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A comparative study of chloroplast morphogenesis in seedlings of some conifers *(Larix decidua, Pinus sylvestris* and *Picea abies)*

En jämförande studie av kloroplastmorfogenesen i groddplantor av några barrträdsarter

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Abstract

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Seedlings of larch, pine and spruce were grown in darkness and some of them were later illuminated for 24 or 48 hours. Chloroplast differentiation in cotyledons of these seedlings was investigated by electron microscopy. In the larch the dark-formed chloroplasts have immature lamellar systems with minute grana formed by 2—3 thylakoids. In the pine the diameters of the grana are much larger but they are still formed by only 2—4 thylakoids. Corresponding spruce chloroplasts have advanced lamellar systems with large grana containing up to about 10 thylakoids. Chloroplasts of larch and pine have a few large prolamellar bodies with a more or less narrow spacing of their structural units, while spruce chloroplasts have many small prolamellar bodies with narrow or wide spacing or a combination of both. All three species have large starch grains in their chloroplasts. After 48 hours of illumination the conifer seedlings possess mature chloroplasts with many prominent grana. Prolamellar bodies are only found in a small fraction of the larch chloroplasts. Chloroplast starch grains are common in the spruce, but rare or absent in the other two species.

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Contents

1 Introduction

Conifer seedlings are able to synthesize chlorophyll in the dark, and this character enables them to develop some kind of chloroplast in the absence of light as analyzed with the electron microscope in *Picea* (von Wettstein 1958, Laudi 1964, Walles 1967, Anikushin 1971), *Pinus* (Camefort 1963, Kawamatu 1967, Nicolić and Bogdanović 1972, Michel-Wolwertz and Bronchart 1974) and *Larix* (Laudi 1964). In *Picea abies,* a *xantha* mutant that is unable to synthesize chlorophyll was found to lack not only chloroplasts but also etioplasts in the dark as well as in the light (Walles 1967). So called *aurea* seedlings, which are heterozygotes for the just mentioned mutant factor, have a reduced chlorophyll content both in the light and in the dark and contain chloroplasts that are less differentiated than those in normal spruce seedlings (Walles 1967). Of other gymnosperms investigated after germination and growth in darkness, *Ephedra* possesses chloroplasts, but *Gnetum* and *Welwitschia* form only etioplasts under these conditions (Laudi and Bonatti 1973).

In the dark, gymnosperm chloroplasts contain prolamellar bodies in addition to grana stacks. More or less prominent starch grains are other characteristic constituents. Published micrographs indicate a considerable variation between different species in the structural organization of these dark-formed chloroplasts. The results from different studies are, however, hard to compare with regard to ultrastructural details because of the variation in growth conditions, in age of seedlings and in preparation methods (e.g. fixation with $KMnO_4$ or OsO_4, with or without prefixation with glutaraldehyde). A meaningful comparison of the fine structure of chloroplasts in different species requires studies of seedlings grown and investigated under identical conditions. For this reason we have undertaken the present examination of mesophyll chloroplasts in dark-grown seedlings of three European members of Pinaceae: larch (*Larix decidua* Mill.), pine (*Pinus sylvestris* L.) and spruce (*Picea abies* (L.) Karst.). Some of these seedlings were illuminated for one or two days to get information on what structural differentiation takes place in the chloroplasts as a consequence of the stimulated chlorophyll synthesis which results from light treatment.

2 Material and methods

For this study were used seeds of *Larix decidua,* collected in Slovakia, CSSR, and of *Pinus sylvestris* and *Picea abies* from south Sweden. We are grateful to Professor M. Simak for the supply of these seeds.

The seeds were immersed into tap water for 12 hours and then planted in sand in plastic boxes. The material was kept in darkness at a temperature of 24—25°C. All handling of the material including the fixations for electron microscopy was done in a dim green safe light.

In one experiment the seedlings were kept in the dark for 15 days. In a second experiment the seedlings were at the age of 13 days transferred to a chamber where they were illuminated with continuous light from fluorescent tubes. Light intensity at the seedling level was about 1500 lux.

Samples were collected from 11 and 13 days old seedling of larch, and from 11, 13 and 15 days old seedlings of pine and spruce. Samples of illuminated plants were taken after 24 and 48 hours of light treatment.

Small segments of cotyledons were fixed in a mixture of glutaraldehyde and formaldehyde (Karnovsky 1965) for 5 hours at room temperature, washed in phosphate buffer and subsequently fixed in 2 per cent OsO_4 in 0.1 M phosphate buffer of pH = 7.0 for 2 hours at 4°C. The material was dehydrated in an acetone series, followed by propylene oxide and embedded in Spurr's epoxy medium (Spurr 1969).

Sections were cut with glass knives on a LKB Ultratome I or with a diamond knife on a Ultratome III. They were stained with 2 % aqueous uranyl acetate and 0.2 % lead citrate (Venable and Coggeshall 1965). For the examination a Hitachi HS-7S electron microscope was used.

3 Results

3.1 Larch

Larch chloroplasts developed in the dark (Figs. 1—3) are usually rather plastic in their shape but most of them are slightly elongated. Between one and four large starch grains appear in each chloroplast profile present in micrographs of 11 days old seedlings (Figs. 1, 3). These grains occupy a considerable volume and can therefore to various degrees alter the shape of the organelle. In 13 days old seedlings the starch grains are generally of smaller size than in younger plants and do not occur in all sections through chloroplasts.

The thylakoid system is poorly developed, usually forming concentric layers (Figs. 2—3). The grana are minute and consist of only two, rarely three, discs (thylakoids). From one to three large prolamellar bodies with narrow spacing of their elements appear in most of the chloroplasts observed (Fig. 1). An incomplete peripheral layer of vesicles (probably interconnected to a reticulum) is often seen beneath the chloroplast envelope (Fig. 1). The stroma contains numerous ribosomes and groups of large plastoglobuli in hexagonal arrangements (Figs. 1—3).

