

Evidence for Programmed Cell Death during Leaf Senescence in Plants

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Cell death caused by senescence of leaves has been thought to be a type of programmed cell death (PCD or apoptosis) for many years, however, no studies at the nuclear level associated with PCD have been reported. In this study, leaf tissue from five different plant species, *Philodendron hastatum*, *Epipremnum aureum*, *Bauhinia purpurea*, *Delonix regia*, and *Butea monosperma* was used to detect the evidence of the PCD. Here, we report the detection of PCD in senescent leaf tissue. DNA ladders, resulting from the cleavage of nuclear DNA into oligonucleosomal fragments in apoptotic cells, were detected by gel electrophoresis and southern hybridization only in senescent (but not in non-senescent) leaves in all five plant species. DNA fragmentation and nuclear DNA condensation were further confirmed by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end in situ labeling (TUNEL) method. Fluorescence was clearly detected in the nuclei of mesophyll cells in senescent leaves. By contrast, there was no TUNEL staining in green healthy leaves. Our results provide direct evidence to support the notion that natural senescence of the leaves is indeed an apoptotic process during plant development.

Key word: Apoptotic cell — DNA fragmentation — Leaf senescence — Programmed cell death — TUNEL.

Programmed cell death (PCD or apoptosis), a phenomenon of cell death followed in a programmed manner, has been intensively described in animals during the past few years (Hengartner and Horvitz 1994, Gerschenson and Totello 1992). It has been thought that PCD may be regulated by mechanisms conserved among eukaryotes (Vaux et al. 1994, Ameisen 1996). In plants, many different pathogen-triggered defense responses (for example, hypersensitive reactions) as well as environmental stress responses resulting in cell death have been proven to follow a death pattern similar to apoptosis in animals by producing the so-called hallmarks of apoptosis including DNA ladders and apoptosis bodies (Greenberg et al. 1994, Dietrich et al. 1994, Vaux and Strasser 1996, Mittler et al. 1995, Wang, H. et al. 1996, Ryerson and Heath 1996, Jones and Dangel 1996, Katsuhara 1997, Pennell and Lamb 1997). Many plant developmental processes that cause cell death, such as senescence of the carpel and petal, xylogenesis, aleurone

deletion, the death of the root cap cells, and somatic embryogenesis, have also been shown to proceed as PCD (Orzaez and Granell 1997a, b, Mittler and Lam 1995, Fukuda 1996, 1997, Wang, M. et al. 1996, Wang, H. et al. 1996, McCabe et al. 1997, Pennell and Lamb 1997).

In plants, senescence of the leaves has also been thought to be a type of PCD for many years (Noodén and Leopold 1978, Gan and Amasino 1997). The mechanism which controls this process must be genetically regulated by endogenous factors such as age and the balance of phytohormones, as well as regulated by environmental signals such as drought and insufficient nutrient supply. At the cellular level, senescence is always accompanied by dehydration of the leaf tissue, increased activity of RNases and proteinases, and diminution of the chloroplasts, resulting in the yellowish color of the leaves (Thomson and Platt-Aloia 1987, Taylor et al. 1993, Bate et al. 1990, Bleeker and Patterson 1997, Gan and Amasino 1997). Although the degradation of protein, lipids, and RNA, and the exportation of nutrients have been observed during the process of senescence, the question of whether cell death during leaf senescence is also associated with other kinds of PCD at the molecular and genetic levels has never been answered. Recently, some studies using the digestion of nuclear DNA into oligonucleosomal fragments as a hallmark have shown some developmental process such as xylogenesis and somatic embryogenesis are PCD (Fukuda 1996, 1997, McCabe et al. 1997). In this paper, we report the detection of DNA fragmentation in naturally senescent leaves from various plant species. Our data prove that senescence of the leaves must share some similar pathways observed in other types of PCD in both plants and animals.

Materials and Methods

Plant material—Leaves were obtained from the following five different plant species: *Delonix regia*, *Bauhinia purpurea*, *Butea monosperma*, *Philodendron hastatum*, and *Epipremnum aureum*. The first three plant species are woody plants, whereas the last two are perennial climbing evergreen plants in the *Araceae* family. Leaves which showed natural dehydration and a yellowish color throughout before being detached from the plants served as senescent leaves. All plants were originally grown on the campus of National Chung Hsing University without any treatments.

