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Abstract

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Keywords

Buchanan Lake Formation, chloroplast, Eocene, fossil, lignite, *Metasequoia*, mummification, thylakoid

Comments

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CYTOLOGICAL AND ULTRASTRUCTURAL PRESERVATION IN EOCENE *METASEQUOIA* LEAVES FROM THE CANADIAN HIGH ARCTIC¹

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The ultrastructural examination by transmission electron microscopy of 45-million-year-old mummified leaves of *Metasequoia* extracted from the Upper Coal member of the Buchanan Lake Formation in Napartulik on Axel Heiberg Island revealed the preservation of intact chloroplasts and chloroplast components. Abundant tanniferous cell inclusions may indicate that the 3-mo period of constant daylight during the Arctic summer induced high concentrations of tannins in the leaf tissues, which may have arrested microbial degradation of the litter. Quantified differences in the extent of chloroplast preservation through a vertical section of the lignite suggest that short-term shifts in the depositional environment took place, perhaps influencing the exposure of the leaf tissues to conditions that would either promote or inhibit decomposition.

Key words: Buchanan Lake Formation; chloroplast; Eocene; fossil; lignite; *Metasequoia*; mummification; thylakoid.

Major portions of the middle-Eocene-age forests (~45 Mybp) of Napartulik, Axel Heiberg Island, in the Canadian High Arctic are preserved as mummifications. Organic rich layers of leafy litter mats or brown coals commonly cap the fining-upward sequences of sandstone, siltstone, and mudstone deposits that comprise the Upper Coal member of the Buchanan Lake Formation. These lignitic layers are primarily composed of exceedingly well-preserved leaves and twigs of the taxodiaceous conifers *Metasequoia* and *Glyptostrobus* (Basinger, 1991), although remains of many other species of pteridophytes, conifers, and angiosperms are present. The autochthonous leaf litter mats in these horizons represent the ancient forest floors of poorly drained floodplains and associated swamps (Ricketts, 1986, 1991; Basinger, 1991). The site is unusual in having little apparent chemical or biological alteration of the fertile and vegetative remains (Obst et al., 1991), though the material has been subject to some compression. The exact chemical conditions leading to this degree of preservation are unknown.

Due to the rarity of uncompressed, well-preserved organic remains, there has been little opportunity to conduct detailed studies of ultrastructural preservation in ancient plant tissues. Most recently, compressed leaves of *Rhodomyrtophyllum pasekovicum* from the middle Eocene of the Central Russian Upland revealed chloroplast ultrastructure that has been preserved to the extent that the thylakoid arrangement (internal membranes of the chloroplast) in grana stacks is distinct (Vikulin, 1999). Similarly, Zhilin and Yakoleva (1994) reported on the preservation of virtually all organelles and their internal struc-

ture in Miocene-age leaf compressions of Eucommiaceae from Kazakhstan.

The application of ultrastructural evidence for interpreting paleoenvironmental conditions has been limited to the work of Karl Niklas and his associates (1985) studying Miocene-age angiosperm leaves from the Clarkia and Succor Creek localities of Idaho and Oregon. Research on leaf compressions from these localities provided a basis for assessing and quantifying organelle preservation under diverse paleoecological and depositional conditions via an index of fidelity to the presumed living state (Niklas et al., 1985). Perhaps one of the most surprising findings from this work was that chloroplasts appeared to be preferentially preserved over nuclei and that both organelles were rarely preserved in the same cell. Niklas (1983) attributed this phenomenon to protoplast partitioning, whereby the nucleus, which controls the senescence process in a cell, becomes isolated from the chloroplasts due to osmotic shock. The presence of intact organelles preserved in ancient leaf tissues implies extremely rapid preservation that not only arrests all catabolic activities, but also appears to prevent microbial attack. This is surprising, considering the processes that take place in leaves either during senescence or following trauma-induced detachment.

The investigation of the ultrastructural preservation of the middle Eocene *Metasequoia* leaves from Axel Heiberg Island consisted of three parts: (1) to assess qualitatively the overall condition of organelle preservation, (2) to determine general features of interest, and (3) to establish properties to which a quantitative assessment could be applied. Thus a statistically significant sample set of *Metasequoia* leaf cross sections, sampled from over a vertical interval from one of the leaf litter mats, was analyzed using transmission electron microscopy (TEM). The objective of the second and third components was to perform a quantitative assessment of chloroplast preservation in the fossil leaves, with particular emphasis on identifying notable differences in the quality of preservation between sampled intervals within the leaf litter mat.

MATERIALS AND METHODS

A 60 mm thick (~250 cm² area) block of frozen *Metasequoia* litter was collected from one of the best preserved leaf litter mats at Napartulik. The

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sample was kept frozen and shipped to the University of Pennsylvania where it was divided into 12 5 mm thick layers. Each layer was floated in a shallow pan containing deionized water and gently agitated to free individual leaves, twigs, and small branches from the muddy matrix. The leaves that appeared to be undamaged and best preserved were collected and re-rinsed with deionized water to remove the clay and other adhering debris. Samples of living *M. glyptostrobooides* previously collected at the Morris Arboretum and refrigerated at 6°C for 3 mo were also prepared for comparison, as were fresh samples of living *M. glyptostrobooides*.