After 24 hours of illumination the chloroplasts appear elongated, bi-convex or plano-convex in shape (Figs. 4, 5). Starch grains are present in some of them. A few of the chloroplasts appear to divide by constriction. The lamellae of the thylakoid (lamellar) system are parallel to the long axis of the chloroplast and contain several small grana formed by 2—5 discs. Small prolamellar bodies with a diffuse substructure may occur in a few of the chloroplasts (Fig. 4). The plastoglobuli are no longer aggregated but dispersed over the organelle.

After 48 hours of illumination the chloroplasts have in most cases no starch grains and appear well differentiated with quite large grana (Figs. 6, 7), although remnants of diffuse prolamellar bodies are still present in a few chloroplasts. At this stage the larch chloroplasts are similar to corresponding organelles in pine (Walles et al. 1973), i.e. they are plano-convex with the thylakoid layers parallel to the curved side and bordering a plano-convex stroma region free from thylakoids, situated adjacent to the flat side of the organelle.

3.2 Pine

The dark-grown pine seedlings have ellipsoidal chloroplasts, each of which contains several elongated starch grains (Figs. 8—11). Plastoglobuli are found at all stages examined. They are usually scattered over the stroma, but in the 13 days old plants (Fig. 10), where their number has increased, they are often assembled into groups like in larch. Plastid ribosomes are common (see Figs. 10, 11).

The thylakoids are arranged in layers, which are parallel to the long axis of the chloroplast. These layers contain grana, formed by 2—4 discs of large diameters. One or two large prolamellar bodies can be seen in most of the chloroplasts present in the micrographs (Figs. 8, 9). Peripheral vesicles may occur below the plastid envelope (Fig. 8).

Chloroplasts that have been illuminated for 24 hours have numerous grana and no prolamellar bodies remain (Fig. 12). Some peripheral vesicles can still occur between the outermost lamellae and the chloroplast envelope. A few plastoglobuli are scattered in the stroma which also contains many ribosomes. Starch grains are absent.

After 48 hours of illumination the chloroplasts are still free from starch (Fig. 13). They have undergone a striking structural development and their appearance is now like that described by Walles et al. (1973) for mesophyll chloroplasts in mature pine needles.

3.3 **Spruce**

The chloroplasts in dark-grown spruce seedlings are characterized by prominent starch grains which occupy a considerable part of the organelle (Figs. 14—17). The chloroplasts are elongated but otherwise often irregular in shape, which to a large extent seems to be due to the voluminous starch grains. The thylakoids form layers, which fundamentally are parallel to the long axis of the chloroplast but a variable fraction of them is generally displaced by starch grains interspersed in the lamellar system. This system contains many grana with up to ten aggregated thylakoids of large diameters (Figs. 17, 18) as well as several small prolamellar bodies. Some of these are widely spaced (Figs. 14, 15), while the rest are narrowly spaced like in larch and pine (Fig. 17). Remarkable is that one part of a prolamellar body can have a wide spacing and the rest of the body a narrow one (Figs. 14, 16). Peripheral vesicles are sometimes found adjacent to the plastid envelope. The stroma contains ribosomes and some plastoglobuli, the later frequently assembled into a number of small groups.

Illumination for 24 hours causes disappearance of the prolamellar bodies and stimulation of grana formation (Fig. 19). In these chloroplasts there are only a few plastoglobuli, which usually are dispersed. Noteworthy is the occurrence of large starch grains. The chloroplasts are more regular in shape than they are in darkness and at least some of them appear more or less plano-convex.

After 48 hours of illumination the chloroplasts are generally plano-convex in shape and the thylakoid system is well developed (Fig. 20). These chloroplasts contain large starch grains.

4 Discussion

The present investigation has revealed a number of conspicuous differences between *Larix decidua, Pinus sylvestris* and *Picea abies* with regard to structural organization of chloroplasts developed in the dark.

According to our results the dark-formed chloroplasts of larch have immature lamellar systems with minute grana, each of which contains only 2—3 thylakoids. In the pine, the lamellar system is more advanced with larger, wider grana formed by 2—4 thylakoids. The best developed lamellar system is found in the spruce, where the large grana may each contain up to about 10 aggregated thylakoids. Differences between the species were also found in the development of prolamellar bodies. The spacing of such bodies can be narrow or wide (cf. Henningsen and Boynton 1969). In the larch and the pine we have only observed narrow spacing, whereas the spruce has both types of spacing, sometimes combined in the same prolamellar body. The individual prolamellar bodies of the larch and the pine are of larger size than those occurring in the spruce, but the number of bodies is higher in spruce than in the other two conifers.

The *Larix* chloroplasts have prominent plastoglobuli assembled into groups. In the pine and the spruce the plastoglobuli may occur in groups or be dispersed in the stroma. The plastoglobuli of spruce are smaller than those present in the other two species.

During the first days of germination the cotyledons of conifers remain inside the embryo cavity, protected from light by the surrounding endosperm. The length of this dark period is evidently influenced by the genotype of the plants and by external factors like temperature and water supply. In *Pinus* it can last for about 10—15 days according to data provided by Camefort (1963) and Michel-Wolwertz and Bronchart (1974). During this period of "dark" growth immature chloroplasts with prolamellar bodies are developed in the mesophyll cells, also in those cases where the germination takes place in the light (Camefort 1963, Durzan et al. 1971, Michel-Wolwertz and Bronchart 1974).

If dark-grown seedlings are illuminated, synthesis of more chlorophyll takes place which stimulates further differentiation of their chloroplasts so that they after a few days will have the structure of mature mesophyll chloroplasts. Such light-induced morphogenesis of conifer chloroplasts has previously been studied by Laudi (1964) and Michel-Wolwertz and Bronchart (1974). Laudi (1964) illuminated dark-grown seedlings of larch and spruce with 3000 lux for 24 hours. He reported that this treatment caused a decrease in the size of the prolamellar bodies, particularly in larch, that the lamellar system grew to produce larger grana and that the starch grains were reduced. Michel-Wolwertz and Bronchart (1974) compared chloroplasts of *Pinus jeffreyi* seedlings grown in darkness and continuous light (1000 lux) respectively. In 12 days old light-grown seedlings the cotyledons were no longer protected by the endosperm and were estimated to have been illuminated for about 48 hours. "Light" chloroplasts had more thylakoids and grana and smaller prolamellar bodies than "dark" chloroplasts of the same age. Starch was still present in the "light" chloroplasts, and their prolamellar bodies were found to keep their regular, crystalline architecture for a few days.

