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Ultrastructure of Mesophyll Cells and Pigment Content in Senescing Leaves of Maize and Barley

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Abstract

Leaf senescence is a genetically regulated stage in the plant life cycle leading to death. Ultrastructural analysis of a particular region of the leaf and even of a particular mesophyll cell can give a clear picture of the time development of the process. In this study we found relations between changes in mesophyll cell ultrastructure and pigment concentration in every region of the leaf during leaf senescence in maize and barley. Our observations demonstrated that each mesophyll cell undergoes a similar senescence sequence of events: a) chromatin condensation, b) degradation of thylakoid membranes and an increase in the number of plastoglobules, c) damage to internal mitochondrial membrane and chloroplast destruction. Degradation of chloroplast structure is not fully correlated with changes in photosynthetic pigment content; chlorophyll and carotenoid content remained at a rather high level in the final stage of chloroplast destruction. We also compared the dynamics of leaf senescence between maize and barley. We showed that changes to the mesophyll cells do not occur at the same time in different parts of the leaf. The senescence damage begins at the base and moves to the top of the leaf. The dynamics of mesophyll cell senescence is different in leaves of both analyzed plant species; in the initial stages, the process was faster in barley whereas in the later stages the process occurred more quickly in maize. At the final stage, the oldest barley mesophyll cells were more damaged than maize cells of the same age.

Key words: Leaf senescence; Ultrastructure; *Hordeum vulgare*; *Zea mays*; Pigment content

Introduction

Leaf senescence is the genetically controlled last phase of leaf ontogenesis leading to death. This process is also considered a type of programmed cell death (PCD) (Greenberg 1996; Beers 1997; Nooden and others 1997; Pennel and Lamb 1997; Yen and Yang 1998; Simeonova and others 2000; Jones 2001).

Various aspects of leaf senescence, for example physiological, biochemical, molecular and anatomical have been studied (for reviews see Stoddart and Thomas 1982; Nooden 1988; Matile 1992; Nooden and Guiamet 1996; Buchanan-Wollaston 1997;

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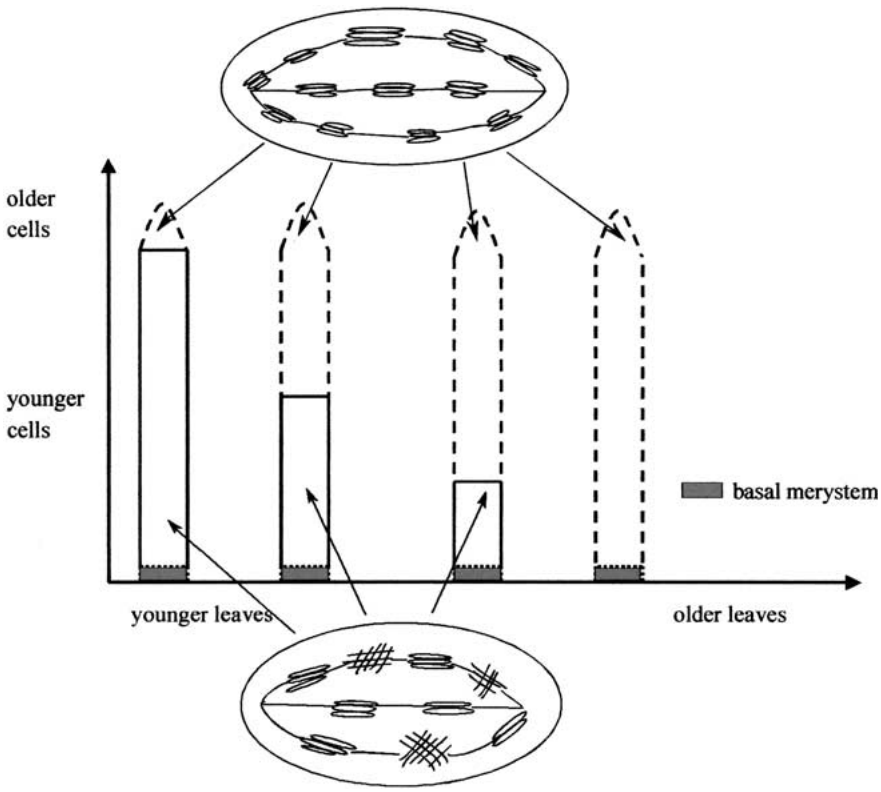

Figure 1. Different stages of chloroplast development in maize leaves of increasing age (based on Rascio and others 1980).

Nooden and others 1997; Jackowski 1998; Quirino and others 2000; Simeonova and Mostowska 2001).

Our previous results proved that internucleosomal nDNA fragmentation, specific for PCD, is one of the earliest symptoms of leaf senescence (Leśniewska and others 2000; Simeonova and others 2000). Also, chloroplast DNA digestion is considered as an early sign (Nii and others 1988; Sodmergen and others 1989; Inada and others 1998, 1999). Both processes precede ultrastructural changes such as chromatin condensation and chloroplast degradation (Nii and others 1988; Kuran 1993; Inada and others 1998, 1999; Simeonova and others 2000).

During leaf senescence ultrastructural changes to chloroplasts, such as swelling of thylakoids and an increased accumulation of plastoglobuli, occur (Barton 1966; Meier and Lichtenthaler 1982; Mostowska 1999). Information about ultrastructural changes to mitochondria during leaf senescence is limited. Changes in mitochondrial membrane potential and the release of cytochrome c from the mitochondria to the cytosol are also considered to be early signals of PCD in plants (Balk and others 1999; Sun and others 1999; Yu and others 2002; Simeonova, personal communication).