DNA extraction and analysis—Genomic DNA was isolated from leaf tissues using a CTAB method (Yang et al. 1995). Leaf tissues were frozen immediately after being collected directly from the plants. About 1.5 g of frozen leaf samples were homogenized in 25 ml extraction buffer (0.35 M Sorbitol, 0.1 M Tris-base, 0.005 M EDTA, adjusted pH to 7.5 with HCl, with 0.02 M

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NaBisulfite added just before use). Samples were centrifuged at full speed for 20 min in a TJ-6 table-top centrifuge and resuspended in 5 ml extraction buffer plus 5 ml nuclear lysis buffer (0.2 M Tris-base, 0.05 M EDTA, 2 M NaCl and 2% [w/v] hexadecyl triethyl ammonium bromide [CTAB; Sigma] and 2 ml 5% sarkosyl, followed by incubation at 65°C for 5 min. Equal volumes (12 ml) chloroform/isoamyl alcohol (24/1) were added and the tubes inverted 30 to 40 times and the samples centrifuged at full speed for 5 min in a TJ-6 table-top centrifuge. DNA was precipitated by adding an equal volume of cold isopropanol to the aqueous supernatant at -20°C overnight. The samples were centrifuged at full speed for 15 min in TJ-6 table-top centrifuge. The pellet was vacuum-dried and resuspended in 1 × Tris-EDTA buffer (0.15 ml TE per g fresh weight of starting material) at 65°C for two h, followed by centrifugation for 10 min to remove insoluble material. This procedure provided about 200 µg DNA/1 g starting material from leaves. About 2 µg genomic DNA from each sample was treated with RNase before running on a 1.5% agarose gel at constant 50 V. The DNA was stained with 0.5 µg ml⁻¹ ethidium bromide and visualized under UV illumination.

For southern hybridization, DNA in 1.5% agarose gel was transferred to a Hybond N membrane (Amersham International, U.K.) following standard protocols, and was stored or subjected to hybridization immediately after being UV-cross linked. Total genomic DNA from green tissue was digested with *EcoRI* and radiolabeled with ³²P to serve as a probe. Hybridization procedures were performed as described below. The membranes were prehybridized for 30 min and hybridized with the ³²P-labeled DNA probe overnight in the same solution (0.25 M Na₂HPO₄, pH 7.2, and 7% SDS), and then washed twice each in solution 1 (20 mM Na₂HPO₄, pH 7.2, and 5% SDS) and solution 2 (20 mM Na₂HPO₄, pH 7.2, and 1% SDS) for 30 min per wash. The membranes were then air-dried, covered with plastic wrap, and autoradiographed.

TUNEL assay and microscopy analysis—For the TUNEL (TdT-mediated dUTP nick end labeling) assay, leaf samples were cut and fixed in 4% paraformaldehyde with a 100 mM phosphate buffer pH 7.2 overnight. The samples were dehydrated through a graded series of ethanol and embedded in paraffin. Sections were cut at 10-micron thickness and mounted on slides. After dewaxing and rehydration, sections were incubated with proteinase K for 15 min at 37°C and rinsed twice with PBS. After drying the area around the sample, the TUNEL reaction mixture (In situ Cell Death Detection Kit, AP, Boehringer Mannheim, Germany) was added to the sample and incubated at 37°C for 60 min. After rinsing 3 times with PBS, samples were examined at 510 to 560 nm as yellow to greenish fluorescence and photographed with a Nikon fluorescence microscope. For nuclei DAPI (4',6-diamidino-2-phenylindole) staining, sections were incubated with 0.001% (w/v) DAPI after dewaxing and rehydration. The DNA were observed with blue fluorescence after excitation with UV light and photographed with a Nikon fluorescence microscope.