Although the leaves retrieved from the litter mats were well preserved and had no external alterations, they were somewhat compressed and dehydrated. To obtain satisfactory results in the embedding and sectioning of the leaf tissues, the leaves had to be rehydrated prior to fixation. The leaves were rehydrated in a 100, 80, 50% alcohol series for 15 min each. After washing in phosphate-buffered saline, the leaves were transferred to a solution of 4% paraformal and 1% glutaraldehyde and left overnight at 4°C. After 12 h, the specimens were transferred to 5% glutaraldehyde for 2 h at 25°C. The solution was then changed, adding 1% tannic acid to the fixative, and left for 4 h. Specimens were then washed three times with 0.1 mol/L sodium cacodylate buffer (NaCaC) at 25°C to remove the glutaraldehyde from the samples prior to adding osmium. The samples were transferred to a glass vial and osmicated with 2% osmium in 0.1 mol/L NaCaC for two 1-h changes. Samples were then washed twice with 0.1 mol/L NaCaC and twice with deionized water, then stained enbloc with 2% aqueous uranyl acetate for 30 min prior to washing three times in deionized water. Dehydration was accomplished in 10-min changes of ethanol (50, 70, 80, 90, and 100%). To improve the infiltration of resin into the leaf tissues the specimens were placed in 1 : 1 ethanol : propylene oxide (PO) for 5 min, followed by two 5-min changes to absolute PO, as suggested by Jones and Rowe (1999). Infiltration was accomplished by successively placing the samples in 3 : 1, 1 : 1, 1 : 3 PO : Spurr's resin (Spurr, 1969) for 45 min each. After a change to 100% Spurr's resin for 1 h, the samples were placed in fresh resin and left overnight at 4°C. The following day, after warming to 25°C, the solution was replaced with fresh resin, using a rotator to infiltrate.

For the fresh and refrigerated leaves of modern *M. glyptostrobooides*, the following protocol, based on the procedure of Meier and Lichtenthaler (1981), was used. After being cut into segments of approximately 2–4 mm², the specimens were placed in 5% glutaraldehyde solution for 2 h at 25°C, and then washed three times with 0.1 mol/L sodium cacodylate buffer at 25°C. Glutaraldehyde was thus removed from the samples before adding osmium. The samples were transferred to a glass vial and osmicated with 2% osmium in 0.1 mol/L NaCaC buffer for two 60-min changes. Samples were then washed two times with the buffer and two times with deionized water. Samples were stained enbloc with 2% aqueous uranyl acetate for 30 min before washing three times in deionized water, then dehydrated in 50, 70, 80, 90, and 100% 10-min changes of ethanol. Infiltration was accomplished by successively placing samples in 3 : 1, 1 : 1, 1 : 3 ethanol : Spurr's resin for 45 min each. After a change to 100% resin for 60 min, samples were placed in fresh Spurr's resin and left overnight at 4°C. The following day, after warming to 25°C, the solution was replaced with fresh resin, using a rotator to infiltrate.

All samples were cured at 70°C for 48 h. Sections were cut to a thickness of 70 nm using an Ultracut S microtome (Leica, Wien, Austria) and mounted on 200-mesh thin bar copper grids. The grids were stained for 15 min at 25°C in 7% uranyl acetate in 50% ethanol, washed in deionized water, and counterstained in bismuth subnitrite for 15 min at 25°C before being washed in deionized water and left to dry.

Our intention was to employ the methods applied to the *Clarkia* leaf compressions for quantifying organelle ultrastructural integrity, thereby providing us with a means for comparison; however, this was not possible, as mesophyll cell wall integrity had not been maintained in the Napartulik samples. Only the epidermal and vascular cells had survived the combined effects of syn- and post-burial decomposition and compression. We therefore had to examine the mesophyll area—consisting of damaged cell wall fragments and organelles suspended in what appeared to be a tannin-rich matrix—as a whole within a set time to control the intensity of investigation. Additionally, we found that the only organelles preserved, with few exceptions, were chloroplasts or chlo-

roplast remnants. The method was amended to quantify the state of chloroplast preservation observed by identifying a number of distinct features that could be tallied on a per-section basis.

Three sections from each leaf and three leaves from each of the 12 5-mm depth intervals were examined, for a total of 108 sections and 36 leaves. Each section was examined for 45 min using a JEM 1010 transmission electron microscope (JEOL, Peabody, Massachusetts, USA) at magnifications up to 150,000×. Chloroplasts and chloroplast constituents were photographed and counted. The dimensions of all intact chloroplasts were measured, as well as the orientation of the long axis, which was always measured at the same magnification. The condition of each chloroplast (or constituent) was recorded in terms of the presence of stacked membranes, grana, associated and disassociated membranes, lipid droplets, amorphous breakdown lipids, and evidence of starch grains (areas clearly once occupied by grains that had deteriorated in place). In order to insure the consistency of these observations, all of the samples were examined and evaluated by the same researcher.

