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DEVELOPMENT AND LIGHT RESPONSE OF LEAVES OF *METASEQUOIA* AND CLOSE RELATIVES

By

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B.S. Beijing Forestry University, China, 2001

A THESIS

Submitted in Partial Fulfillment of the

Requirements for the Degree of

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(in Forestry)

The Graduate School

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August, 2004

Advisory Committee:

Richard Jagels, Professor of Forest Biology, Advisor

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DEVELOPMENT AND LIGHT RESPONSE OF LEAVES OF *METASEQUOIA* AND CLOSE RELATIVES

By Xiaochun Li

Thesis Advisor: Dr. Richard Jagels

An Abstract of the Thesis Presented
in Partial Fulfillment of the Requirements for the
Degree of Master of Science
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Metasequoia glyptostroboides is a useful nearest living relative (NLR) of the Eocene fossil *Metasequoia*. Research on modern *Metasequoia* might give us some clues about its fossil counterpart.

During this study the leaf anatomy of *Metasequoia*, *Glyptostrobus*, *Sequoia* and *Taxodium* was investigated with light microscopy and transmission electron microscopy. *Metasequoia* exhibits several characteristics of typical sciaphilic plants, such as slightly arched outer cell walls in the adaxial epidermal cells, strongly arched outer cell walls in the abaxial epidermal cells, mesophyll composed of spongy cells, chloroplasts with well-developed grana not only in mesophyll cells but in both the adaxial and abaxial epidermis. Based on comparison of leaf morphology and anatomy, we conclude that *Metasequoia* is best adapted to low light intensities, *Sequoia* and *Taxodium* are intermediate, and *Glyptostrobus* is adapted to higher light intensities.

The effects of light intensity on mesophyll plastids of *Metasequoia* leaves were studied with trees grown under different light intensities. *Metasequoia* had the ability to synthesize chlorophyll under complete darkness and was stressed under high light.

These characteristics would provide adaptive advantages for *Metaequoia* to adapt to low intensity, low angle, polar light at their Eocene high latitude paleo-environments, particularly during the polar spring when light levels are exceedingly low. It provides evidence to explain why *Metasequoia* was the dominant tree species in Eocene high latitudes.

The thesis is written as an article to be submitted to the American Journal of Botany.

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1. INTRODUCTION

Plant leaves show great developmental plasticity in adapting to sunny and shady habitats (Arens, 1997; Vogel, 1968). Within species, leaves may develop different morphologies and anatomies to adapt to different light conditions. DeLucia et al. (1996) noted that phenotypically, within and between species, the differences in leaf anatomy are most evident for plants growing in different light regimes. Light intensity can regulate photomorphogenesis and development of plant leaves and chloroplasts (Lichtenthaler et al., 1984). Ustin et al. (2001) noted that the light environment during leaf development can produce varying anatomical characteristics that can affect subsequent physiological functioning of leaves. These changes include changes in structural characteristics of plastids which may affect their function. Studies on how plastids respond to different light regimes are important to understanding photosynthetic performance and adaptation of plant leaves.

Leaves of sciaphilic (shade loving) plants are generally characterized by thinner laminas with thin cuticles, chloroplasts with larger grana stacks, and a higher proportion of spongy (compared to palisade) mesophyll than leaves of heliophilic plants (Boardman, 1977; DeLucia et al., 1996; Ida, 1981; Rabinowitch, 1945; Vogel 1968). The cells comprising the epidermis in the leaves of shade plants often have convex outer walls that may act as lenses focusing light to mesophyll cells (Bone et al., 1985; Lee 1986; Poulson and DeLucia, 1993; Smith et al., 1997; Vogelmann, 1993). Bone et al. (1985) with a computer simulation demonstrated the potential focusing function of the convex epidermal wall. These "focusing" epidermal surfaces, characteristic of many shade-adapted plants, may focus light so that the amount

reaching the chloroplasts of mesophyll cells can be many times more intense than the incident, diffuse ambient light (Lee 1986; Vogelmann, 1993; Vogelmann, 1996).

It is rare for higher plants to develop functioning chloroplasts in epidermal cells. However, epidermal cell chloroplasts are common in the leaves of extreme-shade-adapted ferns (Eames et al., 1925; Lee, 1986) and submerged leaves of water plants (Cutter 1971; Fahn, 1982). Shade adapted species of *Selaginella* have large cup-shaped chloroplasts in epidermal cells (Jagels, 1969; Jagels, 1970; Lee 1986). Submerged leaves of *Ranunculus fluitans* (Cutter, 1971) and seagrass leaves of *Thalassia testudinum*, *Zostera marina* and *Ruppia martima* (Jagels, 1983 and 1989) also have well-developed chloroplasts in the epidermis.

Extant natural stands of *Metasequoia* (*Metasequoia glyptostroboides* Hu et Cheng) are quite restricted: a few trees are found as isolated populations in shady wet areas in eastern Sichuan province, northwestern corner of Hubei province and northwestern Hunan province in China at about 30° N latitude and 108° E longitude (Chu and Cooper, 1951; Shao, 1982). *Metasequoia* has indeterminate shoot growth. With adequate moisture, new leaves are produced throughout the growing season on two kinds of shoots, long persistent and short deciduous. The lamina of the leaves are bifacially flattened, thin, linear and obtusely pointed. The adaxial surface is bright green, with a narrowly grooved midvein; stomata are restricted to the abaxial surface. In autumn, the leaves turn reddish-brown before they are shed with the deciduous branchlets (Jagels and Day, 2004).

Metasequoia is a relict species that was once widely dispersed on lowland wet sites at very high latitudes (up to 80°N) about 45 million years ago (Jagels and Day

2004; Momohara, 1994). At these latitudes, plants received continuous diffuse and low angle light during the growing season (Jagels and Day, 2004). In early spring, when foliage expanded, light levels were below or slightly above the compensation point of photosynthesis (Berner, 1991; Greenwood and Wing, 1995; Jagels and Day, 2004; Pielou, 1994).

Evidence based on morphology, physiology, biochemistry and molecular biology suggests that *Metasequoia* has undergone little evolutionary change since the Tertiary period (Anderson and LePage, 1995; Jagels and Day 2004; Jagels and Equiza, in press; Kuser, 1998; Li et al., 1999; Liu et al., 1999; Vann et al., 2004; Yang and Jin 2000). Thus, *Metasequoia glyptostroboides*, is considered to be a useful nearest living relative (NLR) of the Eocene fossil *Metasequoia* (Jagels and Day, 2004; Jagels and Equiza, in press; Van et al., 2004). Research on modern *Metasequoia* might give us some clues about its fossil counterpart.

Metasequoia glyptostroboides, *Glyptostrobus pensilis* K.Koch, *Sequoia sempervirens* (D.Don) Endlicher and *Taxodium distichum* (L.) Rich are members of the Family Cupresseacae. In addition, the extant natural habitats of these four species share some common characteristics: *Metasequoia* trees are typically found on moist, partially shaded sites such as stream banks in ravines. *Glyptostrobus* trees are confined to swamp-like habitats in the paratropical forests of southeastern China (Dallimore et al., 1967; Vidakovic, 1991) and central highlands of southern Vietnam (Dak Lak Provincial FPD, 1998). *Sequoia* trees are restricted to moist California coastal areas exposed to significant fog during the growing season (Olson et al., 1990; Ornduff, 1998). *Taxodium* grows on saturated and seasonally inundated soils of the

southeastern coastal plains of North America (McWilliams et al., 1998; Wilhite and Toliver, 1990). A comparative study of the leaf anatomy of the four species may give us some clues to better understand the eco-physiology of *Metasequoia*.

Jagels and Day (2004) noted the presence of green chloroplasts in abaxial epidermal cells of *Metasequoia* leaves after examination of freehand sections by light microscopy. In a study of Ida (1981) on the effects of light intensity on the content and composition of leaf pigments, the chlorophyll content of *Metasequoia* foliage was found to increase progressively with reduced light intensities reaching a limit at 7% of natural full light. It has long been known that light is necessary for leaves to turn green in angiosperms but algae and some gymnosperms are able to produce chlorophyll in complete darkness (Bogorad, 1950; Burgerstein, 1900; Ida, 1981; Kirk and Tilney-Bassett, 1967; Laudi and Manzini, 1975; Laudi and Medeghini-Bonatti, 1973; Lewandowska and Őquist, 1980; Selstam and Widell, 1986; Wieckowski and Goodwin, 1967). Laudi and Manzini (1975) demonstrated that *Metasequoia* was able to synthesize chlorophyll in complete darkness and the chlorophyll content in darkened leaves, though much lower than in illuminated ones, was greater than that found in etiolated leaves of other plants. Jagels and Day (2004) proposed that this could provide adaptive advantages in the low light environment of the highest latitudes.

In this study I compared the anatomy of *Metasequoia* leaves with that of *Glyptostrobus*, *Sequoia* and *Taxodium* specifically. Specific questions addressed were (1) do these species have chloroplasts in their epidermal cells and (2) does *Metasequoia* foliage exhibits characteristics consistent with sciaphilic plants.

Additionally I evaluated plastid development in *Metasequoia* leaves, and plastid response to different light intensities was investigated. This information should provide insight into whether the modern species retains uncommon characteristics that were adaptive to temperate high-latitude environments, such as an ability to initiate chlorophyll synthesis under the very low light levels expected in the early spring at high latitudes.

2. MATERIALS AND METHODS

For the comparison of leaf anatomy, *Metasequoia* leaf samples were obtained from eight trees (1-2 years old) growing in a greenhouse at the University of Maine. *Glyptostrobus* leaf samples were collected from 4 young trees (1-2 years old) growing in the same greenhouse. *Sequoia* leaf samples were also collected from young trees (1-2 years old) in the greenhouse. *Taxodium* leaves were collected from a tree (about 20+ years old) on campus. The trees in greenhouse were grown at about 22-30 °C and under natural photoperiod with sufficient water supply. *Metasequoia* leaf samples were collected during the growing season of 2002 and 2003. Collections of *Glyptostrobus* leaf samples were made at the beginning of June in 2003 and in mid-February of 2004. *Sequoia* and *Taxodium* leaves were sampled in early September of 2001.

For developmental studies, *Metasequoia* leaf samples were obtained from 8 young trees growing in the greenhouse at the University of Maine. These trees were grown at temperature ranging from 20°C to 30°C under natural photoperiod with sufficient water. Before sampling, the trees were in winter dormancy. The first samples with emerging buds and partially expanded leaves were collected from the same tree at the same time on January 23 (Day1), about 20 days after bud break. Subsequent collections of expanded leaves were made about every 8-10 days, on February 2 (Day 11), 11 (Day 20), 20 (Day 29) and 28 (Day 37). Mature leaves were collected during July (Day 188). During each collection, about 6-10 leaves of similar size at the same developmental stage were sampled, and 2-3 sections were examined with a microscope.

