Plant Programmed Cell Death, Ethylene and Flower Senescence

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Abstract

Programmed cell death (PCD) applies to cell death that is part of the normal life of multicellular organisms. 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. Most cases of PCD described in animal systems take the form of apoptosis, a cell death process characterised by specific features such as 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. A specific class of cell death- associated cystein proteases (caspases) has been identified. Generally, apoptotic cell death involves a sequence of caspase activation events in which initiator caspases activate down-stream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype.

The occurrence of hallmarks of animal apoptosis was studied in tomato cells treated with the anticancer drug and inducer of apoptosis, camptothecin (cpt). It was shown that cpt-induced cell death is accompanied by nuclear condensation, the appearance of TUNEL-positive nuclei, DNA laddering and formation of DNA-containing (apoptotic) bodies and was greatly inhibited by inhibitors of animal caspases. Together the results indicate that cpt induced a cell death pathway with similarities to caspase-mediated (apoptotic) cell death in animal systems. We used cpt-treated cells to study the possible involvement of ethylene in cell death. Treatment of the cells with relatively high concentrations of ethylene did not have any effect on viability of the cells. However, when ethylene was applied in combination with cpt, a significant increase in cell death was observed as compared to cpt treatment alone. Experiments with inhibitors of ethylene production or ethylene action showed that ethylene is an essential factor mediating cpt-induced cell death.

Flower senescence is accompanied by rapid death of large numbers of cells. In situ DNA degradation was studied in gypsophila petals using TUNEL. We showed that TUNEL positive nuclei appear well before the onset of the increase in ethylene production and visible signs of senescence. The role of PCD in flower senescence is discussed.

INTRODUCTION

According to the general definition of programmed cell death (PCD), it applies to cell death that is part of the normal life of multicellular organisms. 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, cell death resulting from environmental stress (e.g. infection by pathogenic organisms, wounding and low concentrations of toxins) often occurs through controlled disassembly of the cell and can therefore also be termed PCD. Most cases of PCD described in animal systems take the form of apoptosis, a cell death process characterised by specific features such as 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). Inappropriate apoptosis has been implicated in many human diseases, including a number of birth defects, ischemic vascular diseases (e.g. heart attack and stroke), neurodegenerative diseases (e.g. Alzheimer's and Parkinson's diseases), autoimmune diseases (e.g. reumatoide arthritis), AIDS and diabetes mellitus type I.

There are numerous examples of cell death during plant development that conform to the general definition of PCD such as cell death during xylogenesis, aerenchyma formation, plant reproductive processes, leaf and petal senescence, and endosperm development. Furthermore, cell death in response to pathogen attack, and in response to a variety of abiotic factors such as ozone and UV radiation also fall within the definition of PCD. A number of morphological similarities were found between animal cells undergoing apoptosis and dying plant cells, including condensation and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation and the formation of DNA-containing (apoptotic-like) bodies (de Jong et al., 2000; Wang et al., 1996).

This paper describes studies on apoptotic cell death in tomato cells and senescing flower petals.

MATERIALS AND METHODS

For details on methods see de Jong et al. 2000; 2002.

RESULTS AND DISCUSSION

Apoptotic Cell Death in Tomato Cells

Camptothecin (cpt) is an anticancer drug that induces apoptosis in different types of animal cells. In tomato suspension cells cpt (5 µM) caused cell death within 10-12 h of treatment (Fig. 1). Cell death was accompanied by nuclear condensation, the appearance of TUNEL-positive nuclei (Fig. 2) and DNA laddering (Fig. 3).

Although not in all the cases of plant cell death such typical apoptotic hallmarks have been established, the observations do suggest the existence of an apoptotic machinery in plant cells and its involvement in PCD. In mammalian cells, these typical hall marks of apoptosis are ascribed to caspase-mediated processing of specific target molecules such as the activation of a caspase activated DNase (CAD) by cleavage of the CAD inhibitory sub-unit (ICAD) (Enari et al., 1998). It is tempting to speculate that comparable proteolytic events cause the apoptotic phenotype observed in dying plant cells.