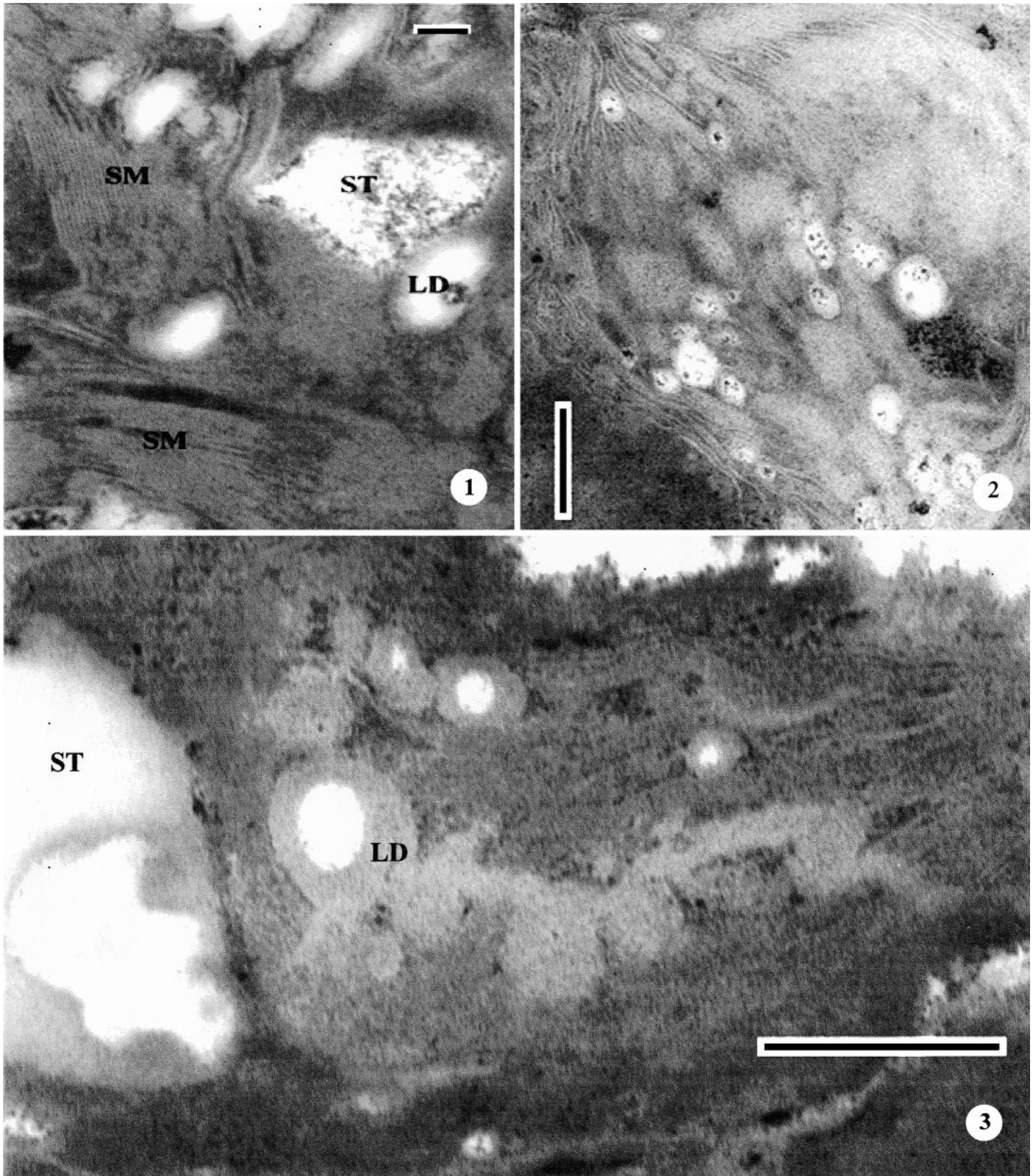
Chloroplasts were characterized as being “intact and having discernible thylakoid geometry” (Fig. 1) if the quality of preservation was sufficient to allow the determination of the number of thylakoids per granum, the degree of grana stacking, and the width of grana stacks. Additionally, the general shape of the individual chloroplast had to be evident; that is to say, the stroma-grana thylakoid system must have been segregated in an obvious manner from its surroundings. In some cases, these ultrastructural characteristics were evident and clearly measurable, but they were in an area consisting of numerous closely spaced chloroplasts, packed so that the membranes separating the individual chloroplasts were not discernible. Chloroplasts were described as being “intact and having evident ultrastructure” if the inner membrane system was evident throughout or in portions, but the quality of preservation was insufficient to allow the determination of the characteristics related to thylakoid geometry (Fig. 2).

Chloroplast membranes were classified in one of four states. “Stacked membranes” (Fig. 1) were considered to be present in any area with a clear lamellar orientation to the internal membranes associated with the chloroplast. In the best case, thylakoids were clearly appressed members of a grana stack, but generally these membranes were likely to have been stromal thylakoids that became closely associated only after the vesiculation of grana stacks, which allowed for the reduction of the gap between stromal thylakoids. Chloroplast membranes were termed “associated” in any case where they were part of a clearly delineated organelle. Membranes were termed “disassociated” in any case where they were randomly dispersed and could not be considered part of any organelle. Chloroplast membranes were termed “vesicular” in cases where portions of either single double-membranes or stacks of membranes had broken off into vesicles—in cross section, small circular bodies with no contents. In many cases, the stages of the vesiculation process were evident.

