmentation, formation of apoptotic bodies, degradation of cell contents in lysosome of other cells
II	Autophagy	Formation of autophagosomes and autolysosomes, degradation of cell contents in same cell, nuclear condensation, DNA degradation and nuclear fragmentation
III	Non-lysosomal	Shrinkage of cytoplasm, no degradation of cell contents, nuclear morphological changes

Table 2. Examples of nuclear changes observed in senescing petals. DNA degradation and laddering are generally observed using DNA gel electrophoresis; in situ DNA degradation by TUNEL; chromatin condensation and nuclear fragmentation by light, fluorescence or transmission electron microscopy.

Species	Observations	Reference
Alstroemeria	DNA laddering	Wagstaff et al., 2003
Antirrhinum	Nuclear shrinkage, chromatin condensation, DNA degradation, no DNA laddering, nuclear fragmentation	Yamada et al., 2006b
Argyranthemum	Nuclear shrinkage, chromatin condensation, DNA degradation, no DNA laddering, nuclear fragmentation	Yamada et al., 2006b
Freesia	DNA degradation, in situ DNA degradation	Yamada et al., 2002
Gladiolus	Nuclear degradation, DNA laddering	Yamada et al., 2004
Gypsophila	DNA degradation, in situ DNA degradation	Hoeberichts et al., 2005
Ipomoea	Chromatin condensation, DNA degradation, no DNA laddering, Nuclear fragmentation	Yamada et al., 2006a
Iris	DNA degradation, no DNA laddering	van der Kop et al., 2003
Nicotiana	Nuclear fragmentation	Serafini-Fracassini et al., 2002
Pea	DNA laddering, in situ DNA degradation	Orzaez and Grannel, 1997
Petunia	Laddering, in situ DNA degradation Nuclear shrinkage, chromatin condensation, no nuclear fragmentation, no DNA laddering	Xu and Hanson, 2000; Langston et al., 2005 Yamada et al., 2006b

Figures

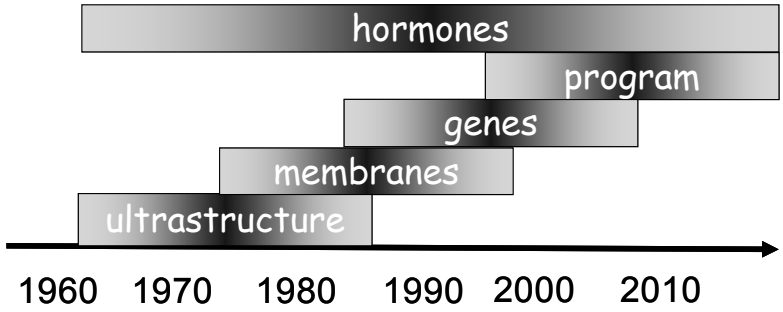

Fig. 1. A rough picture of the changing focus of petal senescence research (see text).

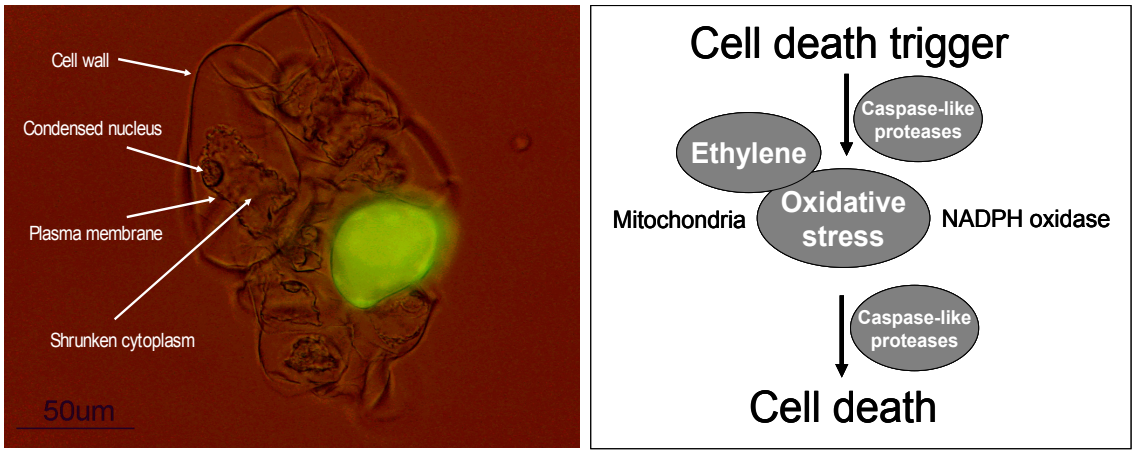

Fig. 2. Cell death in cultured plant cells treated with death-inducing chemicals. Left panel: tomato cell cluster treated with cadmium salt showing fluorescent living cell and non-fluorescent dead cells. Dead cells show shrunken cytoplasm and condensed nuclei. Right panel: Simplified scheme of cell death pathway in suspension cultures cells (see text).