Other processes such as loss of chlorophyll, reduction of RNA, DNA contents, breakdown of proteins such as ribulose-1,5-bisphosphate carboxylase/oxygenase, and loss of photosynthetic capability follow ultrastructural changes (Jiang and others 1993; Kuran 1993; Smart and others 1995; Scheumann and others 1999).

It is known that leaf ontogenesis proceeds asynchronously within the same leaf blade. Therefore a full analysis of senescence requires a description of this process at different regions of the leaf. Ultrastructural analysis of mesophyll cells from each region of the leaf at different points in the senescence progression can provide the most valuable information on the senescence process.

Our studies concern two monocotyledon species: maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.). In both plant species, leaf differentiation proceeds from the apical region of the leaf blade to the base of the leaf (Rascio and others 1980) (Figure 1), as characteristic for monocotyledons, making these leaves appear to be a good model. We proved that leaf senescence proceeds in the same manner as leaf development: from the top of the leaf to the base. However, these two plant species, because of photosynthetic processes, belong to two different types of plants: C_4 and C_3, respectively and may therefore differ in the dynamics of leaf differentiation.

In this study we found correlations between changes in mesophyll cell ultrastructure (chromatin

condensation and chloroplast degradation in particular) and pigment concentration in every region of the leaf during subsequent steps of leaf senescence of maize and barley. Based on these results, we compared the dynamics of leaf senescence of these two plant species.

Materials and Methods

Plant Material and Growth Conditions

Seeds of *Zea mays* L. *var*. Oleńka and *Hordeum vulgare* L. *var* Rataj were soaked for 24 h in water, then placed on wet filter paper in darkness at room temperature. Germinated seeds were sown in soil in flower pots and transferred to a growth chamber. They were illuminated for 16 h per day with continuous fluorescent light (intensity 45 W/m^2). The temperature was 24°C in the light and 22°C in the darkness. Basal, middle and apical thirds of second leaves of 10- and 20-day old seedlings were analyzed. By 30 days, only basal regions of leaves were analyzed.

Electron Microscopy

Samples of each developmental stage were taken for electron microscopy. Samples about 1–4 mm^2 in area were cut from each part of the leaf. The material was fixed in 1.6% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 for 2 h, washed in buffer and placed in 1% OsO_4 at 4°C in 20 μM cacodylate buffer for about 12 h. The specimens, dehydrated in a graded acetone series, were embedded in a low viscosity epoxy resin (Spurr 1969) and cut on a Leica UCT ultramicrotome. Sections stained with uranyl acetate and lead citrate (Reynolds 1963) were examined with a JEM 1200EX electron microscope. All experiments were repeated three times.

Pigment Determination

To determine chlorophyll (Chl) a, b and carotenoid (Car) content, 0.5 g of fresh maize and barley leaves were taken from each part of the leaves and homogenized with 100% acetone chilled down to +4°C and then extracted with 25 ml of 80% acetone. The Chl and Car concentrations were measured spectrophotometrically using a Shimadzu UV-160A spectrophotometer at 663, 646 and 470 nm, respectively. Pigment concentration was calculated according to Lichtenthaler and Wellburn (1983). Chl and Car content were expressed in μg/g fresh weight.

Chl and Car content measurements were repeated 3–4 times, and mean values and standard deviations were calculated.

Results

Second leaves of 10- and 20-day-old maize (*Zea mays* L.) and barley (*Hordeum vulgare* L.) are fully developed and green from their base to the top. The basal region of the second leaves of 30-day-old seedlings of both plant species are yellowish and the other regions are completely withered.

Ultrastructure of Maize Mesophyll Cells

Mesophyll cells of the basal, middle and apical regions of the fully green second leaves of 10-day-old maize seedlings contain a large, central vacuole and dense cytoplasm adjacent to the plasma membrane (Figure 2, Panel 1). Chloroplasts in these cells are fully differentiated with 16 thylakoids per granum on average. Small plastoglobules are dispersed in chloroplasts of the middle and apical regions of the leaves only (Figure 2, Panel 2). Mesophyll cells of the basal, middle and apical regions of the leaves contain nuclei with dispersed chromatin (Figure 2, Panel 3), and also mitochondria with richly-invaginated internal membranes (Figure 2, Panel 4).

Mesophyll cell ultrastructure in older, 20-day-old maize seedlings does not differ significantly from that of the younger ones (Figure 2, Panels 1, 3, 4). Slight differences occur in the middle and apical regions of 20-day-old seedlings. Some mesophyll cells from these regions are slightly swollen compared to younger seedlings. The majority of nuclei have dispersed chromatin and only a few of them contain some regions of condensed chromatin. Most of the mitochondria have well-preserved membranes. The major difference can be observed in chloroplasts of the apical regions, which contain 20 thylakoids per granum on average and more plastoglobules than the younger cells (Figure 2, Panel 5). In apical regions of the leaves, starch grains are present in the stroma of mesophyll chloroplasts (Figure 2, Panel 5). The process of senescence has started in the apical regions of leaves of 20-day-old seedlings with initiation of chromatin condensation and an increase in the number of plastoglobules.