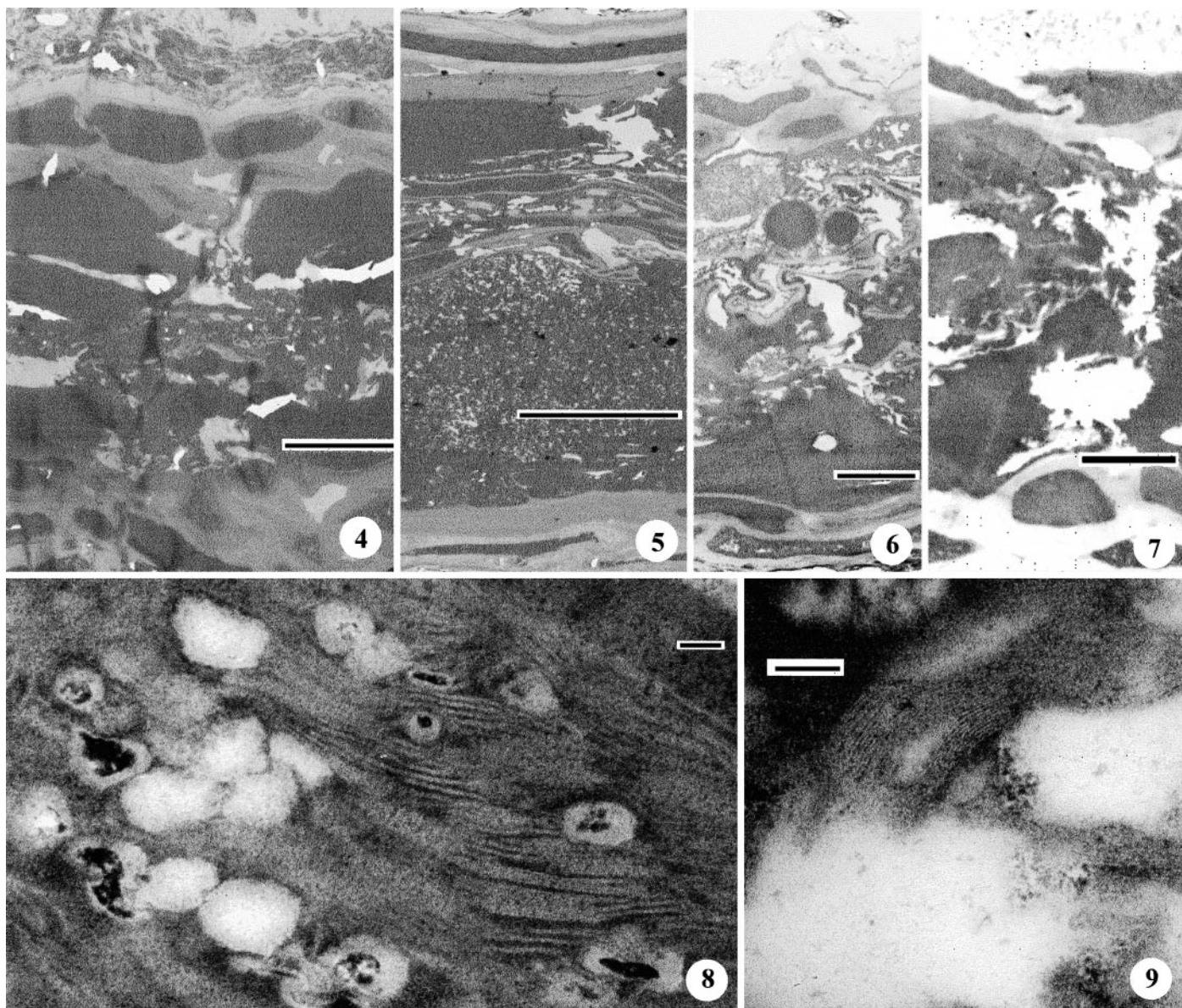
“Starch” (Fig. 3) was considered to be present when an area within a chloroplast body appeared to have been occupied by a starch grain that had presumably decayed in place. Generally, the perimeter of such areas was demarcated by a thin outline of electron dense material, with the thylakoid membranes appearing to bend around it as they would around a starch grain in a fresh green chloroplast. In a few cases, material with the same appearance (under TEM) as an actual starch grain was observed.

“Lipid droplets” (Fig. 3) were considered to be present when appropriately sized, electron-lucent globuli were observed in close association with membranes. In the best-preserved chloroplasts, these droplets typically displayed a small electron dense area near the center. “Breakdown lipids” were counted as being present wherever there was an amorphous cloud of electron lucent material (“negative staining”) that by its size, shape, and association with chloroplast-scale membranes was clearly the result of the decomposition of those membranes. Intact chloroplasts were frequently encountered along the perimeter of large irregular bodies or bands of breakdown lipids. Additionally, portions of stacked membranes could often be discerned at the boundaries of those areas, as if some digestion process originating from the center of a cluster of chloroplasts had taken place.

The number of times each feature was observed for each section was recorded. The maximum cross-sectional thickness of the leaf (the distance from



Figs. 1-3. Chloroplast ultrastructure in Eocene *Metasequoia* leaves from Axel Heiberg Island. **1.** Grana stacks with evident geometry. Stacked membranes, lipid droplets, and starch grain remnants are also present. Scale bar = 100 nm. **2.** Intact fossil chloroplasts with evident ultrastructure. Although stacked membranes are obvious, thylakoid features are too indistinct to measure. Scale bar = 500 nm. **3.** Starch grain. Fossil chloroplast with grana stacks, lipid droplets, and a large starch grain that has lost material at its center. Scale bar = 500 nm. *Figure Abbreviations:* SM, stacked membranes; LD, lipid droplets; ST, starch grain remnants.



Figs. 4–9. Leaf cross-sections and chloroplast ultrastructure in Eocene *Metasequoia* from Axel Heiberg Island. 4–7. Typical cross-sections of fossil *Metasequoia* leaves. Scale bars = 10 μm . 8. Intact fossil chloroplast with evident grana stacks and lipid droplets. Centers of lipid droplets stain positively. Scale bar = 100 nm. 9. Stacked thylakoid membranes at the periphery of a large area of amorphous breakdown of lipids. Scale bar = 100 nm.

the upper to lower cuticle at the thickest part of the leaf) was also measured. This data was subjected to individual nested ANOVAs for each feature and a nested multivariate analysis of variance (MANOVA) to assess whether there was a significant difference in preservation between litter layers and between individual leaves. The between-leaf effect was considered to be nested within the layer effect. The within-leaf (between cross section) variance was used as the residual (error) mean square. That is, the between-interval mean square was tested against the between-leaf (within interval) mean square, while the between-leaf mean square was tested against the between-section (within leaf) mean square.

Chloroplast shape (CS) was determined as the length-to-width ratio, and means and standard deviations calculated for each section and leaf. The standard deviation of the orientation of each chloroplast within the section was also calculated. This was used to detect whether the observed shape of the fossil chloroplasts was reflective of its original geometry or was due to compression-related deformation. A low standard deviation in the angles of chlo-

roplast orientation within a leaf section would indicate that the shape was likely due to compression because directional stress would tend to reorient all chloroplasts, such that the long axes would be perpendicular to the direction of stress. A high standard deviation of the angles of orientation would indicate that the orientation was essentially random, and thus the observed geometry was not produced by compression. Similar measurements were made on randomly chosen chloroplasts in sections of an equivalent number of fresh green leaves of *M. glyptostroboidea* for purposes of comparison.