To observe leaf plastid responses to different light regimes in *Metasequoia*, two experiments were established. In the first experiment, 8 young *Metasequoia* trees were grown in the greenhouse at the University of Maine under three different light environments: natural ambient high light (The maximum light intensity reached inside the greenhouse was about 700-1500 μmol $m^{-2}s^{-1}$), moderate shade (about 10% of ambient light inside the greenhouse) and dense shade (about 1.5% of ambient light inside the greenhouse). One or two layers of 90% black interception black shade cloth provided shade. The shade cloth was installed in early February. Samples were collected in June and July of 2003. During each collection, about 6-10 leaves with similar size at the same developmental stage were sampled and 2-3 sections were examined microscopically.

In the second experiment, nine *Metasequoia* trees were grown in growth chambers under three treatments with three light intensities modified with time: (1) complete darkness, (2) low light intensity (about 10-15 μmol $m^{-2}s^{-1}$) and (3) moderate light intensity (about 150 μmol $m^{-2}s^{-1}$). In the lighted treatments, photoperiod was increased every week to imitate seasonal changes at the high latitudes. The light source included both cool-white fluorescent and incandescent lamps. Three dormant seedlings were given each treatment. The seedlings had previously been stored for 3 months in a 4°C dark chamber to maintain dormancy. Temperature in the growth chambers was $\pm$ 25°C and soil moisture was maintained near field capacity.

The experiment began on June 25. Leaf samples from the complete darkness treatment were collected on July 28, 2003, 33 days after the experiment was initiated. The leaves were collected from expanding buds. Leaf samples from the low and

moderate light intensity treatment (11 hour day-length) were collected on July18, 2003, 23 days after treatment was initiated. The leaves were enlarging but were not totally expanded. During each collection, about 6-10 leaves of similar size at the same developmental stage were sampled and 2-3 sections were examined microscopically.

Microscopy:

Immediately after collection, the mid-portion of leaves was cross-sectioned into 1 mm wide segments while immersed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0). Segments were vacuum-infiltrated until they sank and were fixed overnight in fresh fixative at 0-4°C. Specimens were subsequently buffer-washed for 4 changes (1 hour per change) on ice, post-fixed overnight in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.0) at 0-4°C. They were then distilled water-washed for 4 changes (0.5 hour per change) on ice, dehydrated in a graded acetone series, infiltrated through a graded acetone-Spurr's resin mixture and embedded overnight in Spurr's resin at 55-60°C (Spurr, 1969). These procedures were modified from methods of Bozzola and Russell (1999), Gabara et al. (2003), Hayat (2000) and Laudi and Manzini (1975).

For light microscopy, thick transverse sections (8-10 μm) were cut on a JB-4 microtome (Ivan Sorvall Inc.) and stained with 1% toluidine blue O (Trump et al., 1961) and examined with an Axioskop light microscope (Zeiss Inc., Germany). Images were digitally captured using a SPOT RT camera (Diagnostic instruments Inc., USA) and associated software.

Ultra-thin transverse sections (50-100 nm) were cut on a MT 2-B ultra-microtome (Ivan Sorvall Inc., USA), stained with uranyl acetate for 30 minutes and

lead citrate for 15 minutes (Reynolds, 1963), and examined with a Phillips CM10 transmission electron microscope (Phillips, Netherlands) operated at 80KV. Images were captured using a BioScan 792 camera (Gatan Inc., USA) and associated DigitalMicrography software (Gatan, Inc., USA). Digital images were later processed using Adobe Photoshop7.0 software (Adobe Systems Inc, USA) or ACD FotoCanvas Lite 2.0 (ACD System Inc., USA).

3. RESULTS

3.1. Comparative anatomy of *Metasequoia*, *Glyptostrobus*, *Sequoia* and *Taxodium* leaves

3.1.1. Leaf anatomy of *Metasequoia glyptostroboides* Hu et Cheng

The *Metasequoia* leaf has a median resin canal under a single vascular bundle, and two marginal resin canals (Plate 1, Figure 1). The epidermis is a single peripheral layer of cells. The outer walls of epidermal cells are thicker on the adaxial surface than the abaxial surface (Plate 1, Figure 2). A hypodermis is lacking. The leaf interior is composed of spongy mesophyll cells with numerous plastids (Plate 1, Figure 2). The adaxial epidermal cells of *Metasequoia* leaves are characterized by slightly arched outer walls and the abaxial counterparts characterized by strongly arched outer cell walls (Plate 1, Figure 2 and 3; Table 1). Stomata are confined to the abaxial surface where they form 2 bands on either side of the mid-vein (Table 2). Stomata have partially over-arching subsidiary cells but the guard cells are not sunken (Plate 1, Figure 3; Plate 2, Figure 1 and 2; Table 1). The guard cells contain chloroplasts with few grana and large quantities of starch (Plate 2, Figure 3 and 4).

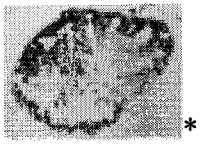
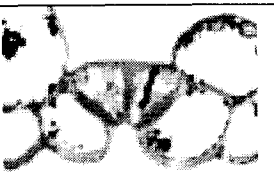
Chloroplasts with grana are found in both the abaxial and adaxial epidermal cells in *Metasequoia* leaves (Table 1) and are located in the parietal cytoplasm adjacent to anticlinal or periclinal interior walls (Plate 3, Figure 1 and 3). The chloroplasts in epidermal cells are smaller than those in mesophyll cells, but they have well-developed grana (Plate 3, Figure 2 and 4). Chloroplast morphology is similar in the adaxial and abaxial epidermis but the plastids are converted to amyloplasts sooner in the adaxial epidermis than the abaxial epidermis.

Table 1. Comparison of anatomical features among *Metasequoia glyptostroboides*, *Glyptostrobus pensilis, Sequoia sempervirens* and *Taxodium distichum.*

Species / Features	***Metasequoia glyptostroboides***	***Glyptostrobus pensilis***	***Sequoia sempervirens***	***Taxodium distichum***
Foliar shape *	Flattened thin, linear needles	Scale-like or needle-like, 4 sided	Linear, generally flattened needles	Linear, generally flattened needles
Foliar longevity	Deciduous	Deciduous	Evergreen	Deciduous
Stomata distribution*	Hypostomatic	Amphistomatic	Amphistomatic	Amphistomatic
Stomata position*	Not sunken, but subsidiary cells raised	Slightly sunken	Slightly sunken	Not sunken, but subsidiary cells raised
Plastids in abaxial epidermis	Chloroplasts present	Mostly amyloplasts	Chloroplasts present	Chloroplasts present
Plastids in adaxial epidermis	Chloroplasts present	Mostly amyloplasts	Chloroplasts present	Chloroplasts present
Adaxial epidermal outer wall	Slightly arched	Not arched, similar for 4 faces	Slightly arched	Slightly arched
Abaxial epidermal outer wall	Strongly arched	Not arched, similar for 4 faces	Strongly arched	Strongly arched

* See Table 2 for further details.

Table 2. Comparison of leaf shape and stomata among *Metasequoia glyptostroboides*, *Glyptostrobus pensilis, Sequoia sempervirens* and *Taxodium distichum.*

Stomata / Leaf shape and leaf size	Stomata on adaxial epidermis	Stomata on abaxial epidermis	Guard and subsidiary cells of abaxial surface
Metasequoia glyptostroboides  *	Abscent	Present; numerous in 2 bands either side of mid-vein	
Glyptostrobus pensilis  *	Present; evenly distributed in 2 bands on each side of mid-vein	Present; evenly distributed in 2 bands on each side of mid-vein	
Sequoia sempervirens  *	Present but rare; generally less than 6 on entire surface, usually near the tip	Present; numerous in 2 wide bands either side of mid-vein	
Taxodium distichum  *	Present but sparse; in 2 single rows either side of mid-vein	Present; numerous in 2 wide bands on either side of mid-vein	

* all same scale.

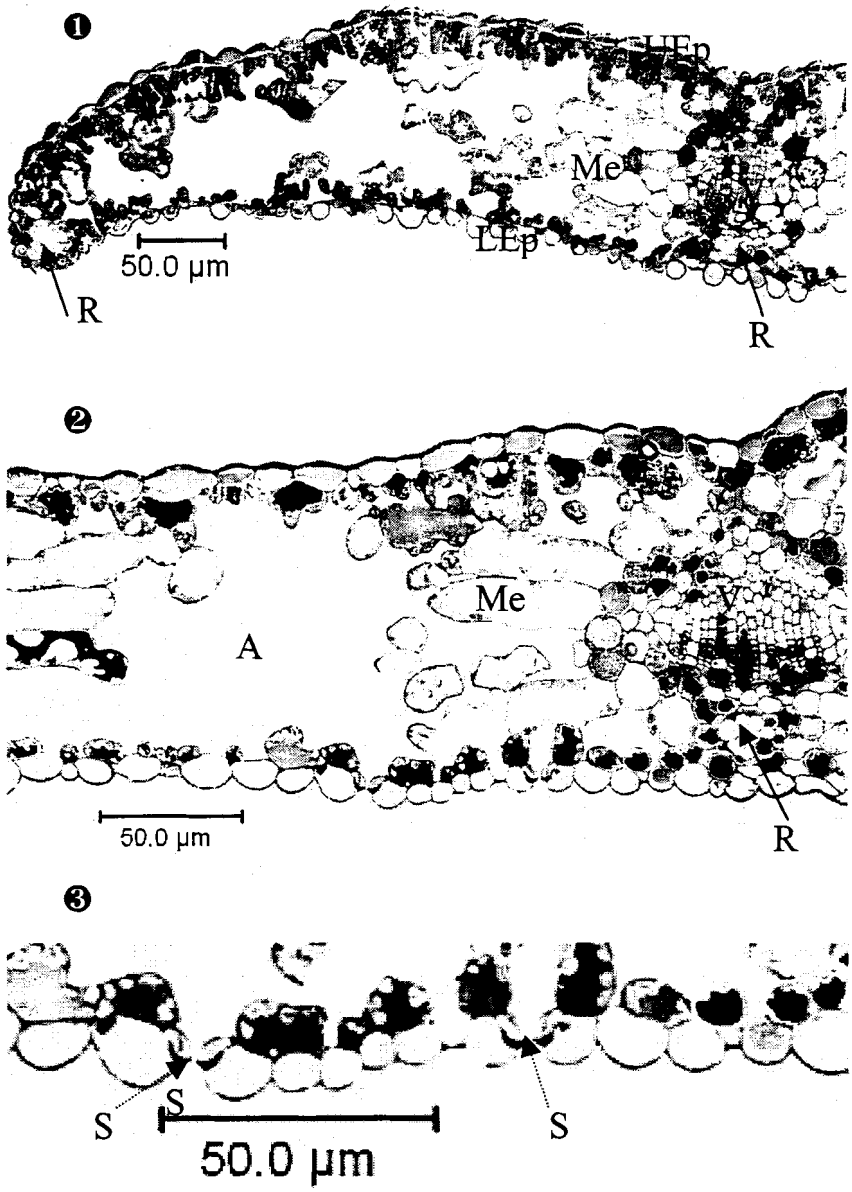


Plate 1. Leaf anatomy of *Metasequoia glyptostroboides*. –Figure 1: A light photomicrograph showing a transverse section of leaf. –Figure 2: A Light photomicrograph showing the thicker outer epidermal cell walls in the adaxial surface and the arched outer epidermal cell walls in the abaxial surface. –Figure 3: A light photomicrograph showing stomata (S) in the abaxial leaf surface.