In the present investigation the dark-grown seedlings were illuminated with 1500 lux for 24 or 48 hours. In this plant material persistence of some prolamellar bodies was observed only in the larch. These prolamellar bodies, which were only found in a minor fraction of the chloroplasts, were very dense with a diffuse fine structure.

After 48 hours of illumination mature chloroplasts occurred in all three species studied. These chloroplasts were generally of a plano-convex shape and similar to mesophyll chloroplasts of pine, the ultrastructure of which has been described in an earlier paper (Walles et al. 1973). We observed also, that in the illuminated seedlings the previously aggregated plastoglobuli became dispersed in the stroma and possibly decreased in number.

The storage material of conifer seeds consists mainly of fat and protein, and the starch content is low (Simola 1974). However, starch is synthesized during germination and the proplastids in young cotyledons of at least some conifer species accumulate prominent starch grains (Camefort 1963, Durzan et al. 1971, Michel-Wolwertz and Bronchart 1974, Simola 1975). Large starch grains are also present in the chloroplasts of dark-grown cotyledons as reported for *Pinus pinea, P. banksiana, P. densiflora, P. jeffreyi, P. nigra, Picea abies, Larix decidua* (Anikushin 1971, Camefort 1963, Durzan et al. 1971, Kawamatu 1967, Laudi 1964, Michel-Wolwertz and Bronchart 1974, Nikolić and Bogdanović 1972, Simola 1975). The present paper adds *P. sylvestris* to the list. When dark-grown spruce seedlings become old, the starch grains are digested (Anikushin 1971).

We have noted some differences between species in the shape, number and behaviour of starch grains. In the larch several large, generally rounded starch grains occur in the chloroplasts present in 11 days old dark-grown seedlings. In the 13 days old plants the number of starch grains has decreased and they are in fact absent from many of the chloroplast profiles observed. After 24 hours of illumination starch grains can still be found in some of the chloroplasts but after 48 hours of light only a few small grains may remain in exceptional cases. In the dark-grown pine seedlings there are several large elongated starch grains in each chloroplast but in the illuminated individuals the starch has disappeared from the chloroplasts. In the spruce a larger amount of starch is synthesized than in larch and pine. In the dark-formed chloroplasts there are several large, rounded starch grains which by causing bulges frequently influence the shape of the organelles. After illumination the number of starch grains has decreased but probably most chloroplasts contain at least one grain.

One of the most important factors determining the differentiation of chloroplasts might be the amount of chlorophyll synthesized in these organelles. Laudi and Fanelli (1964) have shown, that dark-grown seedlings of *Picea* contain far more chlorophyll than corresponding seedlings of *Larix*. However, differences in content of chlorophyll synthesized in darkness can probably not be responsible for all the differences in chloroplast structure that occur between different conifer species. In this connection we want to call attention to the difference between conifer species in number, size, shape and persistence of starch grains in chloroplasts. Those differences can in our opinion not be explained on the basis of variations in amount of chloroplast pigments.

Finally, it is of interest to consider the degree of development of chloroplasts in dark-grown seedlings in relation to the light requirements for normal growth of the species. It is a well-known fact that the spruce belongs to the group of shadow-tolerating trees whereas the pine and the larch belong to the not shadow-tolerating trees (cf. Boysen Jensen 1929). Since a spruce seedling can produce rather differentiated chloroplasts with grana in complete darkness, the light intensities required for formation of a functional photosynthetic apparatus are in this case apparently low. On the other hand, the larch chloroplasts formed in darkness are poorly differentiated and therefore hardly able to do much photosynthesis immediately after illumination of the cotyledons. With regard to chloroplast differentiation in darkness the pine is intermediate between spruce and larch.

Acknowledgements. We are indebted to Mrs. Ulla Afzelius for skilful technical assistance.

5 Sammanfattning

Groddplantor av barrträd förmår syntetisera klorofyll och utveckla kloroplaster också i totalt mörker. Av övriga gymnospermer, vilkas plastidutveckling i mörkret blivit studerad, uppges *Ephedra* förhålla sig som barrträden, medan *Gnetum* och *Welwitschia* i likhet med angiosperma växter kräver ljus för att kunna bilda kloroplaster. "Mörkerkloroplasterna" har en speciell struktur. De innehåller nämligen såväl grana (staplar av skivlika klorofyllförande tylakoider) som prolamellar-kroppar, dvs. strukturella bildningar som karakteriserar etiolerade plastider. Även stärkelsekorn brukar förekomma i dessa kloroplaster. Elektronmikroskopiska undersökningar har visat, att det föreligger markanta skillnader mellan olika barrträdsarter i avseende på graden av differentiering hos deras "mörker-kloroplaster".

Vi har studerat kloroplastutvecklingen i hjärtblad av europeisk lärk, tall och gran. Försöksplantorna fick gro och växa i en mörklagd odlingskammare, varefter några individer vid 13 dagars ålder överfördes till en upplyst kammare och analyserades efter 24 och 48 timmars belysning.

Hos lärk har "mörker-kloroplasterna" ett svagt utvecklat lamellsystem med små grana, bestående av endast 2—3 tylakoider. Hos motsvarande tallplantor har grana avsevärt större diameter än hos lärk men innehåller endast 2—4 tylakoider. Hos gran har "mörker-kloroplasterna" väl utvecklade lamellsystem innehållande stora grana med upp till tio tylakoider. Kloroplasterna hos lärk och tall innehåller ett fåtal stora prolamellarkroppar uppbyggda av strukturella enheter med en mer eller mindre tät sammanfogning, medan grankloroplasterna har många små prolamellarkroppar med tät eller gles sammanfogning eller i vissa fall en kombination av båda. De undersökta arterna har stora stärkelsekorn, vilka är långsträckta hos tall men mer rundade i lärk och gran.