RESULTS AND DISCUSSION

Qualitative observations on fossil leaf ultrastructure—Typical examples of fossil leaf cross sections are shown in Figs. 4–7. Only cells of the epidermis and vascular bundle were generally intact. The cell walls in the spongy mesophyll and palisade layers were most frequently ruptured, presumably

as the result of senescence-related lysis, post-depositional osmotic shock, or failure under tension resulting from the transfer of compressive stresses from overburden mass. The epidermal cells were distinct, having highly lignified cell walls and tanniferous inclusions filling otherwise hollow cells. When observed by TEM, the tannins were electron dense and appeared dark gray. The epidermal cells were reshaped to varying extents by compressive stress. Commonly vacant, vascular cells also tended to be deformed in a manner characteristic of compressional effects; though the cell walls were intact, the cells were collapsed and flattened. It is of note that where cells were clearly occupied by tannins, the shape change was less extensive. The mesophyll area consisted of remnants of ruptured cell walls suspended within a tanniferous matrix.

Identifiable organelles and organelle components were most frequently located within the mesophyll area and were often in proximity to large regions of amorphous breakdown lipids. At the perimeter of these lipid-rich regions, stacked and vesicular thylakoid membranes were frequently evident, suggesting that the amorphous areas were originally clusters of chloroplasts, with the lipids representing membrane breakdown products. Intact chloroplasts and chloroplast remnants were the most frequently encountered recognizable organelle (or organelle component) and were identified in a majority of the leaves examined.

Tannin-enrichment—The abundance of tannins within the mummified leaves concurs with observations made on other fossil leaf tissues by Niklas and Brown (1981), Vikulin (1999), and Zhilin and Yakoleva (1994), as well as those on modern peats by Cohen and Spackman (1977). However, tannin inclusions were far more abundant in the fossil leaves than in the senescent leaves of *M. glyptostroboides* litter (Schoenhut, 2003). Similarly, in modern litter deposited in an aqueous environment artificially enriched with tannic acid, the epidermal cells were only partially filled with tannin inclusions, and mesophyll cells were sparsely occupied by variably sized tannin bodies (Schoenhut, 2003). This disparity may be attributed to the influence of environmental conditions during the development and senescence of leaves during the Eocene arctic summer. Bussotti et al. (1998) observed that by the end of the growing season, tannins completely filled the vacuoles of the epidermal and palisade mesophyll cells in leaves of trees subject to environmental stresses such as high levels of solar radiation, reduced nutrient availability, or wind-induced high transpiration rates, whereas fewer tannin bodies were present in leaves grown under optimal conditions. The Eocene *Meta-sequoia* were subject to 3 mo of 24-h light during the growing season; this or other stressors may have caused the observed excess of tannins in the fossil leaves.

Condition of preserved chloroplasts and chloroplast components—The chloroplasts were in various states of degradation. The least degraded chloroplasts (i.e., those closest in appearance to chloroplasts in the fresh *M. glyptostroboides* tissues) possessed intact double membrane envelopes and grana stacks, with both stromal and granal thylakoids that were clearly discernible (Fig. 8). Lipid droplets and evidence of starch bodies were frequently present. All membranes stained negatively; however, lipid droplets stained positively near the center, and areas that once were occupied by starch grains had limited positive staining at the periphery. Chloroplasts having high fidelity to the living state were isolated in large, irregular

clusters and in bands. In the chloroplasts that were in a slightly more advanced state of degradation, the grana stacks had fused into lipid-rich globuli. In some presumably less mature globuli, linear traces from the individual thylakoid membranes of the original grana stack were still visible.

Stromal thylakoids “liberated” by the fusion of grana into globuli were observed to (1) become more closely stacked, possibly as the result of dehydration or compression; (2) pinch off into vesicles; (3) swell, become unstacked, and disperse into the stroma or, if the chloroplast envelope had been ruptured, into the tanniferous matrix; or (4) remain relatively unchanged such that the stromal lamellae continued to be in association with the globuli. Closely stacked stromal thylakoids were observed to pinch off into vesicles, which eventually fused into globuli, just as the original grana stacks had. The disassociated membranes were also observed to have broken off into single-layer vesicles.

Large clusters or linear groupings of chloroplasts appeared to have been subject to membrane fusion from the center outward such that distinct granal and stromal thylakoids, and even intact chloroplasts, were evident along the periphery of otherwise occluded, negatively stained areas (Fig. 9). Individual chloroplasts frequently were observed to encompass regions of amorphous breakdown lipids.

Negative staining of chloroplast membranes—Though there have been few studies of cellular ultrastructure in fossil leaf materials, these examinations have consistently observed negative staining of organelle membranes, particularly those of the chloroplast (Niklas et al., 1985; Zhilin and Yakoleva, 1994). The mechanism behind this alteration of the staining properties of these membranes has yet to be established. Zhilin and Yakoleva (1994), who described organelle preservation comparable to the living state in Miocene leaf compressions of *Eucommia palaeoulmoides*, suggested that the negative contrast was due to the replacement of organic substances with minerals. These researchers also observed positive staining in membranes not associated with organelles, but rather located near lipid-like sediments.

Alternatively, based on observations from an extensive TEM study of leaf compressions of numerous taxa from the Miocene *Clarkia* deposits, Niklas et al. (1985) suggested that dehydration was the mechanism responsible for negative staining. When applied to modern tissues, dehydration followed by tannic acid fixation results in a negative contrast (Niklas et al., 1985). As described by Luzzati and Husson (1962), 80% dehydration caused the rearrangement of phospholipid components in membranes into hexagonal arrays, reducing the number of sites for reactions with fixatives such as osmium tetroxide or tannic acid and thus resulting in negative staining (Niklas et al., 1985). Senescence, detachment, or other types of trauma may also produce negative staining; however, chloroplast ultrastructure, as evinced in the *Clarkia* samples, did not typically survive senescence (Niklas et al., 1985).