Abbreviations: A= air space; LEp= abaxial epidermis; Me= mesophyll; R= resin canal; S= stomata; V= vascular bundle; UEp= adaxial epidermis.

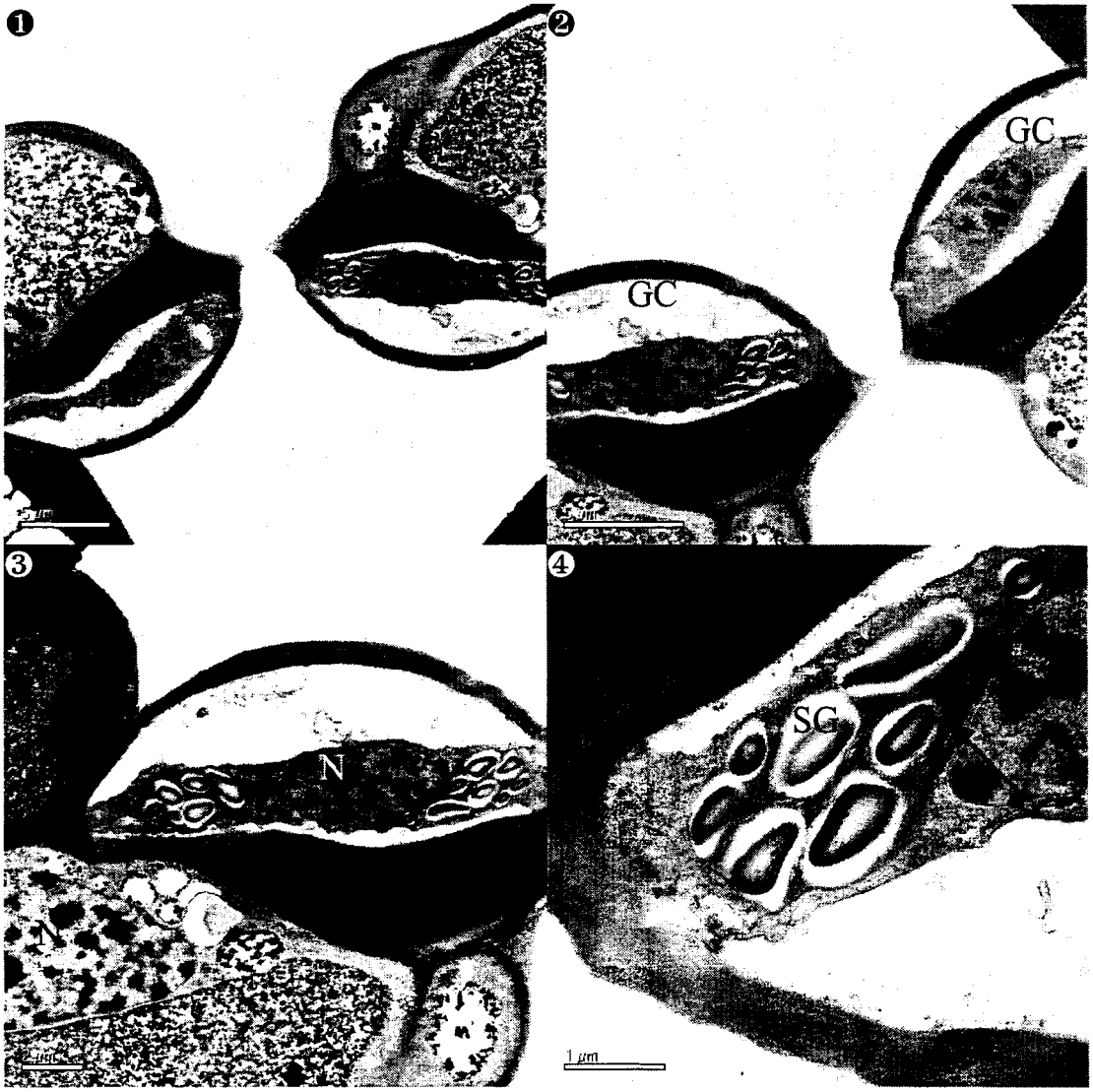


Plate 2. Leaf stomata of *Metasequoia glyptostroboides*. –Figure 1 and 2: Electron photomicrographs showing stomata. –Figure 3 and 4: Electron photomicrographs showing large starch grains and few grana (Arrow) in the plastids in guard cells.

Abbreviations: GC= guard cell; N= nucleus; SG= starch grain.

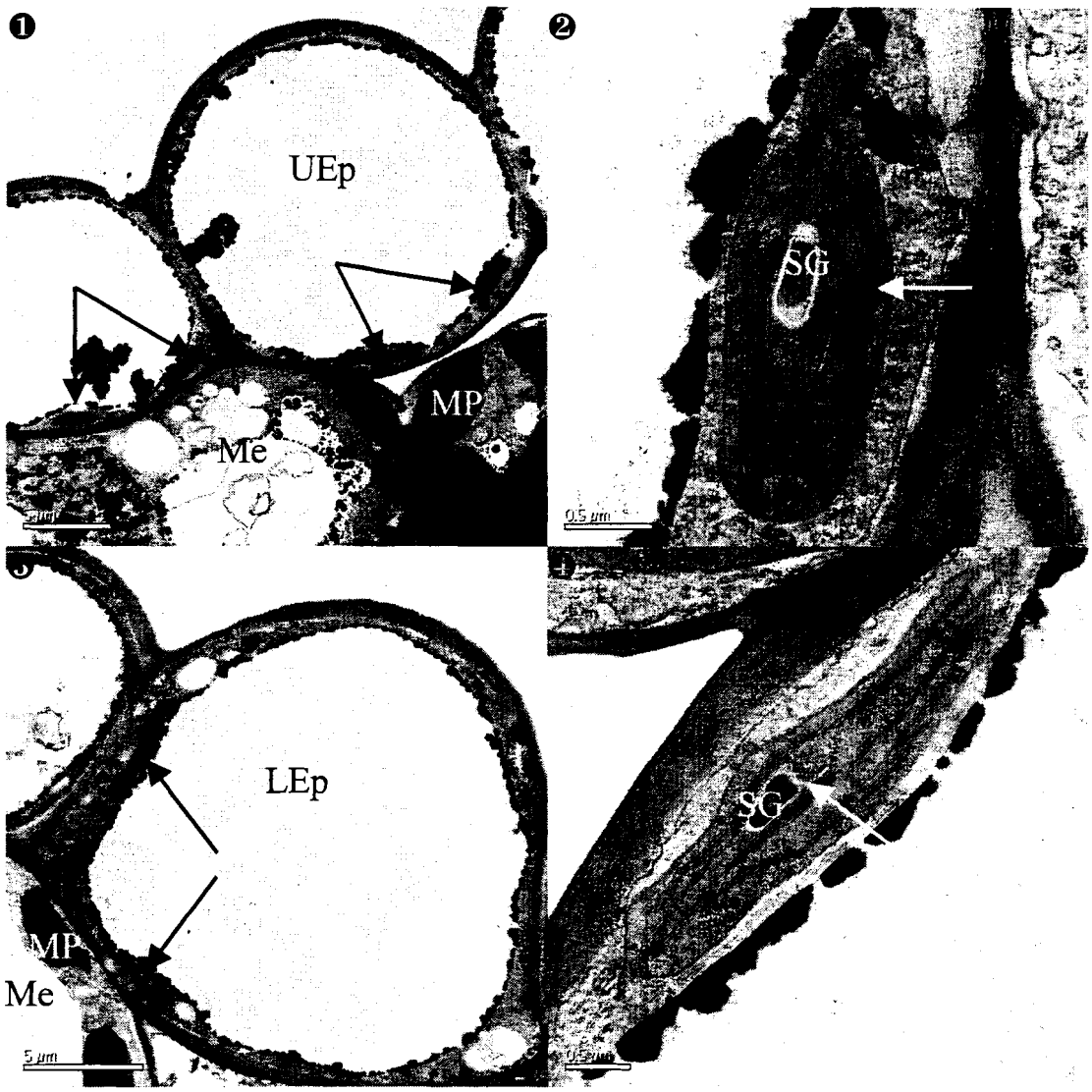


Plate 3. Chloroplasts in adaxial and abaxial surfaces of *Metasequoia glyptostroboides* leaf. –Figure 1: An electron photomicrograph showing small chloroplasts (Black arrow) in the adaxial epidermis. –Figure 2: An electron photomicrograph showing a chloroplast with grana (White arrow) in the adaxial epidermis with a higher magnification. –Figure 3: An electron photomicrograph showing small chloroplasts (Black arrow) in the abaxial epidermis. –Figure 4: An electron photomicrograph showing a chloroplast with grana (White arrow) in the abaxial epidermis with a higher magnification.

Abbreviations: LEp= abaxial epidermis; Me= mesophyll; MP= mesophyll plastid; SG= starch grain; UEp= adaxial epidermis.

3.1.2. Leaf anatomy of *Glyptostrobus pensilis* K.Koch

Glyptostrobus has two kinds of shoots with long persistent branchlets and short annual deciduous branchlets. The leaves on the short shoots are needle-like. *Glyptostrobus* needles are quadrangular in transverse section (Plate 4, Figure 1; Table 2). The single-layer epidermis is thin with no subtending hypodermis. The epidermal cells on all four surfaces have non-arched outer walls, similar in shape (Plate 4, Figure 1; Table 1). The leaf interior has a multi-layered mesophyll composed with palisade and spongy cells, a central vein and resin canals. Stomata are found evenly distributed in 2 bands on each side of the mid-vein on all four surfaces and are slightly sunken (Plate 4, Figure 1; Table 1 and 2).

Amyloplasts with large starch grains, lipid droplets and a few reduced grana are found in all epidermal cells (Plate 4, Figure 2, 3 and 4; Table 1). These amyloplasts are smaller than the plastids in the mesophyll cells.