Results

Detection of DNA ladders increase during leaf senescence—During the early stage of PCD, specific endonucleases attack nuclear DNA in the internucleosomal linker regions between nucleosomal cores, resulting the production of double-stranded, low molecular weight oligonucleosomal DNA fragments about 180 to 200 bp in size (Cohen 1993). To test if leaf senescence is correlated to PCD,

the detection of DNA laddering in the senescent leaf is necessary. Leaves at different developmental stages, from green, yellow, yellow/brown, brown to completely dry, were collected from *Bauhinia purpurea* plants as shown in Fig. 1A. The results indicate that DNA was detected from non-senescent green leaves at high molecular weight without any DNA laddering (Fig. 1B, lane 1). Low molecular weight DNA began to be detected in yellow leaves (Fig. 1B, lane 2); however, no clear DNA fragmentation can be visualized in agarose gel in this stage. The ladders of DNA fragments which differ by less than 200 bp were clearly detected in the yellow/brown leaf (Fig. 1B, lane 3), and the detection of DNA laddering increased significantly in the senescent brown leaf, as shown in Fig. 1B, lane 4. In the dried leaf, the DNA became a smear and the laddering was reduced to become unvisualized in the agarose gel (Fig. 1B, lane 5), suggesting a random breakdown of nuclear DNA in this stage. The result indicates that DNA fragmentation is accompanied by the diminution of the chloroplast and the dehydration of the leaf during senescence. Our result reveals that the degree of yellowness in the leaf tissues corresponds to the degree of DNA fragmentation. The cells gradually died when the leaf changes from green to yellow to brown. The fact that the DNA was smeared in dry leaf indicates most of the cells in this leaf tissue were dead, and the DNA was completely degraded rather than undergoing internucleosomal fragmentation.

Detection of DNA ladders in the senescent leaf in five different plant species—To examine whether DNA fragmentation during leaf senescence is a common phenomenon in other plant species, DNA from green and yellow to brown leaf tissues of four other different plant species (Fig. 2), *Delonix regia*, *Butea monosperma*, *Philodendron hastatum*, and *Epipremnum aureum*, was isolated and analyzed. As shown in Fig. 3A, ladders of DNA fragments similar to those observed in *Bauhinia purpurea*, were detected in senescent yellow/brown leaves from all four plants tested. As controls in Fig. 3A, DNA was detected at high molecular weight without any DNA laddering in green, nonsenescent leaves. The results show that the DNA laddering also increases when the leaf changes to browner color, and are very consistent among five different plant species. The DNA laddering observed from agarose gel were confirmed by southern analysis as shown in Fig. 3B. The DNA laddering again were only detected in senescent leaves and were absent from nonsenescent leaves in all plant species tested. The result indicates that DNA ladder structures are present during senescence in different kinds of leaf of various shapes or sizes from various plant species. The result strongly indicates that the senescence of the leaf during plant development is a type of PCD.

DNA fragmentation is detected in the mesophyll layer within the senescent leaf—To further confirm the DNA fragmentation during senescence of the leaf, an in situ