The ultrastructure of mesophyll cells of basal regions of 30-day-old maize seedlings has evidently

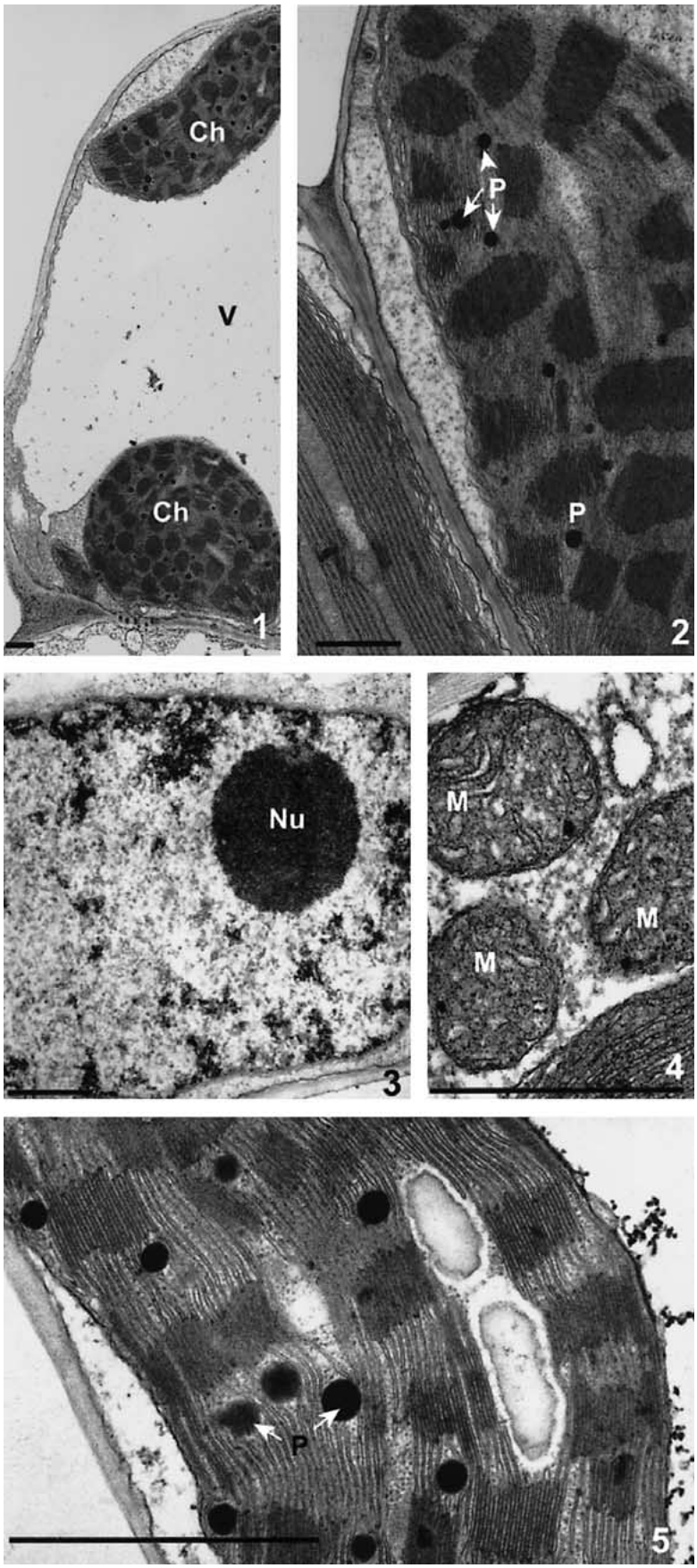

Figure 2. Panels 1–5. Ultrastructure of mesophyll cells of maize second leaves. **Panels 1–4.** Mesophyll cells from 10-day-old plants. **Panel 1.** Mesophyll cell with central vacuole and chloroplasts. **Panel 2.** Differentiated chloroplast with single plastoglobules from apical part. **Panel 3.** Nucleus with dispersed chromatin, from the basal region. **Panel 4.** Mitochondria with richly invaginated internal membranes. **Panel 5.** Differentiated chloroplast in mesophyll cell from apical part of leaf of 20-day-old plant. *Ch*, chloroplast; *G*, granum; *M*, mitochondrium; *Nu*, nucleolus; *P*, plastoglobules; *V*, vacuole; *Bar*, 1 μm.