3.1.3. Leaf anatomy of *Sequoia sempervirens* (D.Don) Endlicher

Sequoia leaves are evergreen. They are linear and flattened. The epidermis consists of one layer of cells, and there is no hypodermis. The mesophyll is differentiated into obvious multi-layered spongy cells and one-layer of palisade cells (Plate 5, Figure 1). In divergence with previous report (Chaurvedi, 1998), slightly sunken stomata are found on both the adaxial and abaxial surfaces. Stomata are extremely rare on the adaxial surface with generally less than 6 stomata on entire surface, usually near the tip. Numerous stomata in 2 wide bands on either side of the mid-vein are observed on the abaxial surface (Table 1 and 2). The adaxial surface has

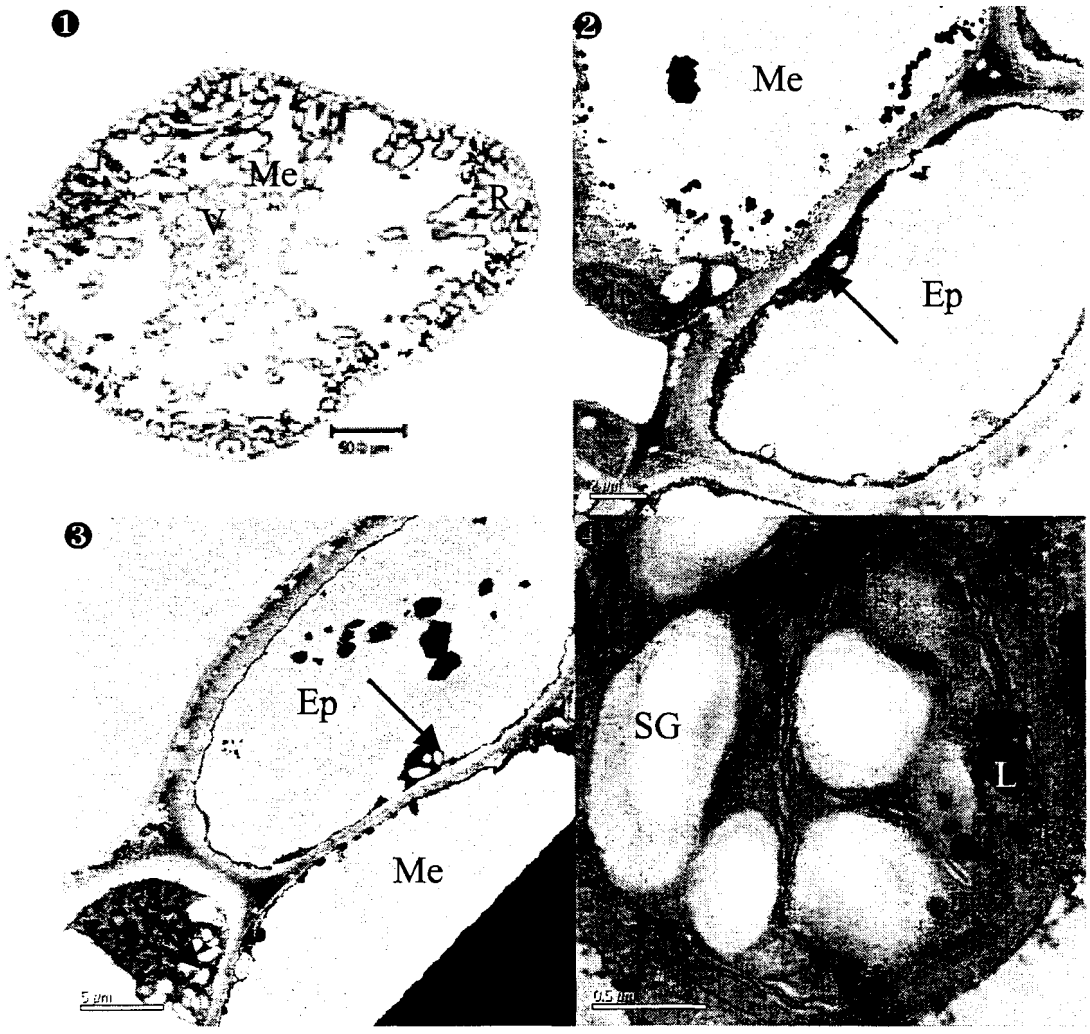


Plate 4. Anatomy of *Glyptostrobus pensilis* leaf. -Figure 1: Transverse section of leaf with stomata on the four epidermal surfaces. –Figure 2 and 3: Electron photomicrographs showing amyloplasts (Black arrows) in epidermal cells. –Figure 4: An electron photomicrograph showing the details of an amyloplast in leaf epidermal cells.

Abbreviations: Ep= epidermis; L= lipid droplet; Me= mesophyll; MP= mesophyll plastid; R= resin canal; SG= starch grain, V=vascular bundle.

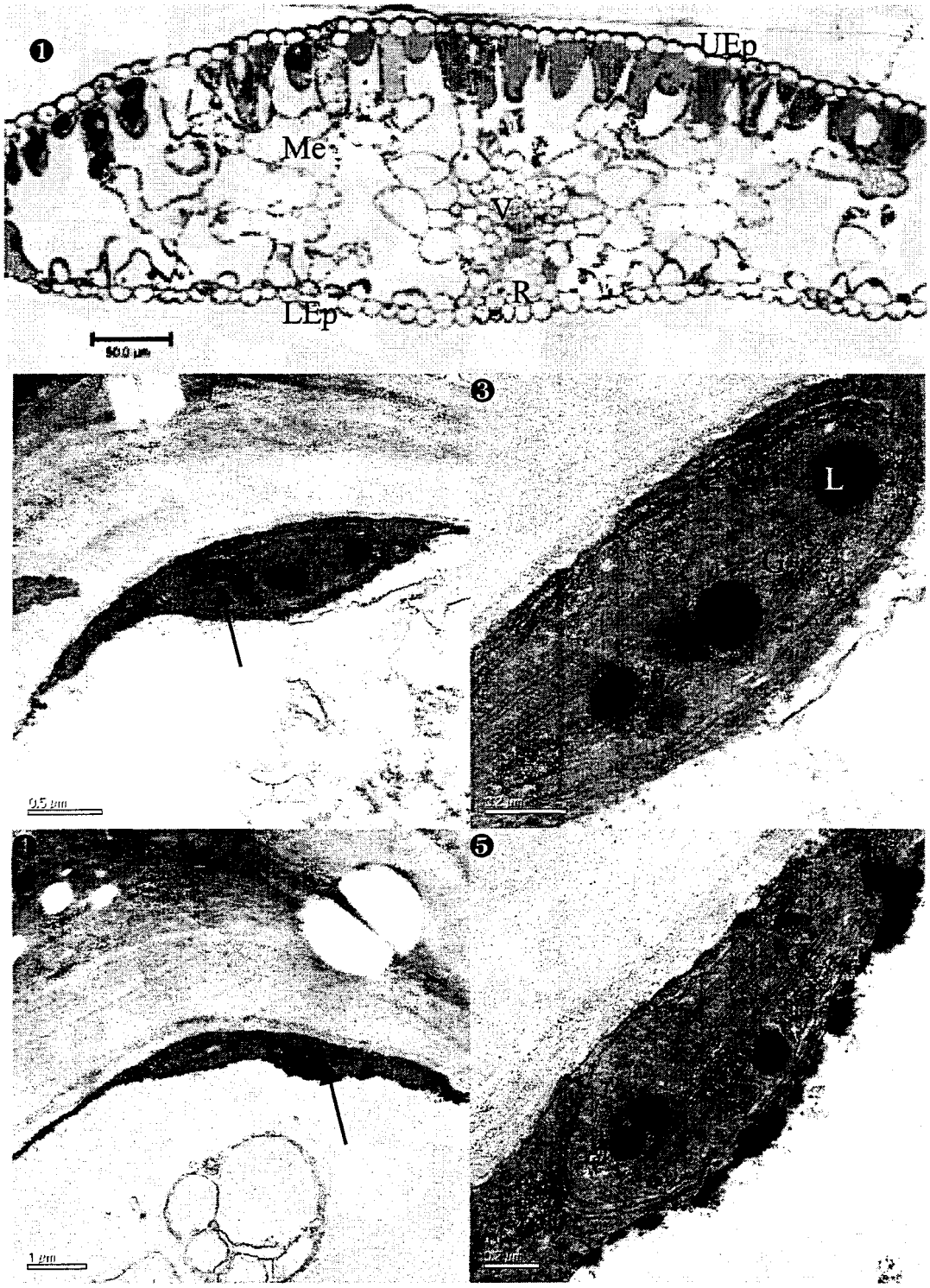


Plate 5. Anatomy of *Sequoia sempervirens* leaf. –Figure 1. Leaf transverse section. –Figure 2 and 3. Chloroplast (Black arrow) in the adaxial epidermis. –Figure 4 and 5. Chloroplast in the abaxial epidermis.
Abbreviations: G= grana; L= lipid droplet; LEp= abaxial epidermis; Me= mesophyll; R= resin canal; V= vascular bundle; UEp= adaxial epidermis.

slightly arched outer cell walls of epidermal cells while the abaxial surface has more strongly arched walls (Plate 5, Figure1; Table 1).

Chloroplasts with grana are found in both abaxial (Plate 5, Figure 2 and 3; Table 1) and adaxial (Plate5, Figure 4 and 5; Table 1) epidermal cells in *Sequoia* leaves, with characteristics similar to those found in *Metasquoia*.

3.1.4. Leaf anatomy of *Taxodium distichum* (L.) Rich

The flattened linear leaves of *Taxodium* are deciduous. The epidermis is composed of a single layer, lacking a hypodermis (Plate 6, Figure 1). The mesophyll consists of one-layer of palisade cells and several-layers of irregularly shaped spongy cells (Plate 6, Figure1). Stomata are found on both the adaxial and abaxial surfaces and they have over arching subsidiary cells but as in *Metasequoia* the guard cells are not sunken (Table 1 and 2). There are few stomata in a single row either side of the mid-vein in the adaxial epidermis and many in two wide bands on either side of the mid-vein in the abaxial epidermis (Table 1 and 2). Slightly arched outer cell walls are found in epidermal cells on the adaxial surface and strongly arched outer cell walls are found in the abaxial surface (Plate 6, Figure 1; Table 1). Chloroplasts with grana are also found in both the adaxial (Plate 6, Figure 2 and 3; Table 1) and abaxial (Plate 6, Figure 4 and 5; Table 1) epidermal cells.