Efter 48 timmars belysning har plantorna välutvecklade kloroplaster, vilka vanligen har formen av en planokonvex lins. Lamellsystemet innehåller ett flertal stora grana, ofta bildade av många tylakoider, medan prolamellarkroppar endast påträffats hos lärk, där de dock är ovanliga. I dessa försöksplantor förekommer kloroplaststärkelse allmänt hos gran, är sällsynt hos lärk och saknas hos tall.

Det kan vara av intresse att jämföra de här erhållna resultaten beträffande kloroplastutvecklingen med de olika ljuskraven hos de undersökta barrträdsarterna. Granen är som bekant skuggfördragande medan tall och lärk icke tål kraftig beskuggning. Redan i mörkret kan groddplantor av gran utveckla kloroplaster som har ett avancerat lamellsystem med talrika grana. Hos lärk däremot är de kloroplaster, som bildas i mörkret, mycket outvecklade med endast ett fåtal, små grana. Sannolikt krävs en relativt långvarig och kraftig belysning för att lärkens fotosyntesapparat skall differentieras så pass mycket att den kan utföra någon nämnvärd assimilation. Vad gäller kloroplastutvecklingen i mörker är tallen intermediär mellan gran och lärk.

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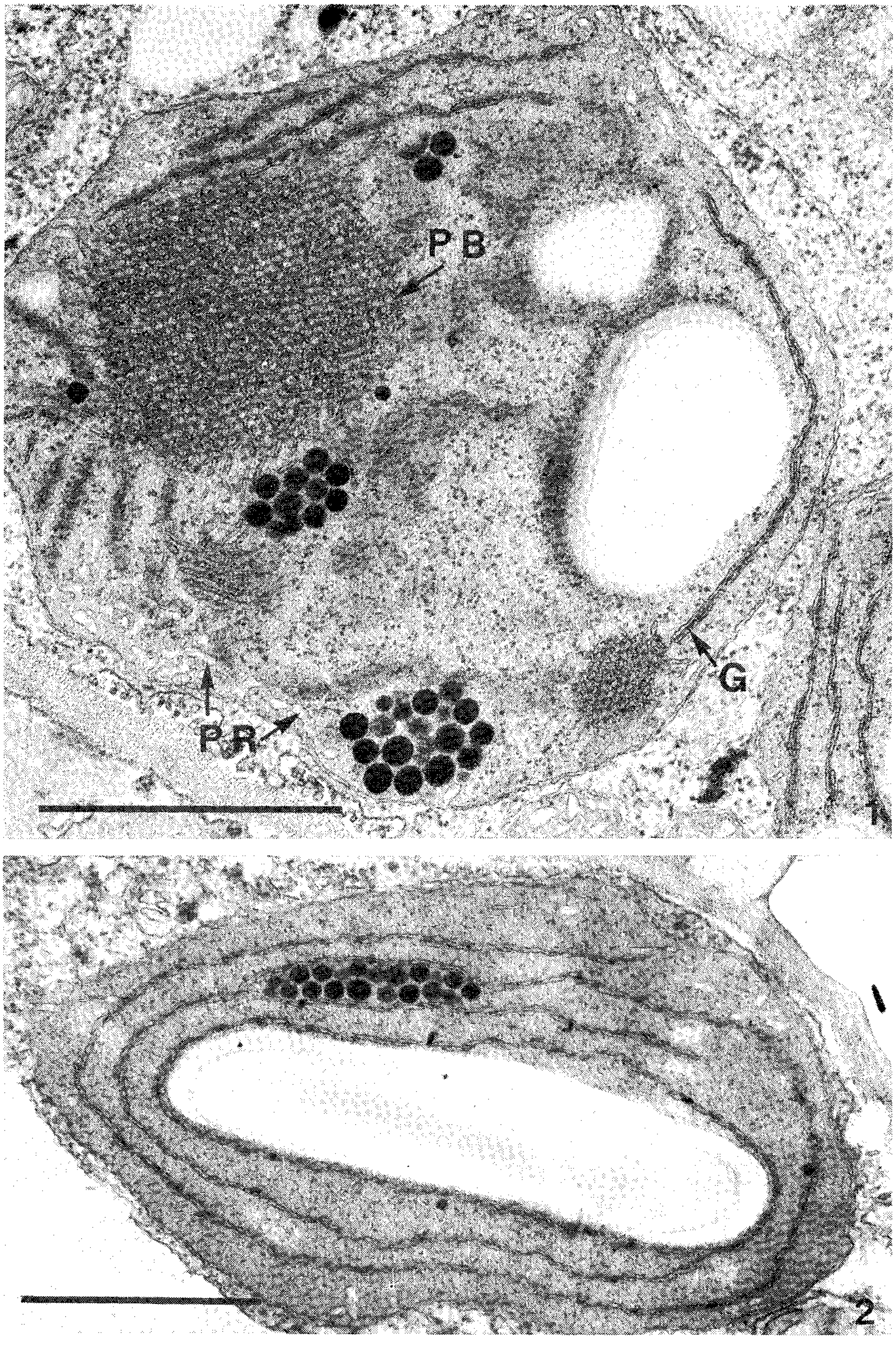
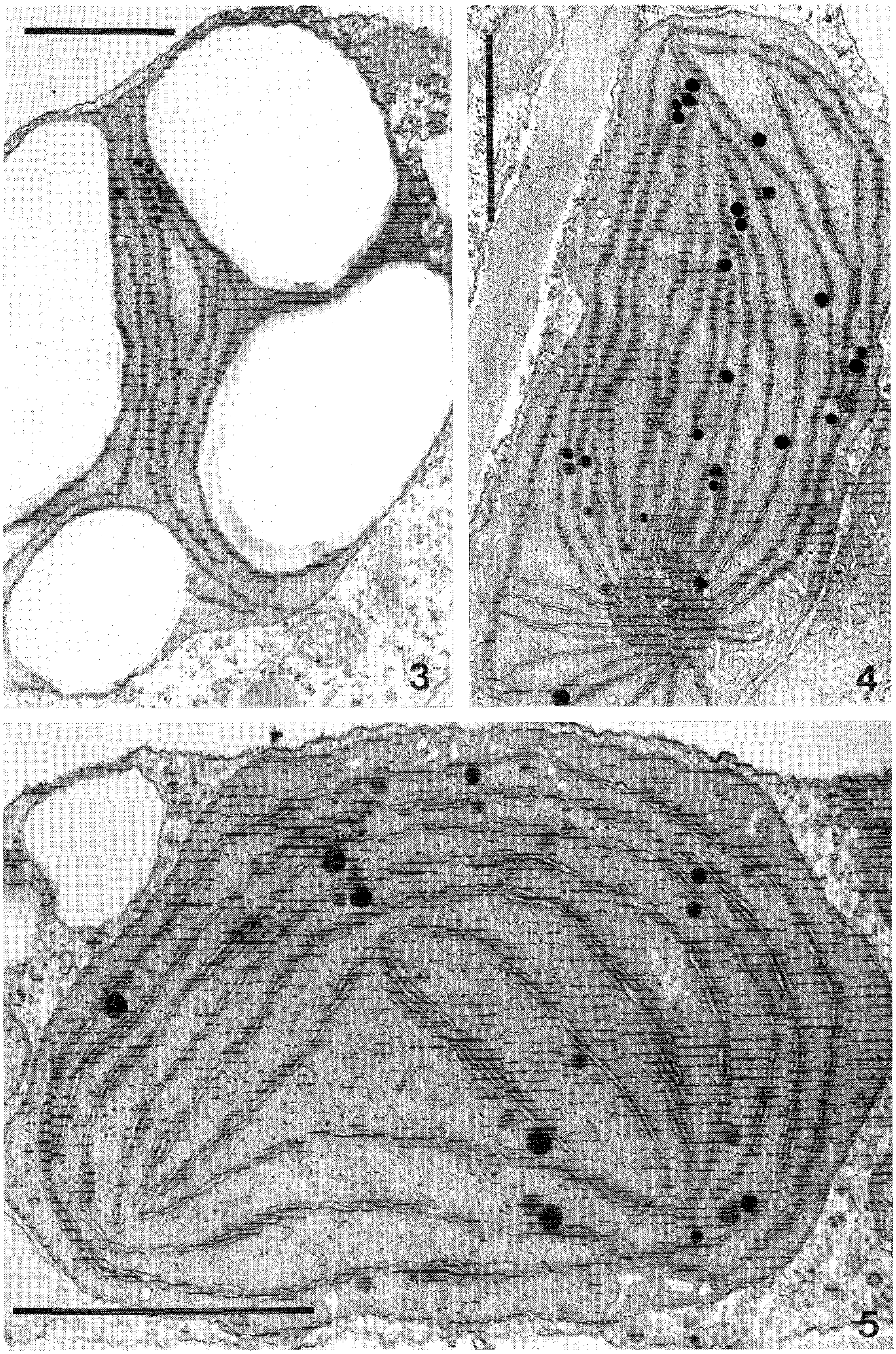
Figures