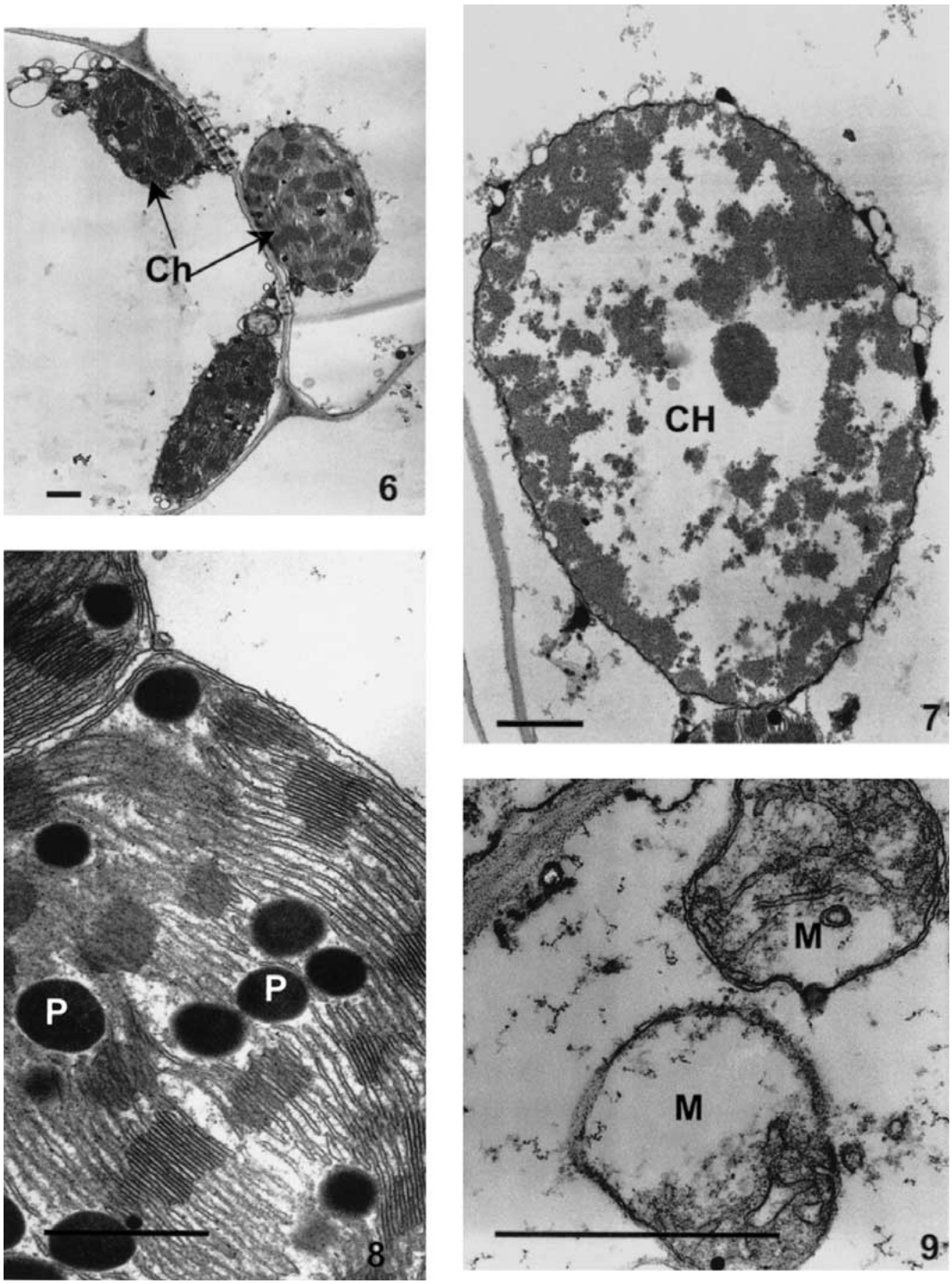

Figure 3. Panels 6–9. Ultrastructure of mesophyll cells of basal parts of second leaves of 30-day-old maize plants. **Panel 6.** Mesophyll cells with swollen and disrupted cytoplasmic membranes. **Panel 7.** Nucleus with condensed chromatin. **Panel 8.** Chloroplast with large numerous plastoglobules. **Panel 9.** Mitochondria with degraded internal membranes. *Ch*, chloroplast; *CH*, condensed chromatin; *G*, granum; *M*, mitochondrium; *P*, plastoglobules; *V*, vacuole; *Bar*, 1 μm.

changed as compared to the younger ones. Cytoplasmic membranes: plasma membranes, tonoplasts and also membranes of the mitochondrial and chloroplast envelope are disrupted in many cells (Figure 3, Panel 6). Nuclei contain many regions of condensed chromatin (Figure 3, Panel 7). Chloroplasts have large plastoglobules and thylakoid membranes are sometimes dilated (Figure 3, Panel