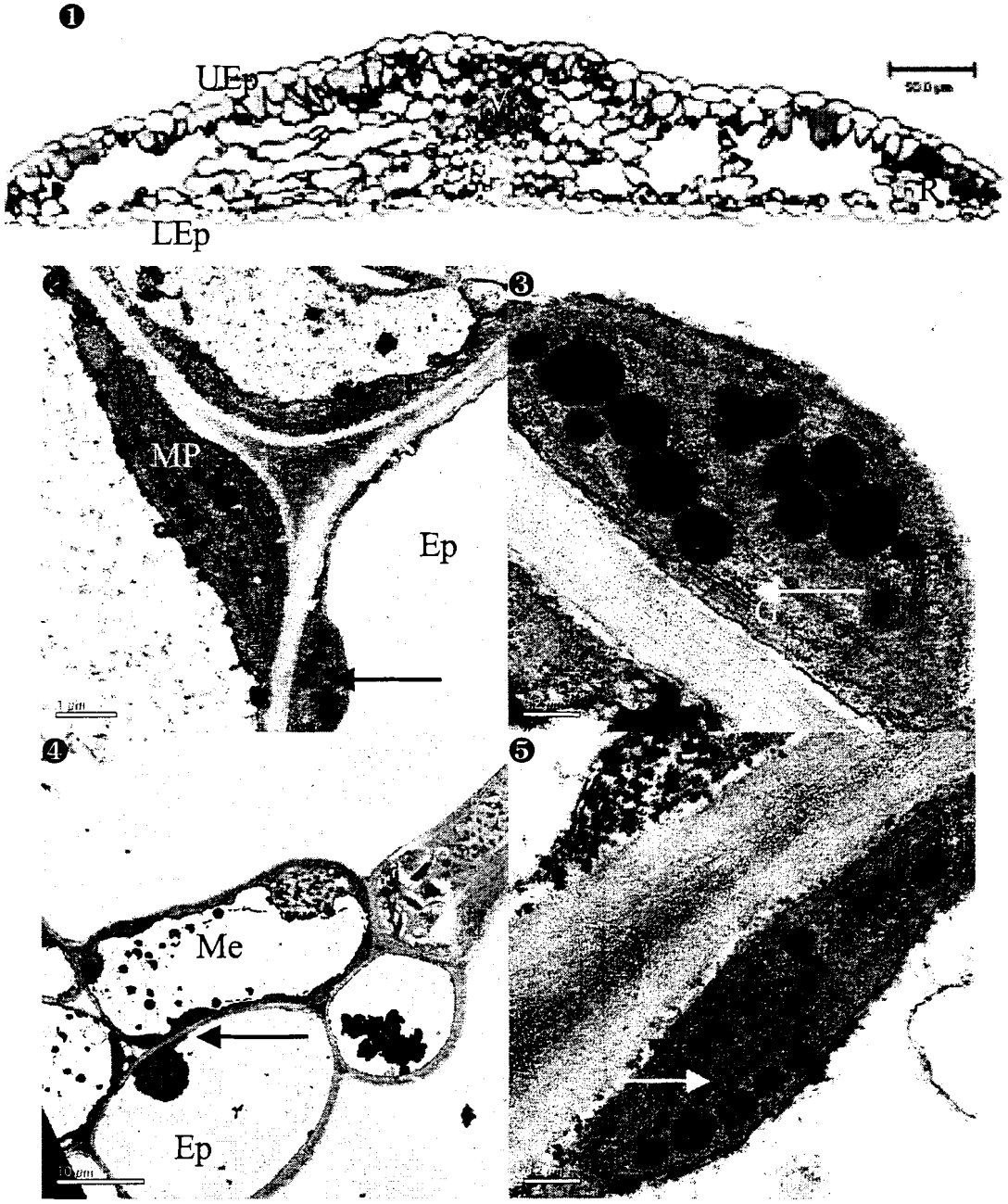


Plate. 6. Anatomy of *Taxodium distichum* leaf. –Figure 1: Leaf transverse section. –Figure 2 and 3: Electron photomicrographs showing chloroplasts (Black arrow) with grana (White arrow) in the adaxial epidermis. –Figure 4 and 5: Electron photomicrographs showing chloroplasts (Black arrow) with grana (White arrow) in the abaxial epidermis.

Abbreviations: Ep= epidermis; G= grana; L= lipid droplet; LEp= abaxial epidermis; Me= mesophyll; MP= mesophyll plastid; R= resin canal; V= vascular bundle; UEp= adaxial epidermis.

3.2. Development of mesophyll plastids in *Metasequoia* leaves

Mesophyll cells of leaves from emerging buds (Day 1) had several small vacuoles and plastids with prolamellar bodies and attached developing membrane system (Plate 7, Figure 5 and 6; Table 3). Some mesophyll cell chloroplasts had long stroma thylakoids (Plate 7, Figure1 and 2; Table 3) and some had grana with up to 9 thylakoids per granum (Plate 7, Figure 3 and Table 3). The mesophyll chloroplasts did not contain starch grains although starch grains were found in epidermal cell chloroplasts (Plate 7, Figure 4; Table 3).

On Day 1, samples were taken from both emergent buds and partially expanded leaves on the same tree. The partially expanded leaves had more intercellular spaces around the mesophyll cells than seen in emerging buds. In addition, more stacked grana (up to 15 thylakoids per granum) were present (Plate 8, Figure 2 and 3; Table 3), but few starch grains or lipid droplets were visible at this stage (Plate 8, Figure 1 and 3; Table 3). Prolamellar bodies were no longer observed.

The mesophyll cell chloroplasts in fully expanded leaves sampled on Day 11 showed a typical well-developed thylakoid system (Plate 8, Figure 4, 5 and 6). The number of grana had increased and each contained up to 20 thylakoids per granum (Plate 8, Figure 5 and 6; Table 3). Starch grains and lipid droplets were still rare (Table 3).

Starch grains and lipid droplets were more common in the mesophyll chloroplasts in expanded leaves sampled on Day 20 (Plate 9, Figure 1; Table 3) and Day 29 (Plate 9, Figure 2; Table 3). In mesophyll chloroplasts of leaves sampled on

Plate 7. Plastid development in the mesophyll of *Metasequoia glyptostroboides* leaves I: Mesophyll plastids in *M.glyptostroboides* emerging buds sampled on Day 1. – Figure 1-2: Mesophyll chloroplasts with long stroma thylakoids (ST). –Figure 3-4: Prolamellar bodies (P) in mesophyll cells with developing thylakoid membrane systems. –Figure 5: Mesophyll chloroplasts with grana. –Figure 6: Epidermal chloroplasts with grana and starch grains.

Abbreviations: G= grana; L= lipid droplet; Me= mesophyll; N= nucleus; P= prolamellar body; V= vacuole; SG= starch grain; ST= stroma thylakoid.

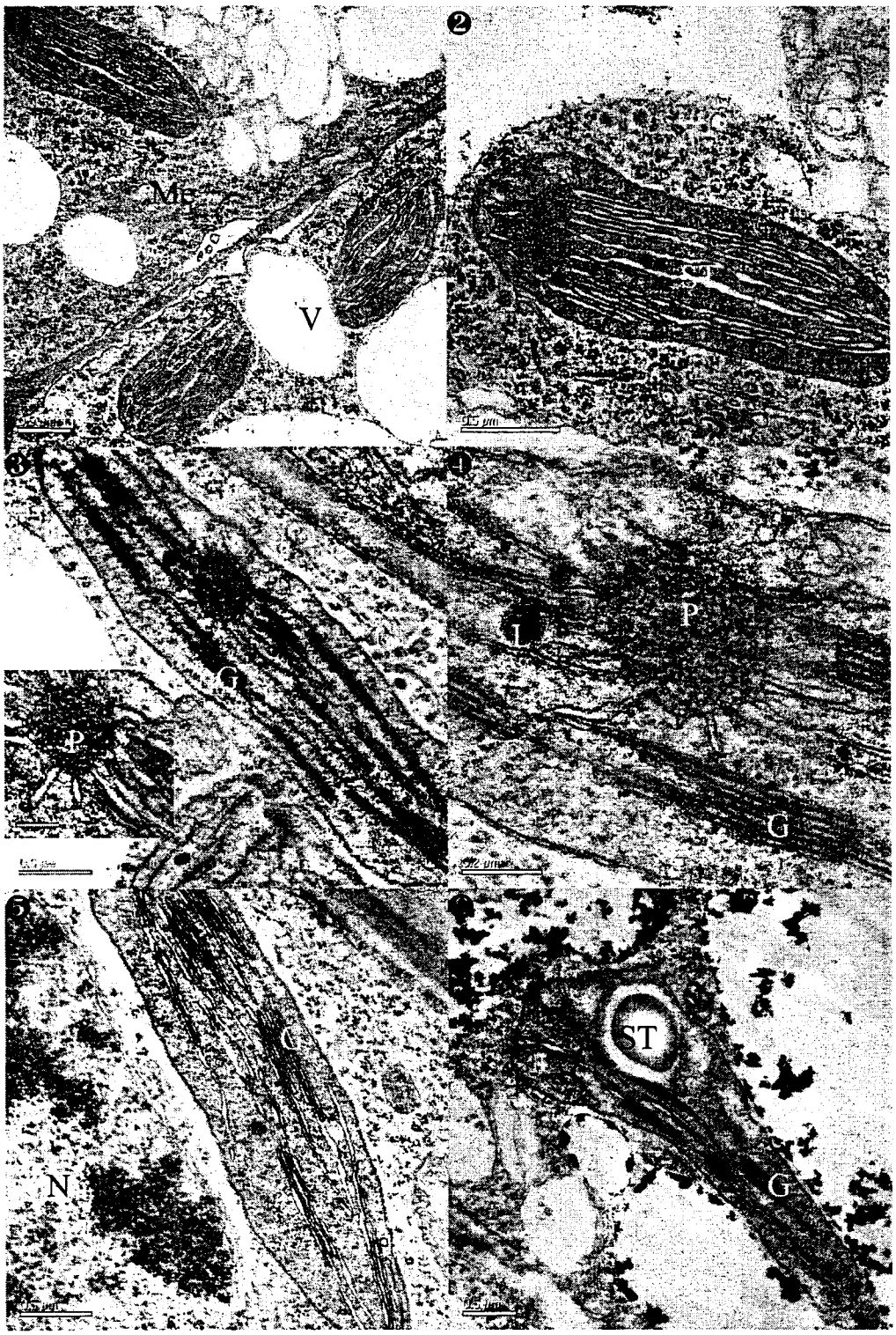

1
Me
V
2
G
3
G
P
4
L
P
G
5
G
N
6
ST
G

Plate 8. Plastid development in the mesophyll of *Metasequoia glyptostroboides* leaves II. –Figure 1-3: Mesophyll chloroplasts (C) with grana (G) in partially expanded leaves sampled on Day 1. –Figure 4 -6: Mesophyll chloroplasts with well-developed thylakoid membrane systems in expanded leaves sampled on Day 11.

Abbreviations: C= chloroplast; G= grana; N= nucleus.