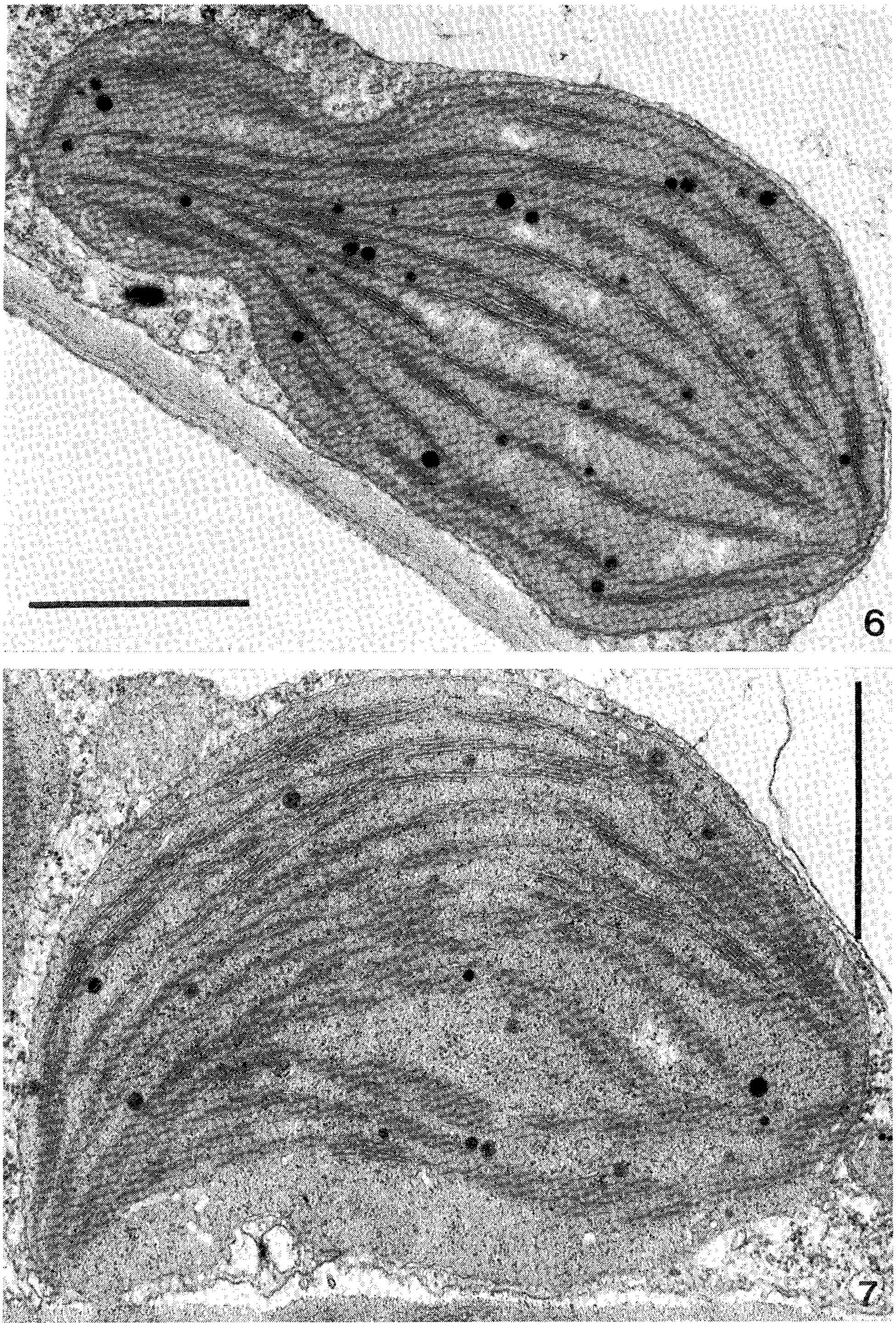
In all figures the scale indicates 1 μm



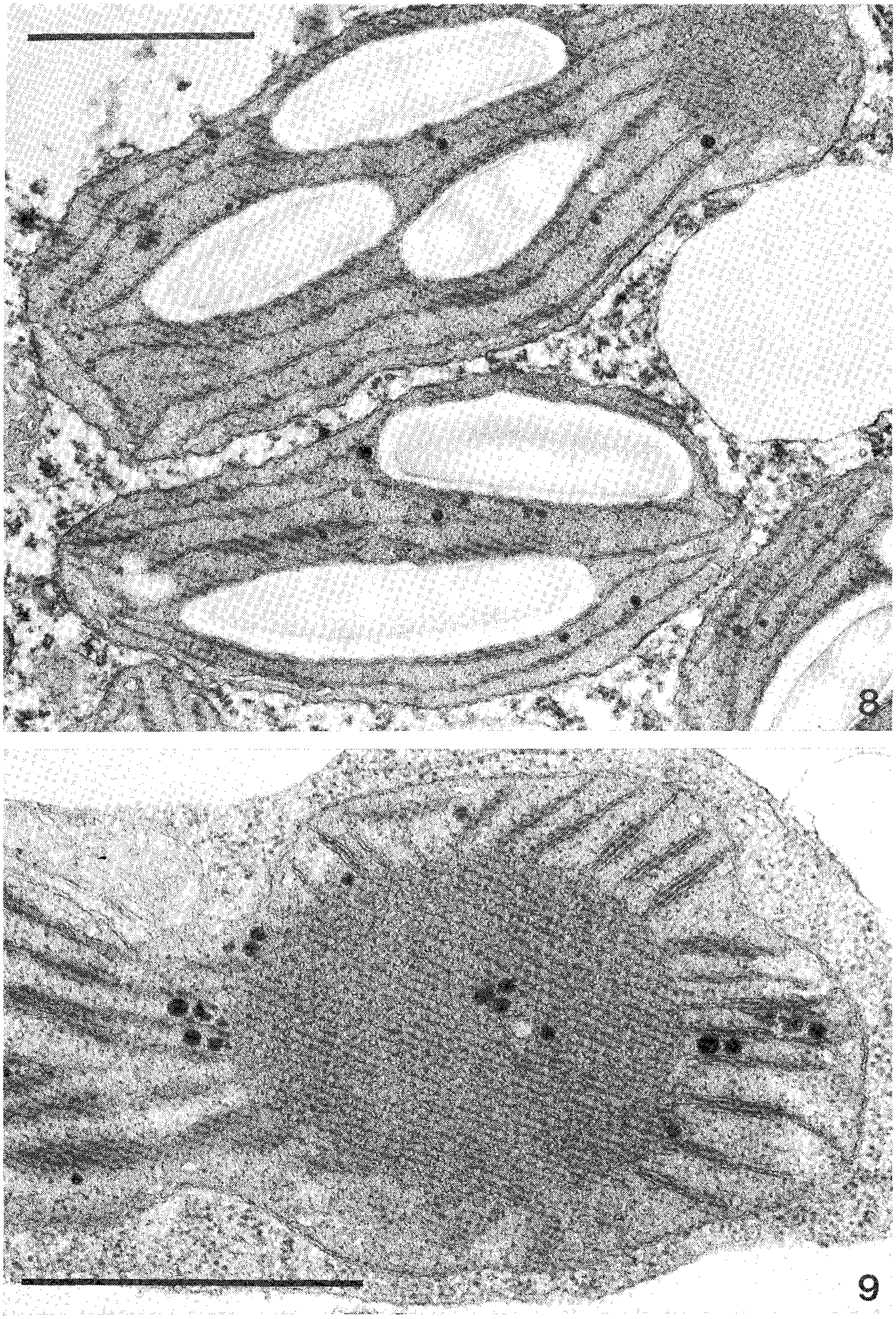
Figures 1—2. Plastids of dark-grown seedlings of larch, 11 days (Fig. 1) and 13 days (Fig. 2) old. These plastids are poorly differentiated, having large prolamellar bodies (PB, Fig. 1) and minute grana (G) formed by two discs. A reticulum (PR) occurs inside the plastid envelope (Fig. 1). In the stroma appear ribosomes, groups of plastoglobuli and starch grains.