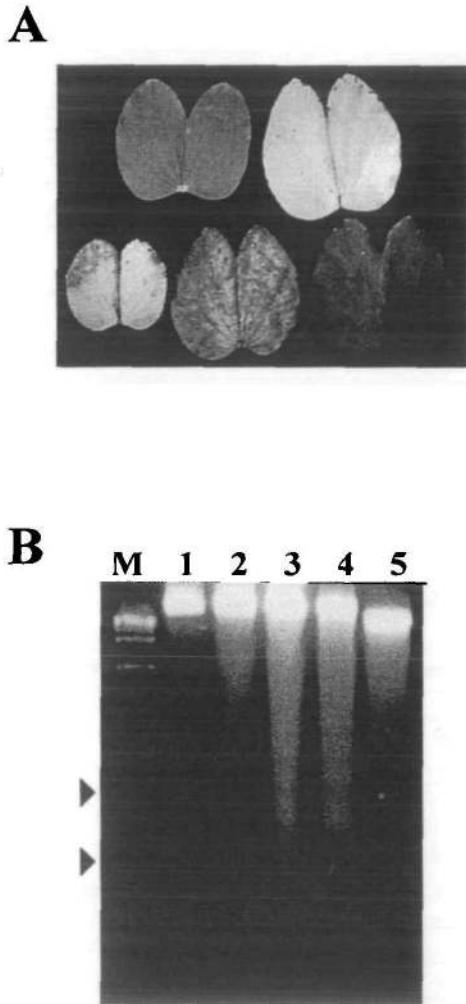


Fig. 1 Leaf and DNA samples from *Bauhinia purpurea*. (A) Top left, a green leaf; top right, a yellow leaf; bottom left, a yellow/brown (more yellow than brown); bottom middle, a brown leaf; and bottom right, a completely dried leaf. (B) DNA isolated from different leaf samples. M, 1 kb ladder marker; Lane 1, DNA isolated from green leaf; Lane 2, DNA isolated from yellow leaf; Lane 3, DNA isolated from yellow/brown leaf; Lane 4, DNA isolated from brown leaf; and Lane 5, DNA isolated from a completely dried leaf. 2 μ g uncut DNA treated with RNase was loaded into each lane and size fractionated in 1.5% (w/v) agarose gel. The arrowheads indicates 250- and 500-bp DNA markers.

detection assay, TUNEL assay was used. Cleavage of genomic DNA into oligonucleosomal fragments yields many single-strand breaks (nicks). These DNA breaks generate free 3'-OH termini which can be labeled with fluorescein-dUTP catalyzed by terminal deoxynucleotidyl transferase (TdT). The appearance and the strength of the fluorescence visualized can serve as an index of DNA breakage in particular cells which undergo PCD at the cellular level.

The fluorescence was clearly detected in the nuclei

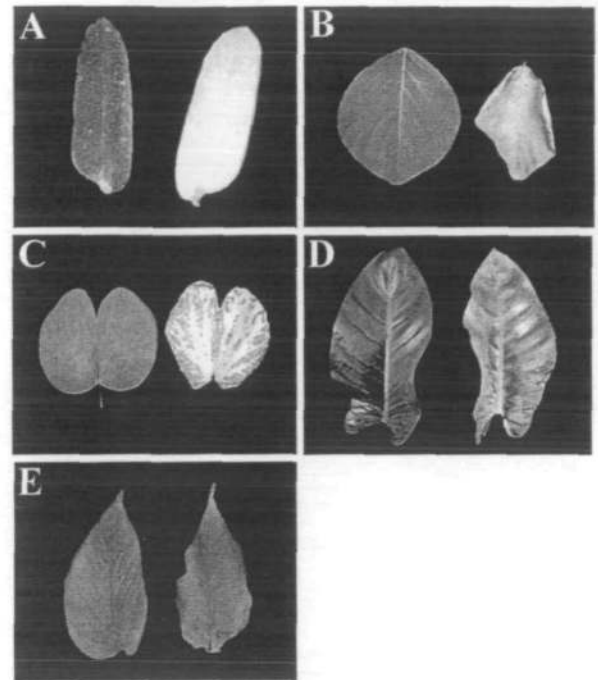


Fig. 2 Green and senescent leaf samples from different plant species. (A) Left, a green leaf; right, a senescent leaf of *Delonix regia*. (B) Left, a green leaf; right, a senescent leaf of *Butea monosperma*. (C) Left, a green leaf; right, a senescent leaf of *Bauhinia purpurea*. (D) Left, a green leaf; right, a senescent leaf of *Philodendron hastatum*. (E) Left, a green leaf; right, a senescent leaf of *Epipremnum aureum*.

of cells in the senescent leaves of plants tested, as shown in Fig. 4E, H, K, and N. The nuclei strongly labeled by TUNEL in Fig. 4F, I, L, and O indicate the DNA breakage associated with PCD is taking place in these cells during this stage of detection. By contrast, there was no TUNEL staining in the non-senescent green healthy leaf, as shown in Fig. 4D, G, J, and M, indicating the nuclei of the cells in the green leaves remain intact. As controls, nuclei from both senescent and non-senescent leaves were clearly stained by DAPI staining, as shown in Fig. 4A, B, and C. The nuclei of cells in senescent leaves were obviously condensed and much smaller (Fig. 4B) than those of non-senescent green healthy leaves (Fig. 4A). Since the condensation of nuclei is an important morphological trait characteristic of apoptosis, our result in nuclei condensation in senescent leaves indicates that leaf senescence is a type of PCD. The result of double labelling with DAPI and TUNEL, as shown in Fig. 4C and F confirm the nuclei of cells in senescent leaves are the places strongly labeled by the TUNEL.