During senescence, or after any treatment resulting in the production of free radicals, long-chain neutral lipid products of lipid peroxidation initiate the transformation of membrane lipids from a liquid-crystalline phase to a hexagonally ordered gel phase (Thompson et al., 1983). The presence of both phases increases the permeability of the membrane (Thompson et al., 1983), but, as just mentioned, decreases the number of sites for reaction during fixation and staining. Chilling also produces this phase change (Bishop, 1983; Thompson et al., 1983);

TABLE 1. Overall preservation quality in fossil leaf tissues (independent of depth in profile).

Features of chloroplasts or remnants	Total number ^a	Percentage of sections with feature	Percentage of leaves with feature	Percentage of layers with feature
Discernible thylakoid geometry	30	13	28	50
Evident ultrastructure	80	24	42	67
Stacked/appressed membranes	405	72	97	100
Evidence of displaced starch grains	127	43	64	92
Associated membranes	621	94	100	100
Lipid droplets	175	47	69	92
Vesicular membranes	157	54	81	92
Disassociated membranes	196	61	86	100
Amorphous breakdown lipids	767	98	100	100
No evidence of chloroplast-related structures	n/a	1.85	0	0

^a The total number of times that the chloroplast feature was observed in all 108 sections of fossil 36 leaves from 12 different layers of lignite sampled over a vertical profile.

senescence or trauma-induced processes effectively increase the temperature at which the transition occurs. In ultrathin leaf sections examined as part of this study, intact chloroplasts of fresh *M. glyptostrobooides* leaves that had been detached and refrigerated at 6°C for 3 mo also had negative staining of the thylakoid membranes.

Additionally, thylakoid membranes were negatively stained in preparations of intact chloroplasts isolated from ground tissue by centrifugation, while isolated thylakoids from broken chloroplasts stain positively (Hooper, 1984). This suggests that stress-produced radicals may originate in the stroma of the chloroplast, whether from dehydration, osmotic shock, or aging. This is consistent with the observation in very well-preserved fossil chloroplasts that, while the thylakoid membranes and lipid droplets are consistently electron lucent, centers of lipid droplets frequently have a small electron dense area at the center (Fig. 8), suggesting that the transformation originates at the surface of the droplet in contact with the stroma and progresses toward the core.

Quantitative analysis of chloroplast preservation—Table 1 lists the chloroplast features that were assessed for the fossil leaves and the percentage of samples in which their presence was observed. The features are arranged such that those at the top of the table represent the highest fidelity relative to living *M. glyptostrobooides* leaves, whereas those at the bottom represent the least.

Intact chloroplasts were observed in 42% of the fossil leaves examined and in 67% of the 5-mm intervals. In 28% of the leaves, the chloroplasts were so well preserved that features related to thylakoid geometry (degree of grana stacking, number of thylakoids per granum, stack width) were measurable. Stacked membranes were observed in 97% of the fossil leaf samples. Only 2% of the leaf sections lacked any evidence of chloroplasts or related structures.

The number of times each chloroplast feature was present in a given leaf section was used to test for significant differences between intervals in the litter sample and between individual leaves. When each feature was considered separately, only the presence of vesicular membranes (VES) revealed a significant difference ($P < 0.05$) between intervals, whereas there was a significant difference ($P < 0.01$) between individual leaves for each individual feature in the univariate analyses. The presence of both stacked membranes (SM) and lipid droplets (LD) showed a marginally significant difference between layers ($P < 0.10$).

When the features were considered simultaneously in mul-

tivariate analyses, the difference between intervals was found to be significant (for a 5% type I error), and, as before, the leaf effect was found to be highly significant ($P < 0.01$). An additional multivariate analysis was performed using only the three features whose least squares means best distinguished between intervals. When only the presence of stacked membranes (SM), vesicular membranes (VES), and lipid droplets (LD) was considered, both the interval effect and leaf effect were found to be significant ($P < 0.01$).

The mean frequencies of the presence of these three chloroplast features—SM, VES, and LD—per vertical 5-mm interval within the leaf litter mat revealed variations in the quality of leaf preservation over time. A series of statistical contrasts were performed to assess whether the observed variations between vertical intervals were significant; both individual layers and sequences of layers were tested. The interval between 5 mm and 35 mm was significantly different from the rest of the layers ($P < 0.01$ by single variant analysis using SM, $P < 0.05$ by multivariate analysis). The interval between 35 mm and 50 mm (the trough in the oscillation) was also significantly different from the other layers ($P < 0.01$ by single variant analysis using “stacked membranes,” $P < 0.05$ by multivariate analysis). The interval between 50 mm and 60 mm (the ascending portion of the oscillation) differed significantly from that between 35 mm and 50 mm ($P < 0.05$ by single variant analysis using “stacked membranes”).

The average length-to-width ratio of the chloroplasts present in the fossil leaves of 2.96 was significantly different from the average ratio found from a random sample of fresh leaves of *M. glyptostrobooides*, for which the average length-to-width ratio was 2.47. However, the standard deviations of the angles of orientation for the chloroplasts within a section varied similarly between the modern and fossil samples, indicating that the elliptical shape of the fossil chloroplasts was not the result of compression-related deformation.

Implications of chloroplast preservation—Because the fossil leaf samples were obtained from a thick litter mat, presumably deposited over many hundreds of years by the seasonal deciduous shedding of shoots (cladoptosis), the leaves were assumed to have undergone senescence prior to abscission, and thus would, under conditions of optimum preservation, reflect the ultrastructural state characteristic of senescent leaves.