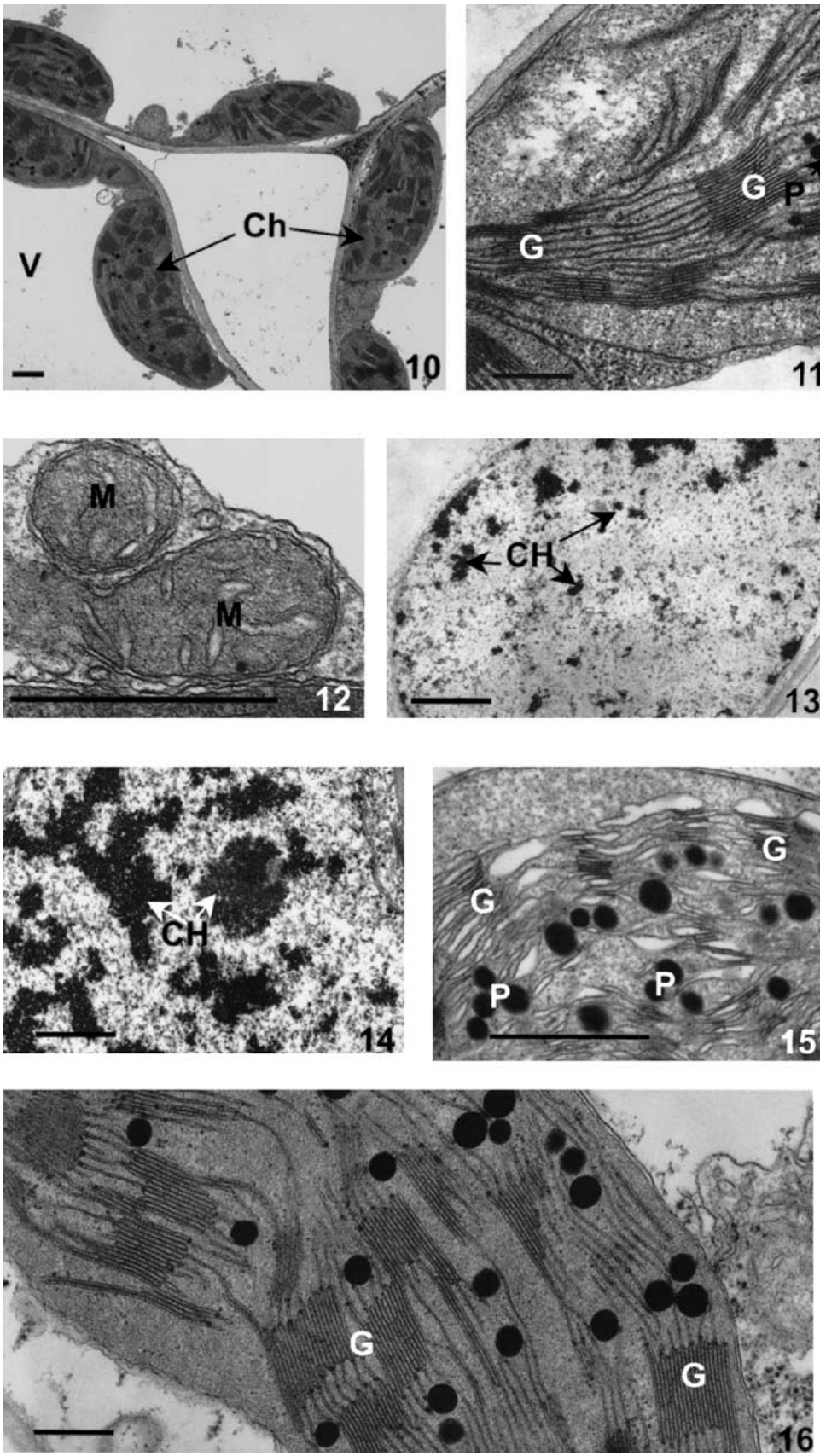

Figure 4. Panels 10–16. Ultrastructure of mesophyll cells of barley second leaves. **Panels 10–14.** Mesophyll cells from 10-day-old plants. **Panel 10.** Mesophyll cell with central vacuole, cytoplasm adjacent to plasmalemma and chloroplasts. **Panel 11.** Differentiated chloroplast with single plastoglobules. **Panel 12.** Mitochondria with richly invaginated internal membranes. **Panel 13.** Nucleus with dispersed chromatin, from basal region of second leaf. **Panel 14.** Nucleus, from apical part of second leaf, with some regions of condensed chromatin. **Panel 15.** Chloroplast from apical part of leaf of 20-day-old plant with degraded thylakoid and increased number of plastoglobules. **Panel 16.** Chloroplast from apical part of leaf of 20-day-old plant with increased number of thylakoid membranes. *Ch*, chloroplast; *CH*, condensed chromatin; *G*, granum; *M*, mitochondrium; *P*, plastoglobules; *V*, vacuole; *Bar*, 1 μm.

8). Some membranes of the chloroplast envelope are preserved but in many chloroplasts they are damaged. Mitochondria have swollen internal membranes (Figure 3, Panel 9).

Ultrastructure of Barley Mesophyll Cells

Basal, middle and apical regions of second leaves of 10-day-old barley seedlings are fully differentiated, contain a large central vacuole and dense cytoplasm adjacent to plasma membranes (Figure 4, Panel 10). Chloroplasts are differentiated with 6 to 12 thylakoids per granum. Chloroplasts contain few plastoglobules and no starch grains (Figure 4, Panel 11). Mitochondria have richly-invaginated internal membranes (Figure 4, Panel 12). Mesophyll cells in the basal regions of leaves contain nuclei with dispersed chromatin (Figure 4, Panel 13), however in apical parts of leaves nuclei contain some regions of condensed chromatin (Figure 4, Panel 14). The process of senescence began in apical parts of leaves of 10-day-old seedlings with chromatin condensation.

Mesophyll cells in 20-day-old barley seedlings became slightly swollen and most of them contain well-preserved tonoplasts and plasmalemma. Most of the chloroplasts, even in the apical regions of leaves, had an intact envelope. The chloroplast structure changed significantly compared to younger tissue. In some chloroplasts, especially in the middle and apical regions, degradation of internal membranes had begun (Figure 4, Panel 15). In others, further formation of new thylakoid membranes was observed (Figure 4, Panel 16). Due to the gradual thylakoid membrane degradation, there was an increase in the number of plastoglobules (Figure 4, Panel 15). Compared to the apical regions of leaves of 10-day-old barley seedlings, there was no change in the process of nuclei chromatin condensation. Nuclei chromatin condensation in all parts of leaves of this age was comparable with nuclei condensation in the apical part of 10-day-old seedlings.

Ultrastructure of the mesophyll cells of the basal regions of leaves of 30-day-old barley seedlings exhibited many characteristics of senescence. Most of the cells were swollen with disrupted tonoplasts and plasmalemma membranes (Figure 5, Panel 17). Chloroplasts had dilated and thylakoid membranes were often disrupted (Figure 5, Panel 18). In most cases, chloroplast structure was damaged, with a disrupted chloroplast envelope. Most chloroplasts contain numerous plastoglobules (Figure 5, Panel 18). Advanced chromatin condensation in the nuclei of mesophyll cells was observed (Figure 5, Panel 19). In most cases, mitochondria had degraded internal membranes (Figure 5, Panel 20). Barley mesophyll cells of the basal regions of leaves of 30-day-old plants were more damaged than similar maize cells.

Pigment Content in Maize Leaves

The content of chlorophyll (Chl) and carotenoids (Car) was the highest in the apical and the lowest in the basal regions of the second leaves of 10-day-old maize seedlings (Figures 6A, B, C). In 20-day-old seedlings, there was a decrease in Chl content in the apical regions of the leaves. This correlated with senescence of chloroplasts that began in the apical parts of these leaves. In 30-day-old leaves, there was a higher Chl content (Figures 6A, B) than in younger leaves. This higher content seems to contradict the ultrastructure of mesophyll cells, which exhibited symptoms of advanced senescence. In basal regions of 30-day-old leaves, there is 5 times more Car than in the basal regions of leaves of the youngest, 10-day-old plants (Figure 6C). The Ch1 a/b ratio remains in the range of 3.4–3.7 within leaf blades of different ages (Figure 6D).