Involvement of Caspase-like Proteases in Plant Cell Death

Caspases belong to a class of specific cystein proteases that show a high degree of specificity with an absolute requirement for cleavage adjacent an aspartic acid residue and a recognition sequence of at least four amino acids N-terminal to this cleavage site. Determination of the tertiary structure of human caspases by crystallography has revealed a unique topology designated as the "caspase-hemoglobinase fold" (Grütter, 2000; Koonin and Aravind, 2002).

Generally, apoptotic cell death involves a sequence of caspase activation events in which initiator caspases activate down-stream executioner caspases that process a variety of target proteins eventually leading to the apoptotic phenotype. Caspases can selectively be inhibited by small peptides, mimicking the substrate recognition site, carrying electrophiles such as aldehydes, nitriles or ketones at their C terminal that react with the active site cystein. In addition, macromolecular proteins such as the cowpox serine proteases inhibitor crmA, members of the inhibitor of apoptosis protein (IAP) family and the broad spectrum caspase inhibitor p35 from baculovirus are able to specifically block caspase activity (Ekert et al., 1999).

Cpt-induced cell death in tomato suspension cells was markedly inhibited by

inhibitors of human caspases. Cell death was effectively inhibited by the human caspase-1 (Ac-YVAD-CHO, Ac-YVAD-CMK) and caspase-3 (Ac-DEVD-CHO) inhibitors as well as by the broad-range caspase inhibitor Z-asp-CH_2- DCB (Fig. 4). Small caspase-unrelated peptides with similar reactive groups that served as negative controls (methoxysuccinyl-Ala-Ala-Pro-Val-CMK, H-Phe-CMK, leupeptin) did not affect cell death in this system. Generally, the caspase inhibitors showed 50% inhibition of Cpt-induced cell death at concentrations between 1 and 10 nM. These data suggest that caspase-like proteases are involved in cell death in plants.

The inhibition of pathogen-induced cell death through heterologous expression of caspase inhibiting macromolecules such as baculovirus Op-IAP and p35 has been reported (Dickman et al., 2001; Hansen, 2000, Del Pozo, and Lam, 2003). These studies also suggest that cell death-associated caspase-like activity is present in plant cells.

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Although the caspases are highly conserved among animal cell types no true homologs have been identified in plants. Recently, iterative homology searches have revealed two new groups of caspase-related cystein proteases designated paracaspases (in e.g. humans and *C. elegans*) and metacaspases (in e.g. fungi and plants) (Koonin and Aravind, 2002; Uren et al., 2000). The (fully sequenced) genome of *Arabidopsis thaliana* contains 9 metacaspase-like sequences. Modelling of the three-dimensional protein structure of metacaspases indicates significant tertiary structure homology to animal caspases (the caspase-hemoglobinase fold) (Uren et al., 2000). Mutational studies in *Trypanosoma brucei* first suggested that metacaspases indeed function as cystein proteinases (Szallies et al., 2002). Recently, it was shown that the only metacaspase present in *Saccharomyces cerevisiae* displays caspase-like proteolytic activity that is activated when yeast is stimulated by hydrogen peroxide to undergo apoptosis (Madeo et al., 2002). The functions of the different plant metacaspases are still unknown. Considering their structural and evolutionary relationship to caspases some of them may exhibit caspase-like activity and may play a role in plant cell death. Alternatively, other, caspase-unrelated, proteases in plants may recognise caspase substrates and may be responsible for apoptotic phenotype in dying plant cells (Woltering et al., 2002).

Ethylene Stimulates Plant Cell Death

In many plant species, ethylene is one of the endogenous signals mediating leaf and flower senescence, a process considered a form of programmed cell death. Cell death induced by plant pathogens is also considered “programmed” and the role of ethylene in pathogen-induced cell death has been evaluated in ethylene insensitive NR-tomatoes. Following infection of these mutants, greatly reduced cell death was observed, indicating ethylene involvement in programmed cell death (Lund et al., 1998). Plant defence reactions can also be elicited by abiotic elicitors, such as ozone (O_3). Ozone forms reactive oxygen species (ROS) in the apoplast and causes the plant cell itself to produce ROS in an oxidative burst. In sensitive plants this leads to the formation of HR-like lesions. Ozone exposure upregulates ethylene biosynthesis and, if ethylene biosynthesis or perception is blocked, the incidence of lesions is reduced. It was therefore suggested that ROS and ethylene together are involved in the induction of cell death in O_3 exposed plants (Overmyer et al., 2000).