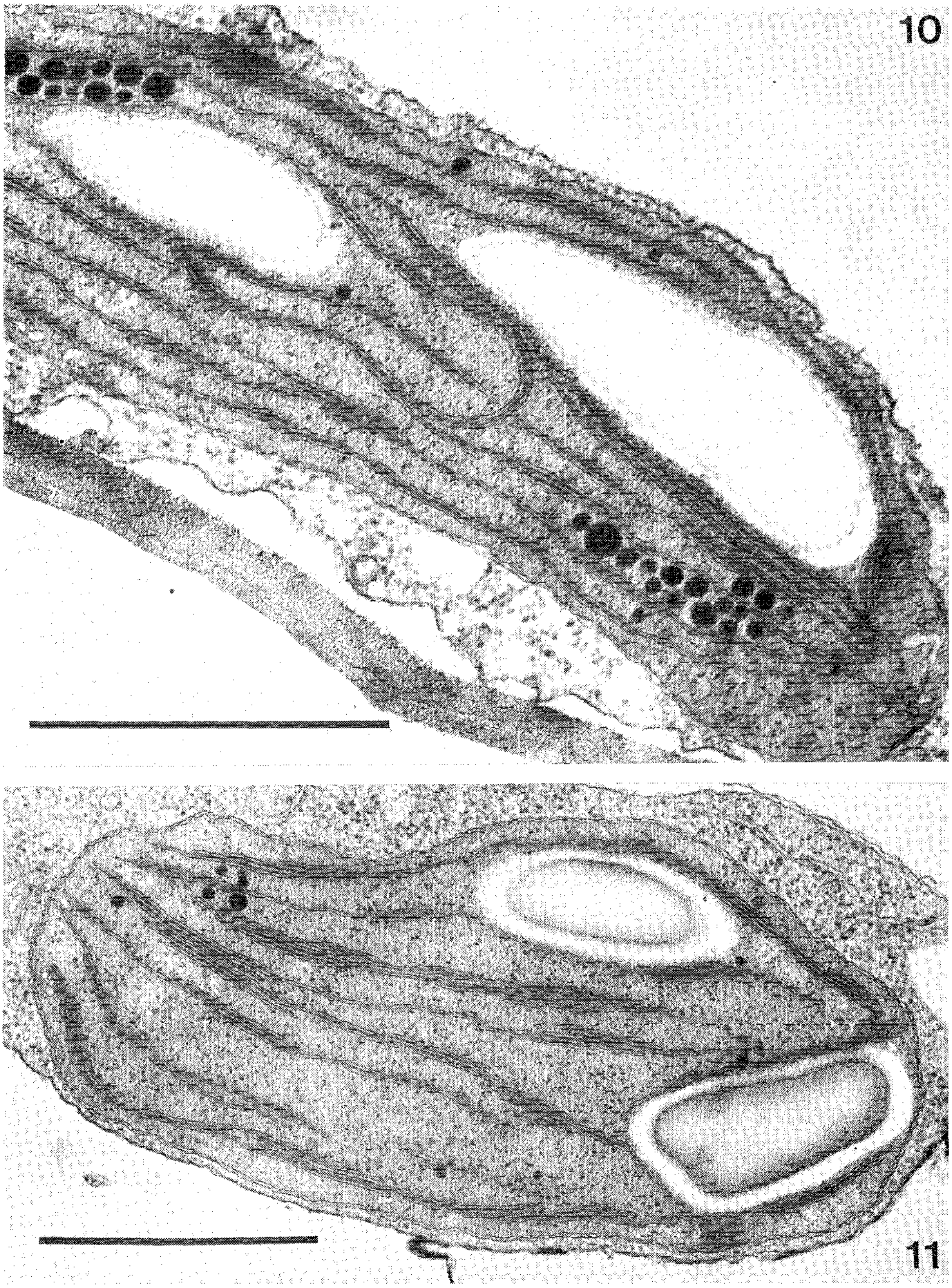
Figures 3—5. Larch chloroplasts. — Fig. 3. Plastid in 11 days old seedling. Note the presence of parallel thylakoid layers with minute grana, plastid ribosomes, aggregated plastoglobuli and large starch grains. — Figs. 4—5. Chloroplasts after 24 hours of illumination showing differentiation of the lamellar system with grana formation, dispersal of plastoglobuli and loss of starch grains. A persisting prolamellar body and a prominent reticulum are seen in the chloroplast of Fig. 4.



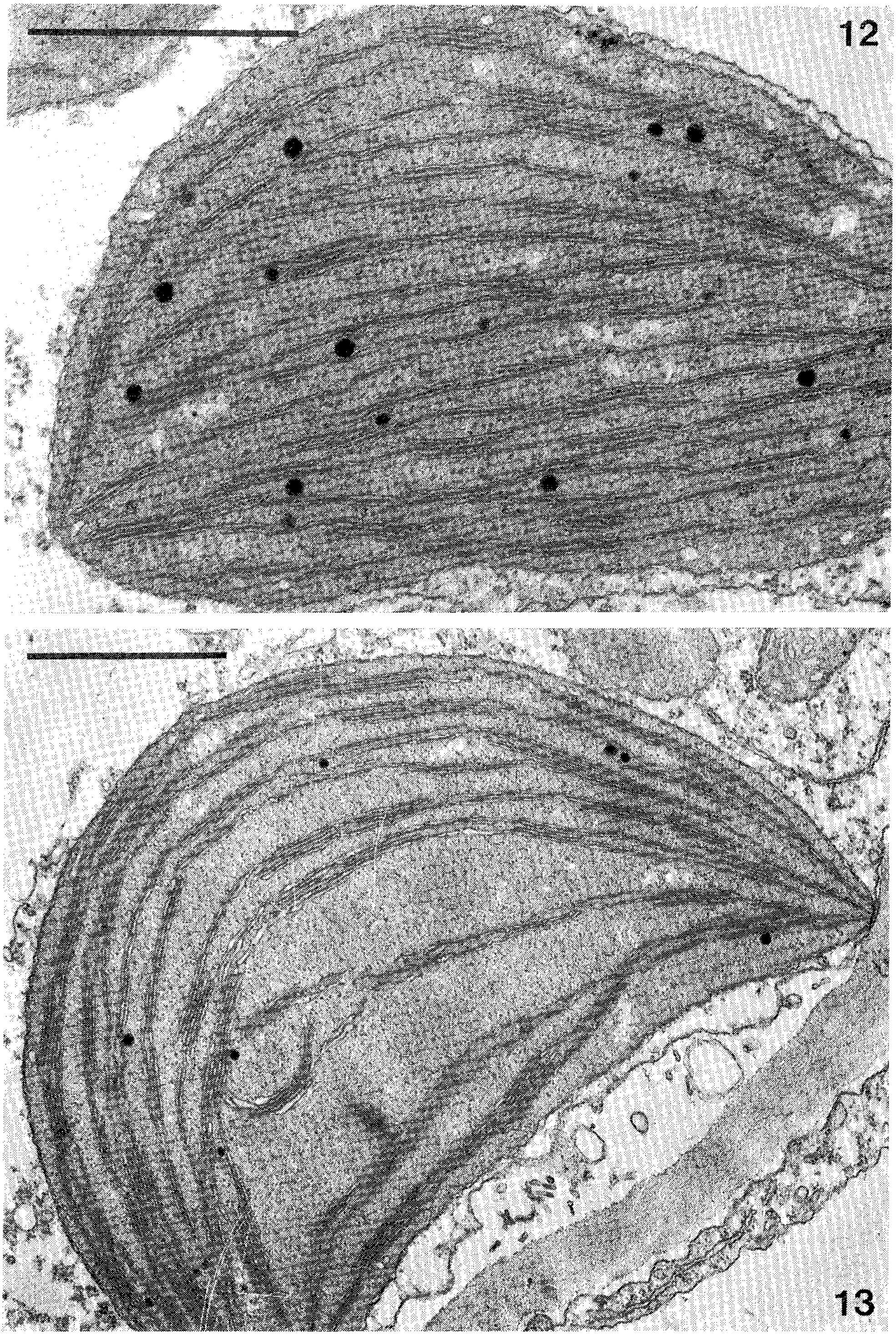
Figures 6—7. Chloroplasts in larch seedlings illuminated for 48 hours. The lamellar system contains numerous grana. Vesicles that are part of a reticulum can still be seen inside the plastid envelope. The plastoglobuli are spread over the stroma. Starch grains are absent.