Pigment Content in Barley Leaves

In both 10- and 20-day-old barley seedlings, Chl content was highest in the apical and lowest in basal regions of the second leaves (Figures 7A, B), although amounts were higher in older than in the younger ones. This is consistent with the ultrastructure of chloroplasts of 20-day-old seedlings where further formation of new thylakoid membranes was observed in some chloroplasts. The basal regions of 30-day-old barley leaves had a Chl content comparable to the second leaves of the youngest seedlings (Figures 7A, B, C). The basal regions of these leaves, similar to maize, have the highest Car content compared to basal regions of all earlier stages (Figure 7C). Car content in barley leaves increases gradually with the age of the seedlings, especially in the middle and basal regions (Figure 7C). The Chl a/b ratio does not differ much upon senescence and remains in the range of 3.0–3.25 within leaf blades of different ages (Figure 7D).

Discussion

In this paper, we demonstrated that each mesophyll cell senescences similarly but that the dynamics of senescence is different in different regions of maize and barley leaves.

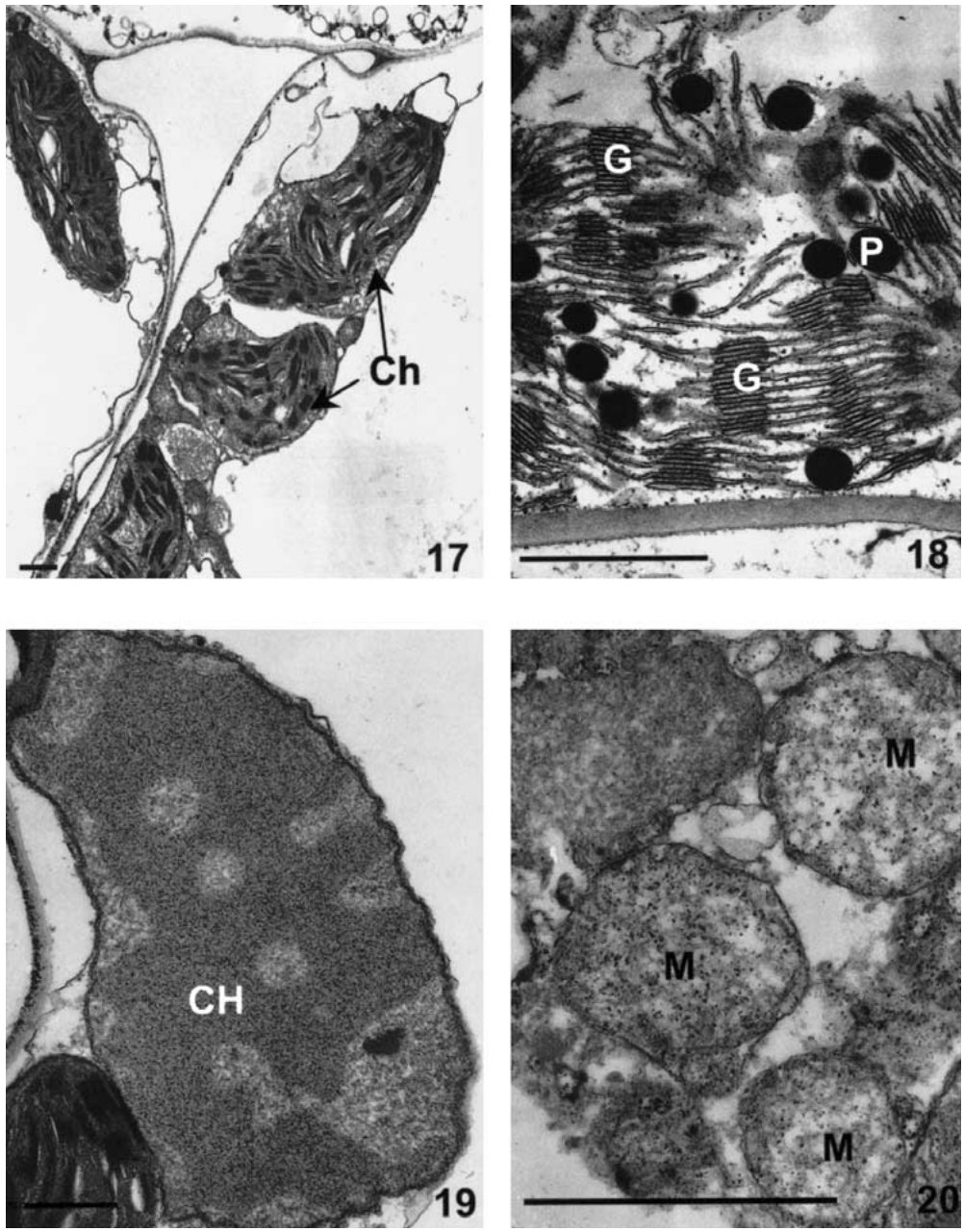

Figure 5. Panels 17–20. Ultrastructure of mesophyll cells of basal parts of second leaves of 30-day-old barley plants. **Panel 17.** Swollen mesophyll cells with disrupted membranes. **Panel 18.** Chloroplast with disoriented and disrupted thylakoid membranes, with numerous plastoglobules and with broken envelope. **Panel 19.** Nucleus with advanced chromatin condensation. **Panel 20.** Mitochondria with degraded internal membranes. *Bar*, 1 μm. *Ch*, chloroplast; *CH*, condensed chromatin; *G*, granum; *M*, mitochondrium; *P*, plastoglobules; *V*, vacuole; *Bar*, 1 μm.

We determined the time sequence of senescence-related events in mesophyll cells in different parts of the leaves of two plant species. The detectable change at the ultrastructural level for mesophyll cells from 10-day-old barley is condensation of chromatin.

Condensation of chromatin was described by many authors with respect to PCD of plants (Kuran 1993; Greenberg 1996; Beers 1997; Nooden and others 1997; Pennel and Lamb 1997; Yen and Yang 1998; Simeonova and others 2000). There is some evidence that chromatin condensation is not associated with DNA cleavage during PCD and that these two processes are regulated differently (Yao and others 2001).