Also in a number of other systems ethylene was closely associated with increased cell death.

- Cell death during aerenchima formation shows features of apoptosis and was found to be dependent on ethylene (Drew et al., 2000; Gunawardena et al., 2001).
- Cell death during maize endosperm development is accompanied by occurrence of DNA ladders and was associated with increases in ethylene production. Cell death could be hastened by treatment with ethylene and blocked by ethylene inhibitors (Young and

Gallie, 2000).
- Death of epidermal cells at the site of adventitious root emergence in submerged rice seedlings was found to precede root emergence and to depend on ethylene (Mergemann and Sauter, 2000).
- Chilling-induced cell death in melon was significantly less in transgenic melons (antisense ACC oxidase) producing only trace amounts of ethylene (Ben-Amor et al., 1999).
- Cell death induced by the mycotoxin fumonisin B1 involves ethylene signalling in Arabidopsis and tomato (Asai et al., 2000; Moore et al., 1999).
- Lethality at 28 °C in interspecific hybrids *of Nicotiana suaveolens* × *N. tabaccum* is caused by overproduction of ethylene (Yamada and Marubashi, 2003)

As described above, the tomato suspension culture represents a system where apoptotic-like cell death can be induced by chemicals such as camptothecin. We used cpt-treated cells to study the possible involvement of ethylene in cell death. Cpt at concentrations that induced cell death did not stimulate ethylene production. This was not due to inability of the cells to produce ethylene because treatment with e.g. xylanase did induce elevated amounts of ethylene (Woltering et al., 1999). This indicates that the cell death-inducing activity of cpt is not related to increased ethylene biosynthesis.

Treatment of the cells with relatively high concentrations of ethylene did not have any effect on viability of the cells (Fig. 5). Concentrations up to 100 ppm in the headspace, giving about 12 ppm in the liquid phase, were applied during 24 h. In response to this treatment, a significant increase in ACC oxidase activity was measured, indicating that the cells were responsive to the gas (data not shown). Addition of ACC to the nutrient medium, despite its stimulating effect on ethylene production, also did not induce cell death. This shows that ethylene is not a primary trigger of cell death in these cells. However, when ethylene or ACC were applied to cpt-treated cells, a significant increase in cell death was observed as compared to cpt treatment alone (Fig. 5). Experiments with inhibitors of ethylene production (aminoethoxy vinylglycine [AVG]) or ethylene action (silver thiosulphate [STS]) revealed that ethylene is an essential factor mediating cpt-induced cell death (Fig. 5).

The stimulating effect of ethylene on cell death was related to an increased production of cpt-induced hydrogen peroxide. Although ethylene alone did not induce elevated levels of hydrogen peroxide, it markedly stimulated cpt-induced hydrogen peroxide. AVG and STS blocked the effect of cpt on hydrogen peroxide production. These results show that ethylene increased ROS levels and cell death in cpt-treated cells (De Jong et al., 2002).

Programmed Cell Death during Flower Senescence

Senescence of plant parts is generally viewed as a programmed series of events ultimately leading to death of the respective plant part. Senescence in leaves either as a part of normal development or induced by specific triggers such as adverse environmental conditions is generally accompanied by loss of photosynthetic activity, chlorophyll breakdown, degradations of protein, lipids and nucleic acids resulting in the release of nitrogen, phosphorus carbon and other minerals that can be mobilised from the senescing cells. Senescence is an active process requiring gene transcription and translation and generally takes place over a time span of several days. During the process, the integrity of the nuclear DNA must be maintained to drive the process. It has been suggested that the senescing cell actually takes the necessary steps to ensure the maximum life span by degrading and storing in the vacuole the potentially toxic chlorophyll, and by activating genes encoding enzymes involved in removal of ROS (Buchanan-Wollaston and Morris, 2000; Matille et al., 1989).