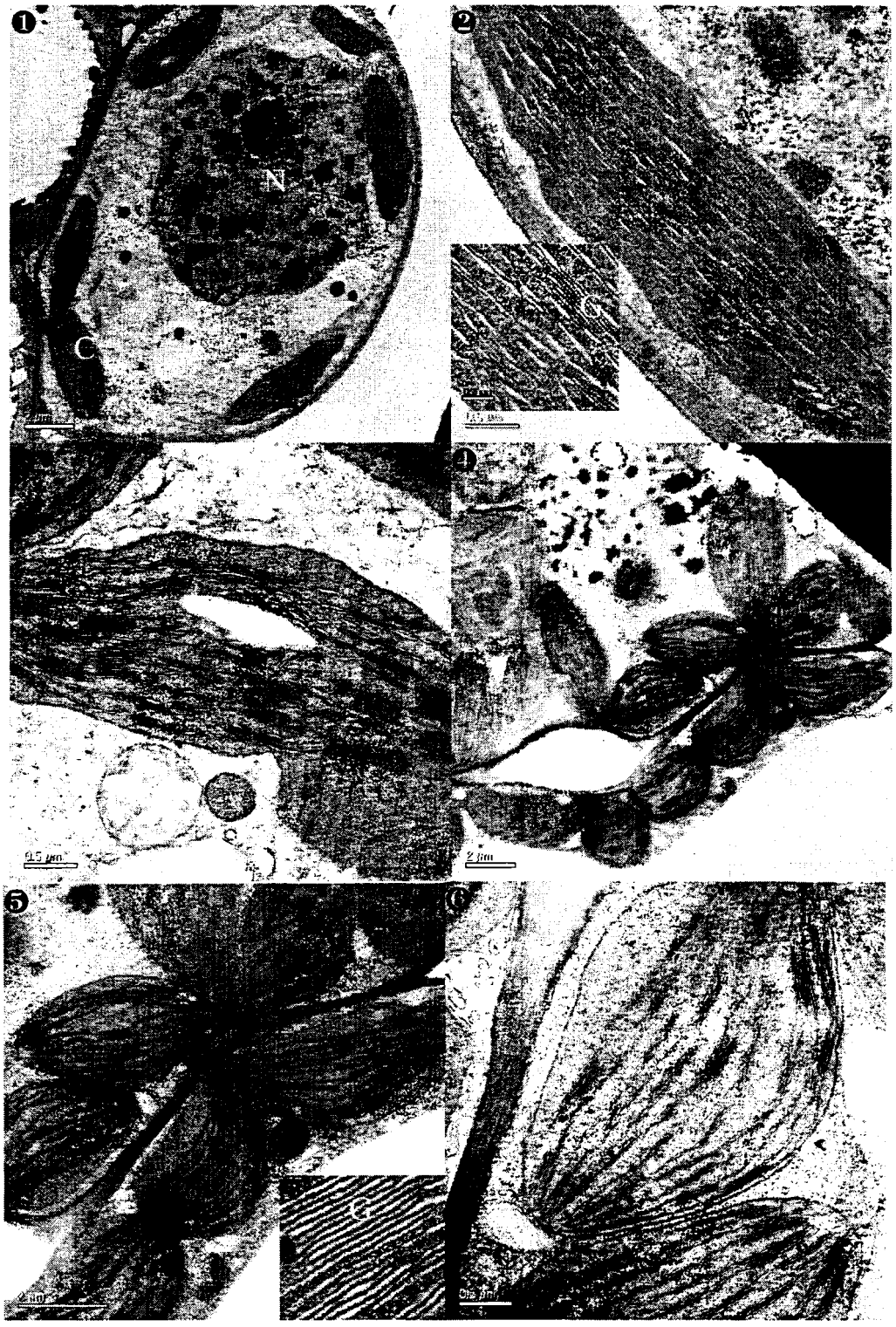

1
N
C
2
G
4
2 µm
5
G
6

Plate 9. Plastid development in the mesophyll of *Metasequoia glyptostroboides* leaves III. –Figure 1: Mesophyll chloroplasts with clear grana (G) and starch grains (ST) in expanded leaves sampled on Day 20. –Figure 2: Mesophyll chloroplasts in expanded leaves sampled on Day 29, with starch grains (ST) and swollen thylakoid membrane systems (Black arrow), showing a very early stage of senescence. –Figure 3-4: Mesophyll plastids in expanded leaves sampled on Day 37, with reduced thylakoid membrane systems (Black arrow) and large starch grains (ST). –Figure 5-6: Mesophyll plastids in expanded leaves sampled on Day 188, with poorly defined thylakoid membrane system and large starch grain (ST).

Abbreviations: G= grana; L= lipid droplet; M= mitochondrion; SG= starch grain.

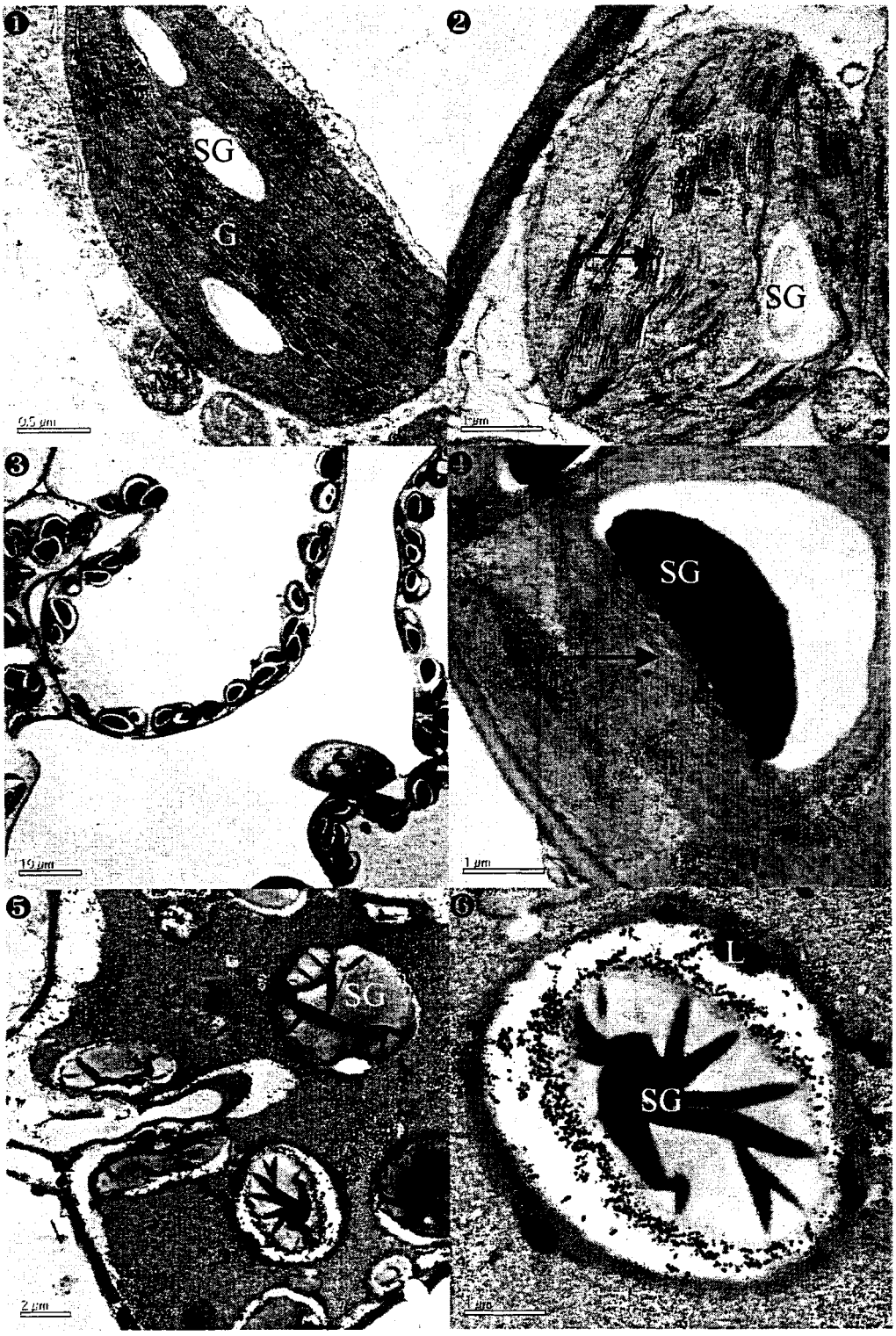
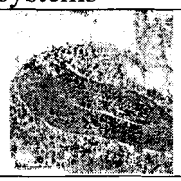
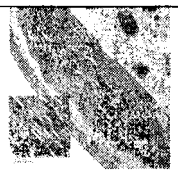
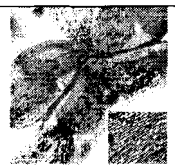
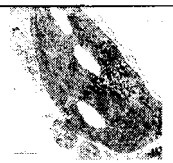
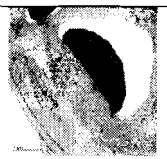
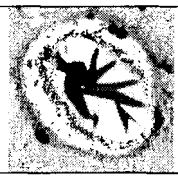

1
SG
G
0.5 µm
2
SG
1 µm
3
1 µm
4
SG
1 µm
5
SG
2 µm
6
L
SG
1 µm

Table 3. Plastid development in mesophyll cells in *Metasequoia glyptostroboides* leaves.

Features \ Leaf stage and collection day	Emerging buds Day 1	Partially expanded leaves Day 1	Expanded leaves Day 11	Expanded leaves Day 20	Expanded leaves Day 29	Expanded leaves Day 37	Fully expanded leaves Day 188
Thylakoid membrane systems	grana up to 9 thylakoids per granum; long stroma thylakoids	up to 15 thylakoids per granum	more grana with up to 20 thylakoids per granum	Similar to day 11	Reduction of grana and stroma thylakoids	Reduction of thylakoid membrane system	Few or no thylakoids
Starch grains	None	Very few	Few	Common	Common	Very common and increasing in size	Larger, occupying most of the plastid space
Lipid droplets	Few	Few	Few	Common	Common	Very common and increasing in size	Very common and increasing in size
Prolamellar body	Present, with developing membrane systems	Absent	Absent	Absent	Absent	Absent	Absent
Representative plastid							

Day 29, the thylakoid membrane system had begun to swell, losing its parallel arrangement, a sign of early senescence (Plate 9, Figure 2; Table 3).

The mesophyll cells of leaves sampled on Day 37 had a single large vacuole surrounded by a thin peripheral cytoplasm containing a nucleus and plastids (Plate 9, Figure 3). The plastids contained numerous large starch grains and lipid droplets (Plate 9, Figure 3 and 4; Table 3). Starch grains occupied large portions of the chloroplasts and the thylakoid membrane system was further reduced in extent (Plate 9, Figure 3 and 4; Table 3).

The mesophyll plastids in mature leaves sampled on Day 188 had large quantities of starch grains and lipid droplets (Plate 9, Figure 5 and 6; Table 3). The thylakoid membrane system was poorly defined and significantly reduced, showing signs of senescence with very large starch grains occupying most of the plastid volume (Plate 9, Figure 5 and 6; Table 3).

3.3. Responses of mesophyll plastids to different light regimes in *Metasequoia* leaves

3.3.1. Response of greenhouse grown *Metasequoia* after 5 months

Under dense shade (about 1.5% ambient light), mesophyll plastids of mature leaves sampled in mid-summer had some swollen membranes, small quantities of starch grains and a few lipid droplets (Plate 10, Figure 1 and 2).

Under moderate shade (about 10% ambient light), mesophyll plastids contained slightly more starch grains and lipid droplets, and the lipid droplets were larger (Plate 10, Figure 3 and 4). The thylakoid membrane systems were nearly gone (Plate 10, Figure 3 and 4)

Under high light (100% ambient light), mesophyll plastids had completely converted to amyloplasts with a single starch grain, and the thylakoid membrane systems were totally gone (Plate 10, Figure 5 and 6; Table 4). Plastids in epidermal cells showed similar responses as those in the mesophyll (Table 4).

3.3.2. Response of *Metasequoia* in growth chamber after 1 month

Under complete darkness, the plastids of mesophyll cells had distinct grana (up to 12 thylakoids per granum) connected by stroma thylakoids (Plate 11, Figure 1 and 2; Table 4). Prolamellar bodies were observed in some of mesophyll cells (Plate 11, Figure 1 and 2; Table 4). No starch grains and few lipid droplets were found. The plastids of epidermal cells were similar to those in mesophyll cells, except that they contained fewer thylakoids per granum (Table 4).