We also report changes in chloroplast ultrastructure associated with senescence. These changes be-

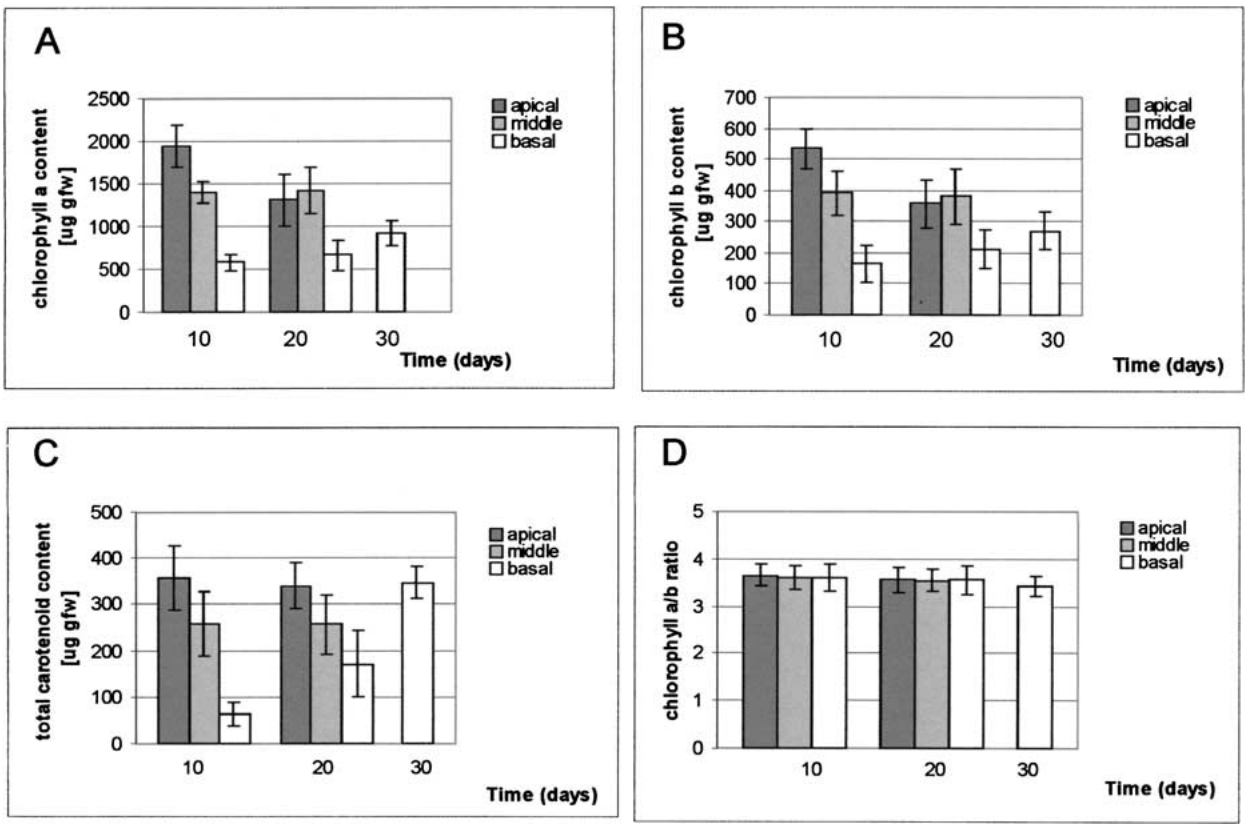

Figure 6. (A-D). Pigment content in apical, middle and basal parts of second leaves of 10- and 20-day-old maize plants and in basal parts of 30-day-old maize plants. (**A**) Chlorophyll a content, (**B**) Chlorophyll b content, (**C**) Carotenoid content, (**D**) Chlorophyll a/b ratio. *Vertical bars* represent the standard error.

gin with an increase in the number of plastoglobules in the middle and apical regions of leaves of 20-day-old barley plants and in apical regions of maize leaves of the same age, suggesting gradual thylakoid degradation. Changes in chloroplasts were, however, different in barley than in maize. In barley, changes were much more profound: gradual thylakoid membrane degradation had begun in some chloroplasts whereas in others further formation of new thylakoid membranes occurred.

The ultrastructural changes in chloroplasts of both species correlated with changes in the level of pigments. In barley, an increased number of photosynthetic membranes corresponded with higher Chl a and b content in 20-day-old seedlings. Thylakoids became swollen and disrupted during senescence in both plant species but in spite of that Chl content remained at a high level. High levels of Chl upon senescence may be caused by high levels of Car. It is well known that Chl is protected against oxidation due to the abundance of Car (Krinsky 1966; Sandmann and others 1993). Reactive oxygen species cause peroxidation of membranes that leads to their destruction during the senescence process. The level of Car may be high enough to protect Chl but insufficient to protect chloroplast membranes against destruction. Nevertheless, chloroplasts in 30-day-old maize leaves, (with high Car content) were less damaged than chloroplasts from barely leaves of the same age (with a lower Car level).

The Chl a/b ratios of about 3.5 in maize and about 3.0 in barley do not significantly change during senescence of maize and barley leaves. Increasing Chl a/b ratio during senescence of barley leaves, due either to faster degradation of Chl b than Chl a or transformation of Chl b into Chl a, was observed by Scheumann and others (1999).

The ultrastructure of chloroplasts during senescence is reminiscent of changes caused by different environmental agents that induce oxidative stress (Mostowska 1999). In oxidative stress, as in senescence, thylakoid swelling and increased numbers of plastoglobules, accompanied by photodestruction of pigments and inhibition of photosynthesis, can lead to damage in the ultrastructure of chloroplasts membranes (Mostowska 1997, 1999).