The process of petal senescence shows similarity to leaf senescence (e.g. extensive breakdown of proteins, DNA and RNA) and mobilisation of nutrients from senescing petals to other parts of the same flower (e.g. the developing ovary) or to other flowers on the same branch has also been observed (e.g. van der Meulen-Muisers, 2000). However,

petal senescence basically performs a different function than leaf senescence. Changes in petal colour and shape and senescence primarily serve as a signal to pollinators. Petal senescence often can be very rapid and although remobilization may take place during senescence, it may not be the most important event. In many flowers petals abscise well before visible senescence and apparently no nutrient mobilisation has taken place (e,g. following pollination). This indicates that petal senescence serves to rapidly discard of an energy-demanding structure rather than to orderly breakdown and remobilize nutrients that are needed elsewhere in the plant.

DNA degradation is a general feature of senescing petals (e.g. van der Kop et al., 2003; Xu and Hanson, 2000); specific DNA degradation yielding a laddering pattern has so far only been observed in a limited number of flowers. In senescing pea, petunia, freesia and alstroemeria petals DNA laddering was detected at a relatively late stage of senescence (Orzaez and Granell, 1997; Xu and Hanson, 2000, Yamada et al. 2001; Wagstaff et al., 2003). Nuclear fragmentation detected by flow cytometry was observed during senescence of freesia flowers (Yamada et al., 2001). This indicates that apoptotic-like cell death occurs during petal senescence.

We investigated the occurrence of DNA degradation during senescence of gypsophila flowers. These flowers produce an ethylene peak just before visible wilting. During flower opening and senescence we observed an increasing amount of TUNEL positive cells, already clearly increased in fully opened flowers that did not show signs of visible wilting (Fig. 6). The TUNEL (TdT mediated dUTP-biotin nick end labeling) method facilitates the in situ labeling of DNA breaks in individual nuclei and relies on the specific binding of terminal deoxynucleotidyl transferease (TdT) to exposed 3'-OH ends of DNA followed by the synthesis of a labeled polydeoxynucleotide molecule. The TUNEL methods preferably labels double strand breaks thought to result from the action of e.g. endo-DNases involved in animal apoptosis (Gravieli et al., 1992).

The early occurrence of TUNEL-positive nuclei is surprising and indicates that DNA is at least partially being degraded and such cells are no longer expected to actively direct e.g. nutrient remobilization processes. Comparable observations on early degenerative changes in petal cells were reported by e.g. van der Kop et al. (2003) and Wagstaff et al. (2003). These authors showed degradation of petal mesophyll cells at early stages of flower senescence in Iris and Alstroemeria. Together these observations support the idea that cell death is an integral part of the developmental program of the petal. Unlike the situation in senescing leaves, where cells stay alive and metabolically active till late in senescence, part of the petal cell die in an early developmental stage. Apparently these cells or their activity is longer necessary to support the petal's primary function i.e. to attract pollinators.

CONCLUSION

Evidence has accumulated that a cell death pathway with similarities to animal apoptosis exists in plants (Danon et al., 2000; Hoeberichts and Woltering, 2003; Lam et al., 1999). Dying plant cells may show typical apoptotic hallmarks indicating that a similar death machinery may be responsible. The striking effect of synthetic and macromolecular caspase inhibitors on cell death in plants and the existence of caspase-related proteases suggest involvement of caspase-like activity in plant cell death (Woltering et al., 2002). The recent findings that ROS play an important role in animal as well as plant cell death further support the view of functional conservation of cell death pathways between animals and plants.

Ethylene was found to be an important mediator of plant cell death although its mode of action is not known. We found that ethylene markedly stimulated cell death and the amount of hydrogen peroxide produced in response to cpt treatment. The general proposition can be made that ethylene apparently increased the oxidative stress invoked by the primary stress (cpt). If we generalise this view, it could explain the stimulatory role of ethylene in symptom expression a number of cases where oxidative stress is believed to induce cell death such as in the effects of temperature extremes, ozone, wounding and

pathogen challenge. The mechanism of ethylene-enhanced oxidative stress is not known and may vary from case to case. Possible modes of action of ethylene in enhanced ROS levels may be the stimulation of NADPH oxidase activating proteins, the down regulation of ROS scavenging pathways or interference with mitochondrial functioning.

Unlike the situation in senescing leaves, where cells stay metabolically active till late in senescence, apoptotic DNA degradation (as detected by TUNEL) in senescing gypsophila petals occurs very early. This indicates programmed cell death is an integral part of the developmental process of the petal to discard of cells that are longer necessary to support the petal's primary function i.e. to attract pollinators.