Under low light intensity (about 10-15 μmol $m^{-2}s^{-1}$), the plastids of mesophyll cells showed a normal photosynthetic apparatus. The grana (up to 16 thylakoids per

Plate 10. Responses of mesophyll plastids of *Metasequoia glyptostroboides* to different greenhouse light regimes. –Figure 1-2: Mesophyll plastids in leaf under dense shade (about 1.5% of ambient light). –Figure 3-4: Mesophyll plastids in leaf under moderate shade (about 10% of ambient light). –Figure 5-6: Mesophyll plastids in leaf under high light (natural ambient light).

Abbreviations: L= lipid droplet; SG= starch grain.

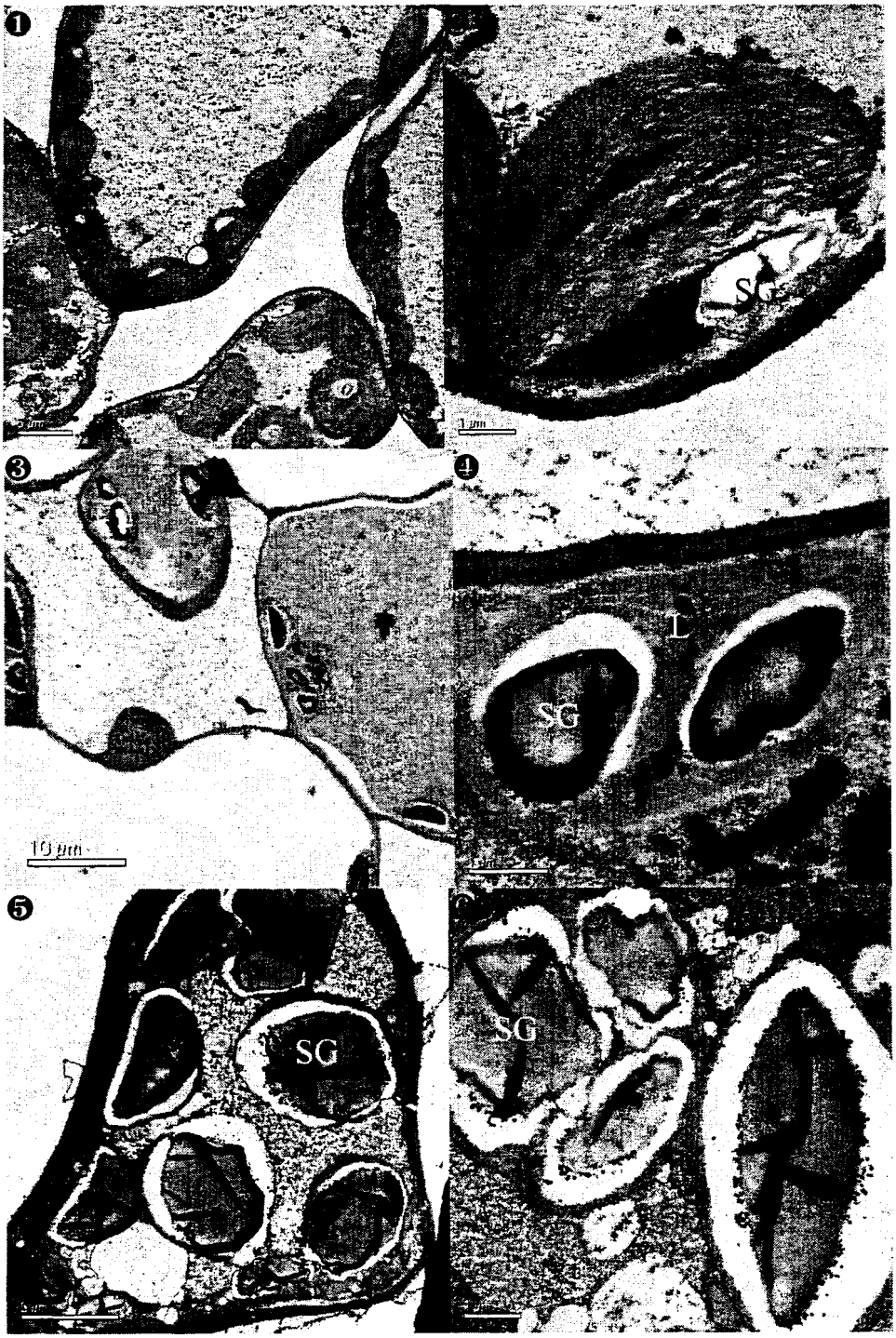

1
SG
1 μm
3
10 μm
4
L
SG
5
SG
SG

Plate 11. Responses of mesophyll plastids of *Metasequoia glyptostroboides* to different light regimes in growth chambers. –Figure 1-2: Mesophyll plastids of leaf in darkness with a prolamellar body (P) shown in Figure 2. –Figure 3-4: Mesophyll plastids of leaf under low light intensity (about 10-15 μmol $m^{-2}s^{-1}$). –Figure 5-6: Mesophyll plastids of leaf under moderate light intensity (about 150 μmol $m^{-2}s^{-1}$).

Abbreviations: G= grana; M= mitochondrion; N= nucleus; P= prolamellar body; SG= starch grain.

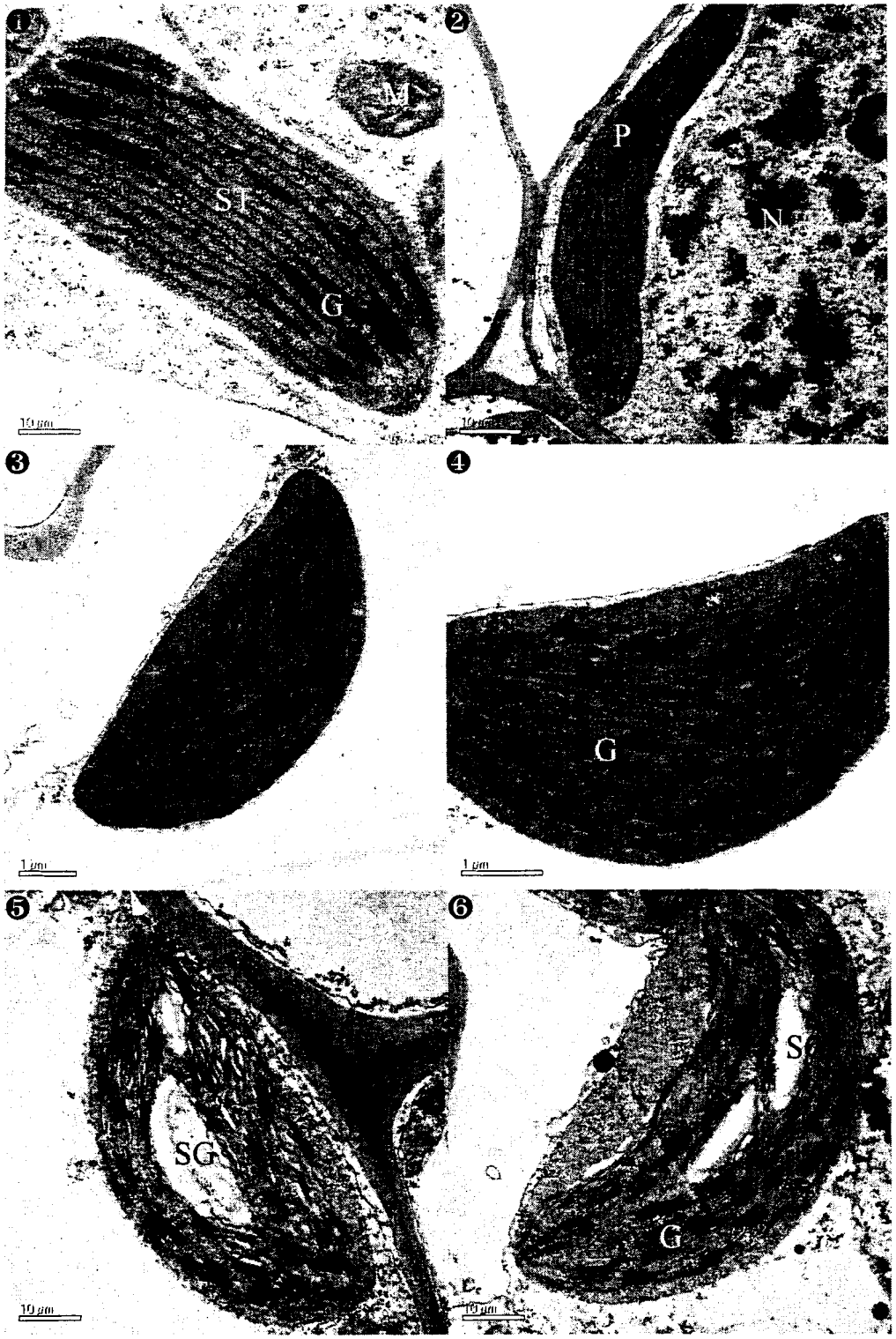
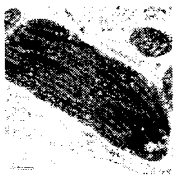
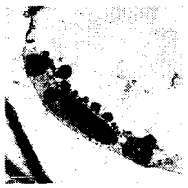
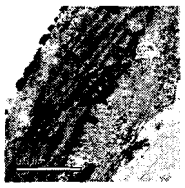
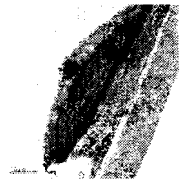
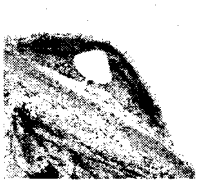
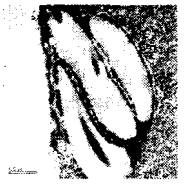

1
M
SI
G
10 μm
2
P
N
10 μm
3
1 μm
4
G
1 μm
5
SG
10 μm
6
S
G
10 μm

Table 4. Responses of plastids to different light regimes in *Metasequoia glyptostroboides* leaves.

Light regimes	Complete darkness	Low light intensity (about 10-15 μmol $m^{-2}s^{-1}$)	Moderate light intensity (about 150 μmol $m^{-2}s^{-1}$)	Natural greenhouse light (about 700- 1500 μmol $m^{-2}s^{-1}$)
Plastid location	Leaves from expanding buds, 33 days treatment	Expanding leaves, 23 days treatment	Expanding leaves, 23 days treatment	Expanded leaves, 188 days treatment
Mesophyll cells	Grana present, up to 12 thylakoids per granum; Prolamellar bodies present; No starch	Thicker grana up to 18 thylakoids per granum; No prolamellar body; No starch; Few lipid droplets	Thylakoid membrane systems swollen; Well developed starch grains and lipid droplets	Thylakoid membrane systems gone; A large single starch grain occupying the entire volume; (Amyloplast)
				
Adaxial epidermal cells	No grana; Long stroma thylakoids; No starch; Prolamellar bodies present	Grana present, up to 7 thylakoids per granum; No starch	Thylakoid membrane system reduced; Large starch grains; (Amyloplast)	Thylakoid membrane system nearly gone; Large starch grains and lipid droplets; (Amyloplast)
				
Abaxial epidermal cells	No grana; Long stroma thylakoids; No starch; Prolamellar bodies present	Grana present, up to 4 thylakoids per granum; No starch	Grana still present; Many starch grains present;	Thylakoid membrane system nearly gone; Large starch grains; (Amyloplast)
				

granum) and the thylakoid membrane systems were well developed (Plate11, Figure3 and 4; Table 4). The plastids in the epidermal cells had fewer thylakoids per granum than those in mesophyll cells. No starch grains and few lipid droplets were found.