Discussion

Although the leaf senescence has been studied for many years, most of the studies have been on the break-

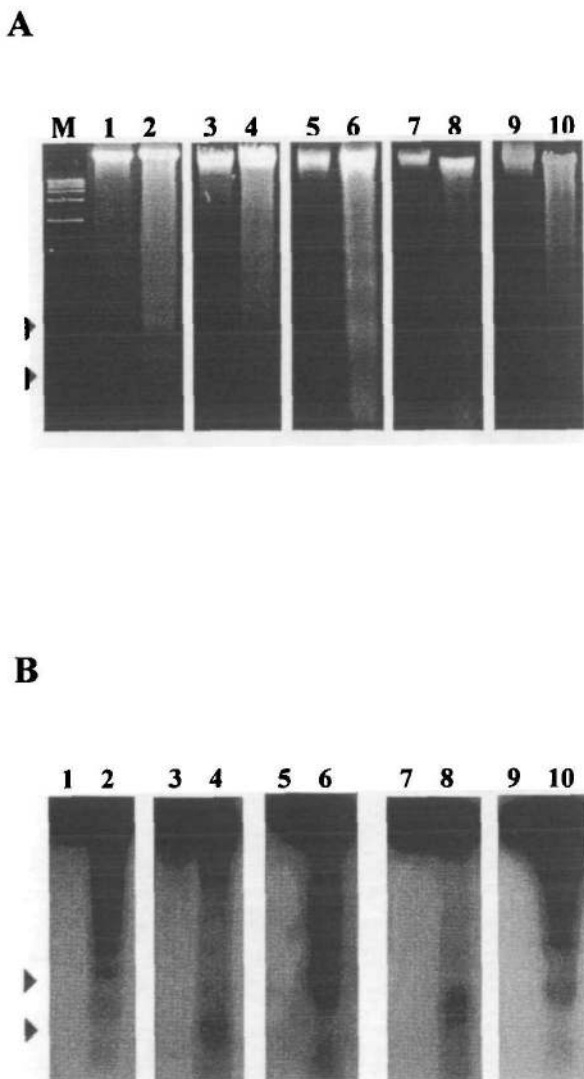


Fig. 3 Detection of DNA laddering in senescent leaves. (A) Analyzed by agarose gel electrophoresis. 2 µg uncut DNA was loaded into each lane and size fractionated in 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination. The arrowheads indicate 250- and 500-bp DNA markers. (B) Analyzed by southern hybridization. *Eco*RI digested total genomic DNA from the green tissue of each plant species was radiolabelled with ^{32}P to hybridize its own DNA blot. The membrane was exposed to X-ray film for 30 min. The arrowheads indicate 250- and 500-bp DNA markers. In both (A) and (B), M, 1 kb ladder marker; Lane 1, DNA isolated from green leaf; Lane 2, DNA isolated from senescent leaf of *Delonix regia*. Lane 3, DNA isolated from green leaf; Lane 4, DNA isolated from senescent leaf of *Butea monosperma*. Lane 5, DNA isolated from green leaf; Lane 6, DNA isolated from senescent leaf of *Bauhinia purpurea*. Lane 7, DNA isolated from green leaf; Lane 8, DNA isolated from senescent leaf of *Philodendron hastatum*. Lane 9, DNA isolated from green leaf; Lane 10, DNA isolated from senescent leaf of *Epipremnum aureum*. Each experiment was repeated at least 3 times in this research.

down of the chloroplast and the changing of proteinase activity (Thomson and Platt-Aloia 1987). Only a few reports were on the nuclear changes such as chromatin condensation and nuclear pycnosis (Bhattacharya et al. 1996). Interestingly, no reports on DNA cleavage associated with PCD at the nuclear level have been published. Our results indicate that at the cellular level, senescence of the leaves takes place in a similar manner as that observed in animals by producing condensed nuclei and yielding fragmented DNA in the dying cell. Since all five plant species tested here show the same result, this suggests PCD is a common process during natural leaf senescence in plants.