Studies on ultrastructural changes within cells during senescence show a highly ordered pattern of degradation that is quite distinct from that observed in leaves prematurely detached due to trauma. In senescing cells, the sequence begins

with a loss of ER and Golgi body membranes, typically followed by a loss of the chloroplast, then the nucleus and finally, the mitochondria deteriorate (Hurkman, 1979; Niklas et al., 1985). Nuclear persistence permits control of these events, while the mitochondria provide energy for enzymatic disassembly. This ordered sequence permits the recovery of a substantial portion of the nutrients contained in the tissues. During this process, the internal chloroplast proteins and structures disintegrate first, leaving behind large plastoglobuli that are thought to be the remnants of the membrane lipids (Burke et al., 1984; Grover and Mohanty, 1993; Larcher, 1995). The plastid then loses its characteristic shape, becoming spherical (Niklas et al., 1985), and ultimately, the outer membrane disintegrates (Hurkman, 1979; Woolhouse, 1984; Grover and Mohanty, 1993). Early disruption of the chloroplasts would release photoactive compounds into the cell, oxidizing the contents and preventing the withdrawal of nutrients (Larcher, 1995).

Alternatively, when fresh leaves are either artificially or naturally detached, the pattern of degeneration observed is quite different. In leaves detached for several days, the chloroplast membranes rupture, releasing their contents into the cytoplasm. The thylakoid membranes, which remain associated with the other chloroplast constituents (e.g., lipid droplets, starch grains), persist long after the other organelles have disappeared (Larcher, 1995). This is consistent with observations made on isolated chloroplasts suspended in water or hypotonic buffer. The chloroplast envelope swells and ruptures, liberating the stromal contents into the medium; the thylakoid system remains intact and associated with osmiophilic globuli (Kirk and Tilney-Bassett, 1978). Under these circumstances, the remnants of the ruptured chloroplast envelope form numerous vesicles (Cline, 1985).

In contrast to expectations, the observed preferential preservation of chloroplasts and chloroplast components in the Napatulik samples is not consistent with the pattern of degradation produced by senescence; instead, it resembles trauma-induced patterns. The elliptical shape of fossil chloroplasts and the presence of intact grana and stacked membranes conflict with the observed effect of senescence on chloroplast ultrastructure. The lack of a preferential orientation in the long axis of the chloroplast indicates that the shape is not a compression artifact, but rather is a result of the thylakoid structure. Although the difference between the means of the length-to-width ratios between fossil and modern samples may be attributed to post-depositional dehydration effects (an intact thylakoid system could reduce the chloroplast width, but not the length), it is more likely due to the fact that, in many cases, the chloroplast envelope had ruptured. The released thylakoid system would tend to be narrower than the chloroplast as a whole, because starch grains and the stroma extend the envelope in the direction perpendicular to the thylakoids. The presence of vesicular membranes is also consistent with the effects of detachment rather than senescence.

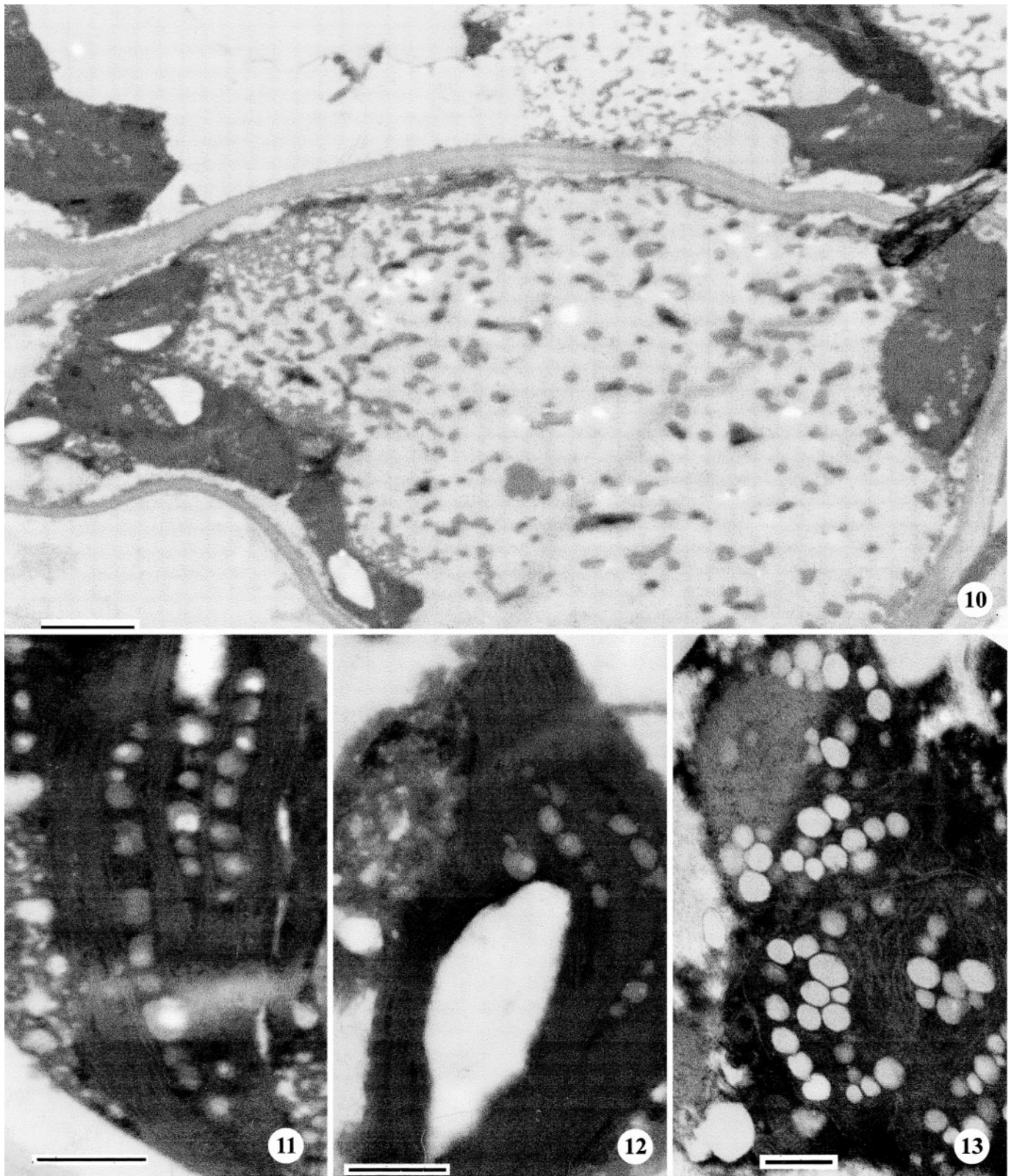
It is crucial to note, however, that studies on leaf senescence and aging have been focused almost exclusively on angiosperms because the deciduous habit is rare among gymnosperms and that the pattern of degradation observed during senescence may be significantly different for a deciduous conifer such as *M. glyptostrobooides* or the Eocene *Metasequoia* of the Canadian High Arctic. Observations of senescent leaves of *M. glyptostrobooides* indicate that elliptical, starch-bearing chloroplasts with intact thylakoid geometry regularly persist,