ACKNOWLEDGEMENT

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Figures

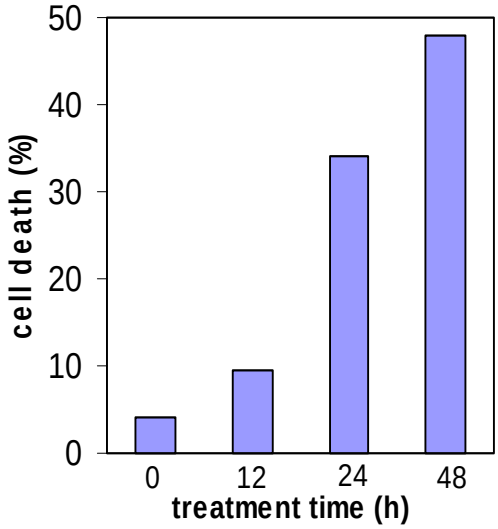

Fig. 1. Induction of cell death in cpt-treated cells (5 µM), as measured by FDA staining.

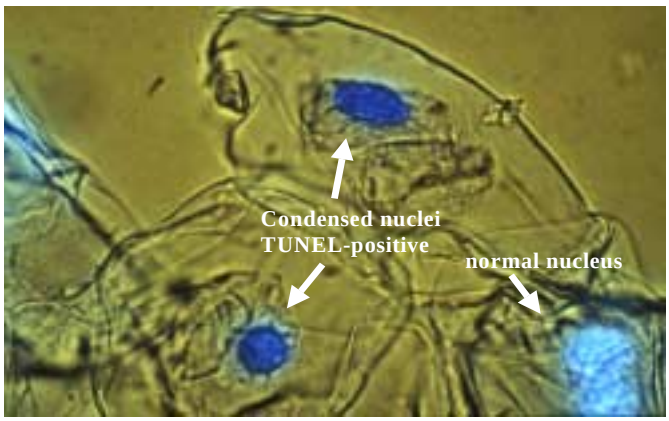

Fig. 2. DNA fragmentation in nuclei of suspension-cultured tomato cells undergoing programmed cell death (PCD), visualised by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) in combination with Hoechst 33258 staining. Using UV illumination, non-apoptotic (TUNEL-negative) nuclei show as bright blue fluorescent spheres, whereas nuclei undergoing PCD (TUNEL-positive) can be recognised by the dark TUNEL staining that interferes with fluorescence.

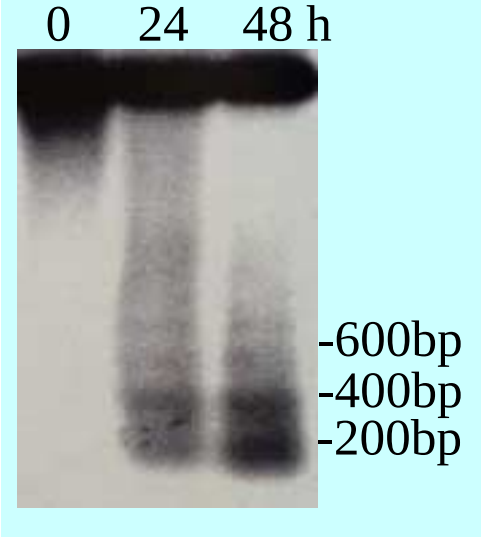

Fig. 3. DNA laddering in cells treated for different periods of time with 5 µM cpt.