One of the last changes observed during senescence is in mitochondria ultrastructure. (There is

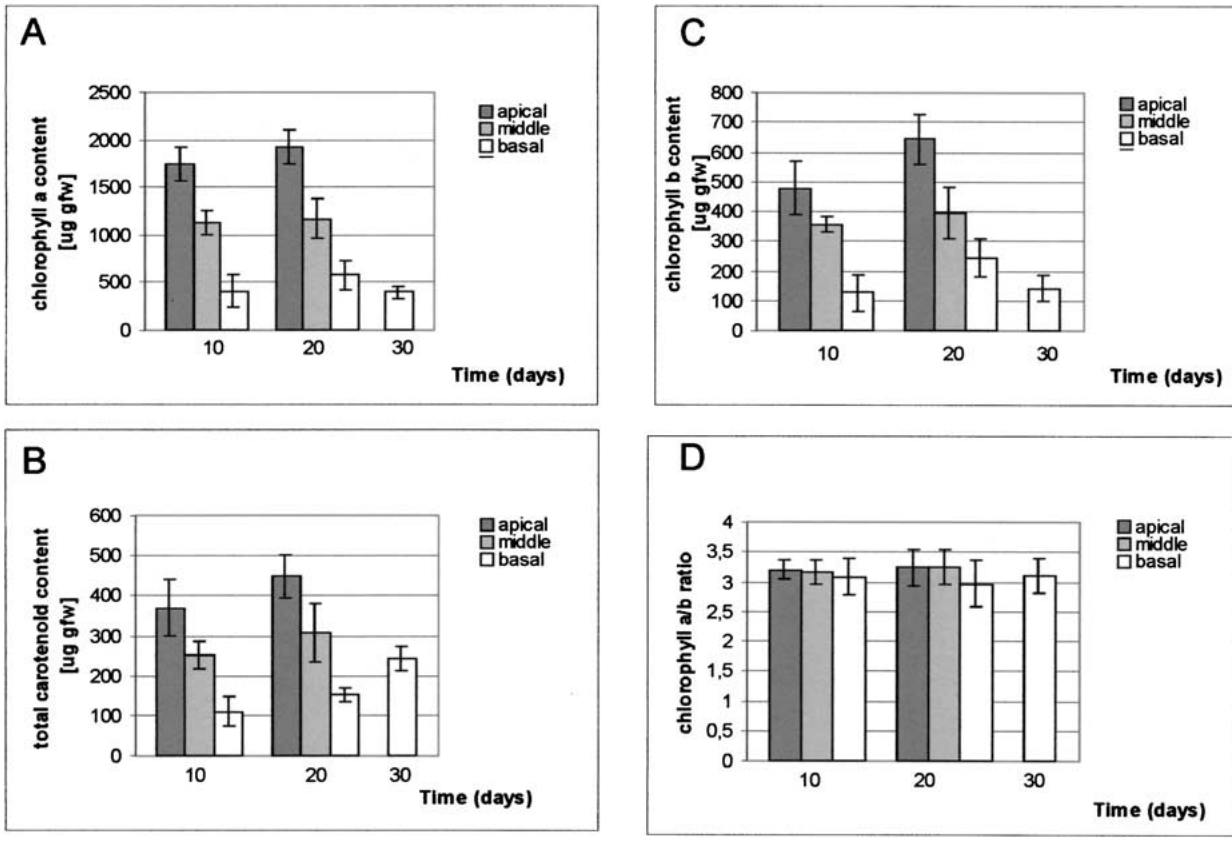

Figure 7. (A-D). Pigment content in apical, middle and basal parts of second leaves of 10-, 20-day-old barley plants and in basal parts of 30-day-old barley plants. (**A**) Chlorophyll a content, (**B**) Chlorophyll b content, (**C**) Carotenoid content, (**D**) Chlorophyll a/b ratio. *Vertical bars* represent the standard error.

little published about mitochondria ultrastructure during leaf senescence). In animals, mitochondria retain their integrity for a long time during apoptosis and play a key role in controlling PCD by releasing cytochrome c into the cytosol, thereby activating caspases (D'Silva and others 1998; Solomon and others 1999; Desagher and Martinou 2000; Jones 2000).

Our results revealed that each mesophyll cell undergoes a similar senescence program and that the time sequence of degrading events is as follows:

1. chromatin condensation
2. degradation of thylakoids and increase in the number of plastoglobules
3. damage of internal mitochondrial membrane and chloroplast destruction

Similar time sequence of senescence events for each mesophyll cell supports the statement that leaf senescence can be considered as a form of PCD. Such a sequence of ultrastructural events is consistent with Nii and others (1988), Inada and others (1998, 1999), and Simeonova and others (2000), but not with Yu and others (2002), who observed changes in mitochondria ultrastructure in Zinnia mesophyll cells prior to vacuole collapse.

We also showed that these changes in ultrastructure do not proceed at the same time in different parts of maize and barely leaves. Mesophyll cells from the basal regions of 10-day-old leaves from both plant species showed no signs of senescence. At the final analyzed senescence age (basal regions of leaves of 30-day-old plants), barley mesophyll cells were more damaged than the maize ones. Leaf senescence started earlier in barley than in maize seedlings and began in apical parts of leaves of 10-day-old seedlings with chromatin condensation. The dynamics of senescence was faster in barley between the ages of 10 and 20 days than between the ages of 20 and 30 days.

In maize leaves the senescence process started later than in barley. It began in the apical regions of 20-day-old seedling leaves with initiation of chromatin condensation. The dynamics of senescence was faster between the ages of 20 and 30 days than between the ages of 10 and 20 days.

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