It has been proposed that the loss of chloroplast integrity occurs before the breakdown of the nucleus during the senescence of the leaf (Thomson and Platt-Aloia 1987). This notion is supported by the observation in this report that DNA fragmentation increased when the leaf became more yellow. Based on our results, in the beginning of the breakdown of the chloroplasts (the leaf starts to turn yellow), the cells should still be alive and the nuclei of the cells remain intact. Therefore, no clear DNA fragmentation can be visualized. Later on, the continuing loss of the integrity of the chloroplast (the leaf became yellow/brown) is obviously correlated to the cleavage of more nuclear DNA into internucleosomal fragmentation as observed in this study. This process leads to extensive cell death and finally turns the whole leaf brownish.

Leaf senescence no doubt is a complex process which is involved in programmed cell death in a large-scale manner. Although the PCD which occurs in leaf senescence causes the same result, i.e. DNA fragmentation, the mechanisms triggering PCD in this pathway may differ from other types of PCD observed in plants or animals. Genetically, several genes associated with senescence of the leaf have been either identified or isolated from various plant species (Oh et al. 1996, 1997, Drake et al. 1996, Smart et al. 1995, Taylor et al. 1993, Buchanan-Wollaston 1994). However, it is not surprising that most of these genes encode enzymes, such as proteinases and RNases and enzymes involved in mobilization, such as malate synthase and acetyl-CoA acyl transferase (Smart 1994, Weaver et al. 1997). Based on the appearance of DNA fragmentation in different stages of senescent leaves observed in this study, the signals which trigger natural leaf senescence should occur very early, before the leaf begins to show the sign of yellowing in which cells have already entered PCD. This is supported by the notion that leaf senescence is initiated by decline of photosynthesis to a certain level, which occurs much earlier than when leaves change color from green to yellow (Gan and Amasino 1997). Since most genes associated with senescence of the leaf are expressed during the latter phases of senescence, further identification of genes involved in the initial step of this process becomes necessary, and should lead to a deeper understanding of its mechanism.