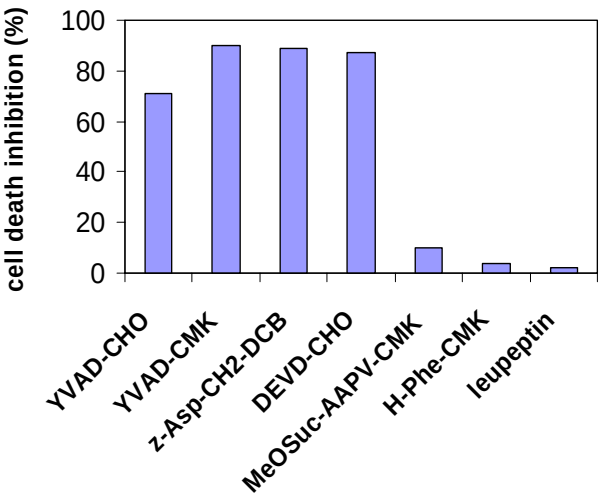

Fig. 4. Effect of caspase inhibitors (YVAD-CHO, YVAD-CMK, z-Asp-CH2-DCB, DEVD-CHO) and caspase unrelated peptide inhibitors (MeOSuc-AAPV-CMK, H-Phe-CMK, leupeptin) on cell death inhibition in cpt-treated tomato suspension cells. Peptide inhibitors at 100 nM were applied simultaneously with 5 µM cpt; cell death was determined by FDA staining 24 h after start of the treatment.

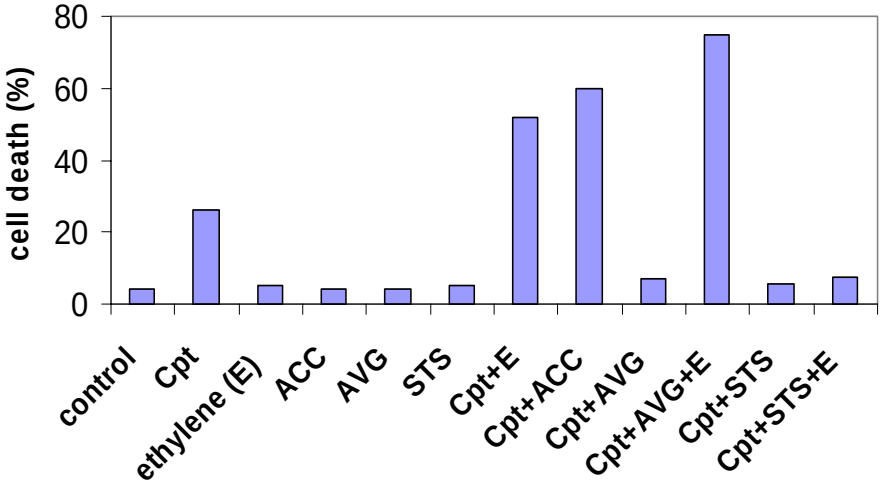

Fig. 5. Effect of various chemicals on cell death in tomato suspension cells. Chemicals were applied at time zero; cell death was determined after 24 h by FDA staining. Cpt, 5 µM; Ethylene, 100 ppm in gas phase; ACC, 10 µM; AVG, 10 µM; STS, 20 µM.

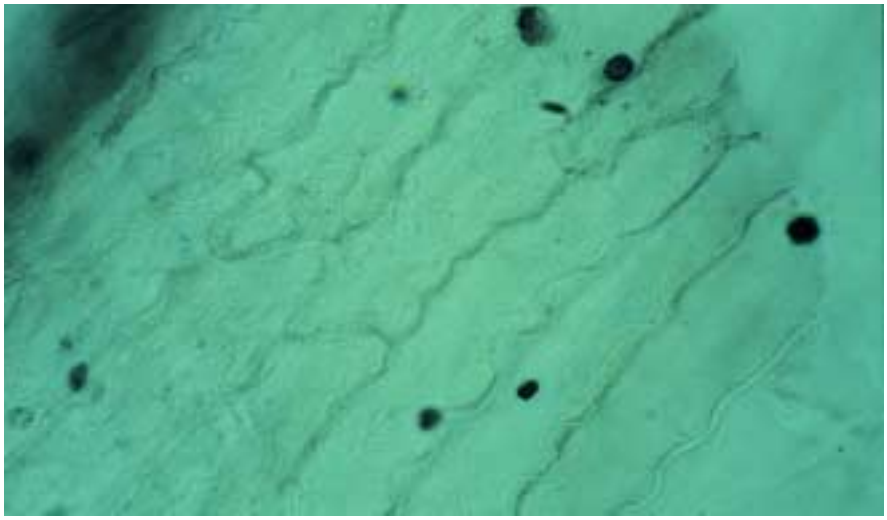

Fig. 6. TUNEL positive nuclei in cells of upper epidermis of petals of fully open *Gypsophila* flowers that show no visible signs of senescence and have not yet started increased ethylene production.