Under moderate light intensity (about 150 μmol $m^{-2}s^{-1}$), the plastids of mesophyll cells had well-developed starch grains and lipid droplets (Plate 11, Figure 5 and 6; Table 4). The membranes were swollen indicating a possible early stage of senescence. The plastids in the adaxial epidermal cells had large starch grains and reduced membranes, characteristic of amyloplasts. The plastids in the abaxial epidermal cells still had some granas (Table 4). Starch grains and lipid droplets were common and well-developed.

4. DISCUSSION

Based on light and electron microscopy *Metasequoia* leaves (growing under all light intensities) have slightly arched outer cell walls in the adaxial epidermal cells, and strongly arched outer cell walls in the abaxial epidermal cells. Mesophyll is composed entirely of spongy cells (Plate1). Chloroplasts with well-developed grana are found not only in mesophyll cells but in both the adaxial and abaxial epidermis (Plate3).

Arched outer epidermal cell walls can act as lenses to collect and focus incident light into the mesophyll cells in the leaf interior, to enhance photosynthesis (Bone et al., 1985; Lee, 1986; Poulson and Delucia, 1993;Poulson and Vogelmann, 1990; Smith et al., 1997). Consistent with previous findings (Lee, 1986; Smith et al., 1997), our studies show that the optical geometry of individual epidermal cells varied with light with the extent of convexity in epidermal cell walls decreasing with increasing light intensity.

The walls of spongy mesophyll cells and the large fraction of air space in the leaf interior (Plate1, Figure 1 and 2) would generate considerable scattering of light, which could help to increase light absorption by chloroplasts within mesophyll cells (Smith et al., 1997).

The choloroplasts in the epidermal cells would be able to absorb low intensity, diffuse light and thus enhance photosynthesis in early leaf development. The chloroplasts in the adaxial epidermis became amyloplasts sooner than those in the abaxial epidermis (Table 4) and both existed for a shorter time period than the

chloroplasts in mesophyll cells. The epidermal cell chloroplasts are functional while light intensities are low, but at high light intensities are converted to amyloplasts.

Plastids were found in the epidermis in the four species studied: *Metasequoia*, *Glyptostrobus*, *Sequoia* and *Taxodium*. Chloroplasts with well-developed grana were found in the epidermis of *Metasequoia*, *Sequoia* and *Taxodium*, while in *Glyptostrobus*, we only found amyloplasts with large starch grains and a few grana.

Slightly arched outer walls in adaxial epidermal cells and strongly arched outer walls in abaxial epidermal cells were found in *Sequoia* and *Taxodium*. While outer walls of epidermal cells in *Glyptostrobus* were not arched on any surface, and the leaves are not flattened as in the other species. Thus, based on leaf morphology, lack or presence of epidermal cell wall arching, and presence or absence of chloroplasts in epidermal cells, we conclude that *Metasequoia* is best adapt to low light intensities, *Sequoia* and *Taxodium* are intermediate, and *Glyptostrobus* is adapted to higher light intensities.

As seen in Table 2, stomata were confined to the abaxial leaf surface in *Metasequoia*. Both *Sequoia* and *Taxodium* have stomata in both adaxial and abaxial leaf surfaces, although in *Sequoia*, stomata are extremely rare in the adaxial epidermis. This is probably why *Sequoia* has been previously reported as hypostomatic (Chaturvedi, 1998). *Glyptostrobus* has a larger number and more even distribution of stomata over the entire leaf surface similar to many evergreen conifer species — and suggestive of a more efficient gas exchange potential under high light intensities (Smith et al., 1997). Based on stomatal distribution and density a continuum from hypostomatic and few stomata to amphistomatic and many stomata

is seen as: *Metasequoia Sequoia Taxodium Glyptostrobus* — a pattern consistent with leaf morphology and chloroplast distribution. Unlike many conifers (evergreen and deciduous) the stomata in all four species are not sunken, although subsidiary cells are slightly raised. These observations suggest adaptation to moist sites— consistent with observed natural distributions.

Mesophyll plastids in *Metasequoia* show a normal development cycle, from proplastids to mature chloroplasts with well-developed grana, to amyloplasts with reduced thylakoid membrane systems (or chromoplasts), to senescent plastids (Kutík, 1998; Whatley, 1978), except for the presence of prolamellar bodies in early developmental stages in the light environment (Plate 7, Figure 5 and 6).

Previously, prolamellar bodies have been reported in the plastids of darkened leaves (Bogorad, 1950; Burgerstein, 1900; Ida, 1981; Kirk and Tilney-Bassett, 1957, 1967; Laudi and Manzini, 1975; Laudi and Medeghini-Bonatti, 1973; Lewandowska and Őquist, 1980; Selstam and Widell, 1986; Wieckowski and Goodwin, 1967). However, we observed prolamellar bodies connected to grana in the early stages of expanding leaves. Although these plants were exposed to full greenhouse daylight, the leaves in the expanding buds probably received much lower light intensities. In a few other studies, the presence of prolamellar bodies (or prolamellar body-like structures) in light-grown plants has been documented: in the inner yellow expanding leaves of spinach (Rascio et al., 1985; and Polettini et al., 1986), *Selaginella* (Lerbs and Eicke, 1974); *Lepidozamia peroffskyana* (Medeghini Bonatti and Baroni Fornasiero, 1990), young leaves in the sea grass *Posidonia oceanica* (Mariani Colombo et al., 1983 a), and in young leaves of the fern *Phyllitis scolopendrium*

(Marinani Colombo et al., 1983b). These studies and our observations are consistent with the statement by Vothknecht et al. (2001) that although prolamellar bodies are primarily confined to etioplasts, they are not restricted to them. The functional significance of prolamellar bodies in low intenisty light is not clear. They may act as transitory accumulation of tubular membranes which can subsequently change into thylakoid membranes (Casadoro and Rascio, 1979; Mariani et al., 1982; Rasacio, Mariani and Orsenigo, 1980; Schnepf, 1980).

A significant observation in our studies on the responses of plastids to different light regimes was the presence of not only prolamellar bodies in developing plastids but also developed chloroplasts with chlorophyll in *Metasequoia* leaves under complete darkness. Previously, chlorophyll synthesis in *Metasequoia* in the dark had been demonstrated by Ida (1981) and Laudi and Manzini (1975). My ultrastructural study confirms that chlorophyll synthesis is linked to grana production in the dark. Although the amount of chlorophyll in the darkened leaves is small, *Metasequoia* has been shown to produce more chlorophyll at all light intensities than *Glyptostrobus*, *Sequoia* and *Taxodium* (Ida, 1981). This would provide a competitive advantage at high latitudes where chlorophyll synthesis could occur under the very low light levels of early spring.

The response was similar in my two experiments designed to determine the effects of light intensities on leaf plastids. Adaptation in response to different light intensity can occur within days of light shift (Jones, 1992), thus, the duration of my treatments (about 5 months for the greenhouse experiment, and about 1 month for the growth chamber experiment) should have been more than sufficient for adaptations to

occur. The results (Plate 10 and Plate11) show a higher proportion of grana and stroma thylakoids, and a smaller number and size of starch grains and lipid droplets with reduced light intensities. Since needle anatomy can reflect needle physiology (Pachepsky et al., 1995), these changes in structure are likely correlated with changes in photosynthetic activity and hence chlorophyll content. Bjőrkman (1981) and Elisa and Ciamporova (1986) suggested that the extensive grana formation in sciaphilic plants may offer a means by which the plants can attain a high chlorophyll content and hence enhance photosynthesis even under limited light irradiance. According to the measurements of Ida (1981), the chlorophyll content per unit leaf area increased with decreased light intensity, with the increase in leaf chlorophyll by shading reached a limit at light intensities of approximately 7% of full sun. In the greenhouse study, I did not measure the chlorophyll content of different light intensities, but the proportion of grana and stroma thylakoids in plastids was higher at 1.5% of ambient light (about 10- 23 μmol $m^{-2}s^{-1}$) than 10% of ambient light (about 70- 150 μmol $m^{-2}s^{-1}$).

Mesophyll cells of *Metasequoia* leaves grown under greenhouse full sun had abundant starch grain accumulation, large lipid droplets, and degeneration of the thylakoid membrane system (Plate 10, Figure 5 and 6). This suggests that the photosynthetic apparatus were stressed under high light intensities. In extreme sciaphilic plants, photosynthesis may light saturate at less than 100 μmol $m^{-2}s^{-1}$ (PAR), which is about 5% of full sunlight (Jones, 1992). *Metasequoia* photosaturates at light intensities between 290-495 μmol $m^{-2}s^{-1}$ (Jagels and Day, 2004). Extremely shade adapted ferns, such as *Adiantum raddianum* and *Asplenium nidus*,

photosaturate at 80-120 μmol $m^{-2}s^{-1}$ (Yeh and Wang, 2000). While *Picea rubens*, a shade tolerant species, photosaturates at 271-803 μmol $m^{-2}s^{-1}$ (Jagels and Day, 2004). *Larix laricina*, a shade intolerant species, photosaturates above 1500 μmol $m^{-2}s^{-1}$ (Jagels and Day, 2004). *Metasequoia* photosaturates at a level intermediate between plants adapted to extreme shade and those that are shade intolerant — but is closer to the former. A shade tolerant species, such as *Picea rubens*, can survive in deep shade for 50 plus years, by having a determinate growth form and evergreen needles that can be retained for many years (Blum, 1990; Davis, 1991). *Metasequoia* is not well adapted to full sun, but does not tolerate deep shade. This is likely due to its deciduous habit and indeterminate growth habit—both of which require significant annual biomass production (Jagels and Day, 2004).

In conclusion, *Metasequoia* leaves exhibit several characteristics of typical sciaphilic plants, such as arched outer epidermal cell walls, mesophyll composed of spongy cells, and the presence of chloroplasts with well-developed grana in the epidermal cells. Combined with the ability to synthesize chlorophyll in complete darkness, these characteristics would provide advantages for *Metasequoia* in adapting to low intensity, low angle, polar light at their Eocene high latitude paleo-environments, particularly during the polar spring when light levels are exceedingly low. These leaf characteristics also provide evidence to explain why *Metasequoia* was the dominant tree species in Eocene high latitudes (Jagels and Day, 2004; Jagels et al., 2003).

Although the other members of the Cupresseaceae compared in this study had some leaf characteristics in common with *Metasequoia*, none had the full range of

low-light capture adaptations found in this relict species. These character differences combined with those previously reported (Jagels and Day, 2004; Jagels and Equiza, In press) provide strong evidence supporting the theory that the unique light environment found at polar latitudes was the primary driving force in the adaptation of species to those environments. The leaf characters of *Metasequoia* are less competitively adaptive to current mid-latitude environment, and hence the likely reason for its relict status.

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