even when the nuclei have also remained intact within a given cell (Figs. 10–12), although gerontoplasts may also be present, as shown in Fig. 13 (Schoenhut, 2003). Following 3 mo of post-depositional decay, chloroplasts of *M. glyptostrobooides* with intact thylakoid stacks were observed in 23 of 36 leaves examined (Schoenhut, 2003). This apparent inefficiency of the senescence process in this species would explain the presence of chloroplasts in the fossil leaves, though not their preferential preservation over other persistent subcellular features.

It is possible that the chloroplast's highly organized internal structure and the tendency for membranes to stain negatively, rendering them readily apparent, increased the probability that the organelle or its constituents would be recognized even in an advanced state of degradation. The abundance of chloroplasts in contrast to nuclei could also contribute to this bias. However, there is evidence that the post-depositional conditions favored chloroplast preservation because circumstances preserving leaf tissue in general from fungal and bacterial attack would also prevent decomposition of chloroplasts in particular.

Mechanism of preservation—Noting that taxa characterized by high, naturally occurring concentrations of tannic acids were frequently found in the best state of preservation, Niklas et al. (1985) attributed the excellent ultrastructural preservation observed in the *Clarkia* leaf compressions to an “auto-fixation” process whereby dehydrated leaves were submerged in standing waters that were rich in tannic acid presumably leached from the bark of partially immersed trunks and roots. Additionally, these authors reported that after dehydration and tannic acid fixation, chloroplast membranes appeared to be more stable than those of nuclei or mitochondria, a finding consistent with the observed preferential preservation of chloroplasts in the *Clarkia* samples. Similarly, Cohen and Spackman (1977) reported a high frequency of tanniferous cell fillings in tree-dominated, autochthonous peats in southern Florida and attributed preferential preservation of *Rhizophora* over *Avicennia* leaves within these peat deposits to the presence of these inclusions. The mechanism proposed for the pristine preservation of leaves deposited within peat deposits was accumulation in standing acidic, tannin-bearing waters; this would arrest or retard degradation, as well as inhibit fungal and bacterial growth (Cohen and Spackman, 1977; Gastaldo and Staub, 1999).

The significant variation in the preservational state of chloroplasts between fossil leaves may reflect differences in the time required for natural fixation processes to occur, during which the degradation characteristic of detachment would proceed. The variation observed between 5-mm intervals, which are likely time-averaged across several years, may reflect long-term changes in depositional conditions or may be the product of small-scale variation in diagenetic conditions. The oscillation in preservational quality between layers appears to have a period of some 50 mm, with about 22.5 mm having above-average preservation and 27.5 mm having below-average preservation. Clearly, a larger data set would be required to determine whether this pattern is actually present throughout the leafy litter mats. Relatively small-scale temporal variations in these deposits have previously been observed (Greenwood and Basinger, 1994). However, in a recent study, Richter (2002) noted significant shifts in the community composition of a floodplain over a 2-m interval using high-resolution sampling techniques. Using an estimated leaf lignite accumulation rate



Figs. 10–13. Chloroplasts and gerontoplasts in senescent *M. glyptostroboides* from southeastern Pennsylvania. 10–12. Intact chloroplasts of “red” *M. glyptostroboides* leaves after a 3-mo decay period. 10. Scale bar = 2 μm . 11–12. Elliptical chloroplasts with intact thylakoids, starch grains, and lipid droplets. Scale bars = 500 nm. 13. Gerontoplasts in “red” *M. glyptostroboides* leaves after a 3-mo decay period. Scale bar = 500 nm.

of 0.8 mm/yr (Kojima et al., 1998), the period of the observed oscillation could be considered equivalent to approximately 63 yr.

In terms of the mechanism of preservation, the findings of the ultrastructural examination of the mummified leaves suggest that the 3-mo period of 24-h light during the Arctic summer induced the production of high concentrations of tannins in the leaf tissues. These polyphenolic flavonoid compounds could in turn have arrested microbial degradation of the litter because tannins have been shown to inhibit bacterial and fungal development. Differences in the quality of preservation through a vertical section of the leaf litter mat indicate that short-term shifts in the depositional environment took place, which perhaps influenced the amount of time during which leaf tissues were exposed to conditions that would promote decomposition.

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