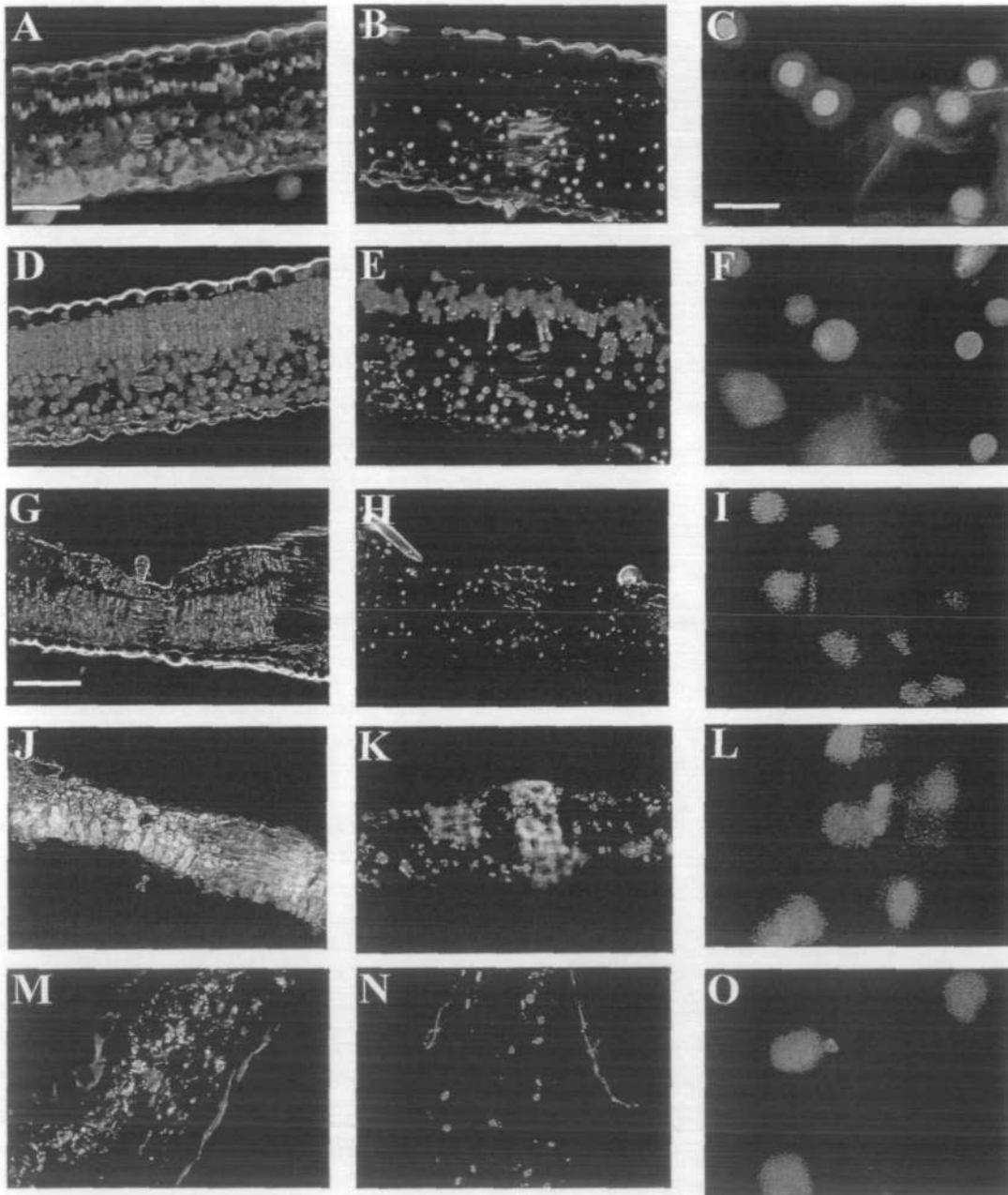


Fig. 4 Detection of DNA cleavage in senescent leaves by the TUNEL method. Leaflets (longitudinal section) from senescent and non-senescent leaf samples of different plant species were stained by TUNEL or DAPI. Each experiment was repeated at least 3 times in this research. (A) to (F), leaf samples from *Delonix regia*. (G) to (I), leaf samples from *Butea monosperma*. (J) to (L), leaf samples from *Bauhinia purpurea*. (M) to (O), leaf samples from *Epipremnum aureum*. (A) and (D) are the same section from a green leaf, whereas (B) and (E) are the same section from a senescent leaf. Both sections were double stained by using the DAPI and TUNEL methods. (A) and (B) were observed after excitation with UV light. Nuclei of mesophyll cells from both green and senescent leaves are indicated by bright blue fluorescence. Nuclei are clearly condensed and smaller in (B) than in (A). (D) and (E) were examined at 510 to 560 nm. Cells from the green leaf (D) containing nonfluorescent nuclei indicate no DNA cleavage. Cells from the senescent leaf (E) containing bright green fluorescent nuclei indicate DNA cleavage. (C) and (F) are enlargements from (B) and (E) respectively. Nuclei of cells from the senescent leaf are positively double-labeled by DAPI and TUNEL. (G), (J), and (M) are sections from a green leaf stained by using the TUNEL method. Cells from the green leaf containing nonfluorescent nuclei indicate no DNA cleavage. (H), (K), and (N) are sections from a senescent leaf stained by using the TUNEL method. (I), (L) and (O) are enlargements from (H), (K), and (N) respectively. DNA cleavage in nuclei of mesophyll cells proceeding PCD in the senescent leaf are indicated by bright green fluorescence. Bar in (A)=20 μm for (A), (B), (D), (E), (M), and (N); Bar in (G)=25 μm for (G), (H), (J), and (K); Bar in (C)=2 μm for (C), (F), (I), (L) and (O).

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