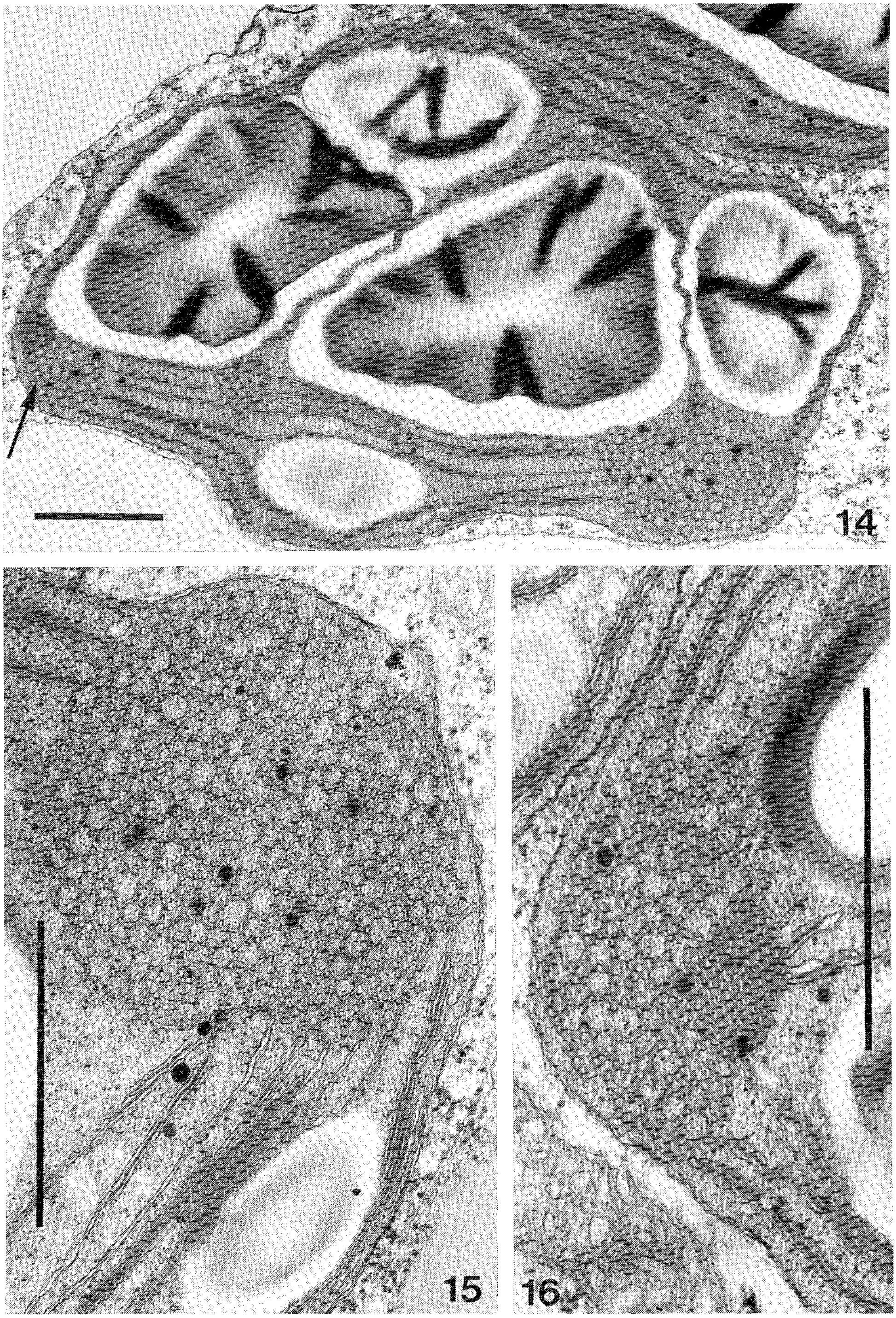
Figures 8—9. Plastids of dark-grown seedlings of pine. Fig. 8 shows some chloroplasts in a 11 days old seedling; Fig. 9 shows part of a chloroplast with a prominent prolamellar body in a 15 days old seedling. In pine the prolamellar bodies are large and narrowly spaced. The grana are elongated and formed by 2—3 discs. A reticulum is present inside the plastid envelope (Fig. 8). The starch grains have a characteristic elongated shape.



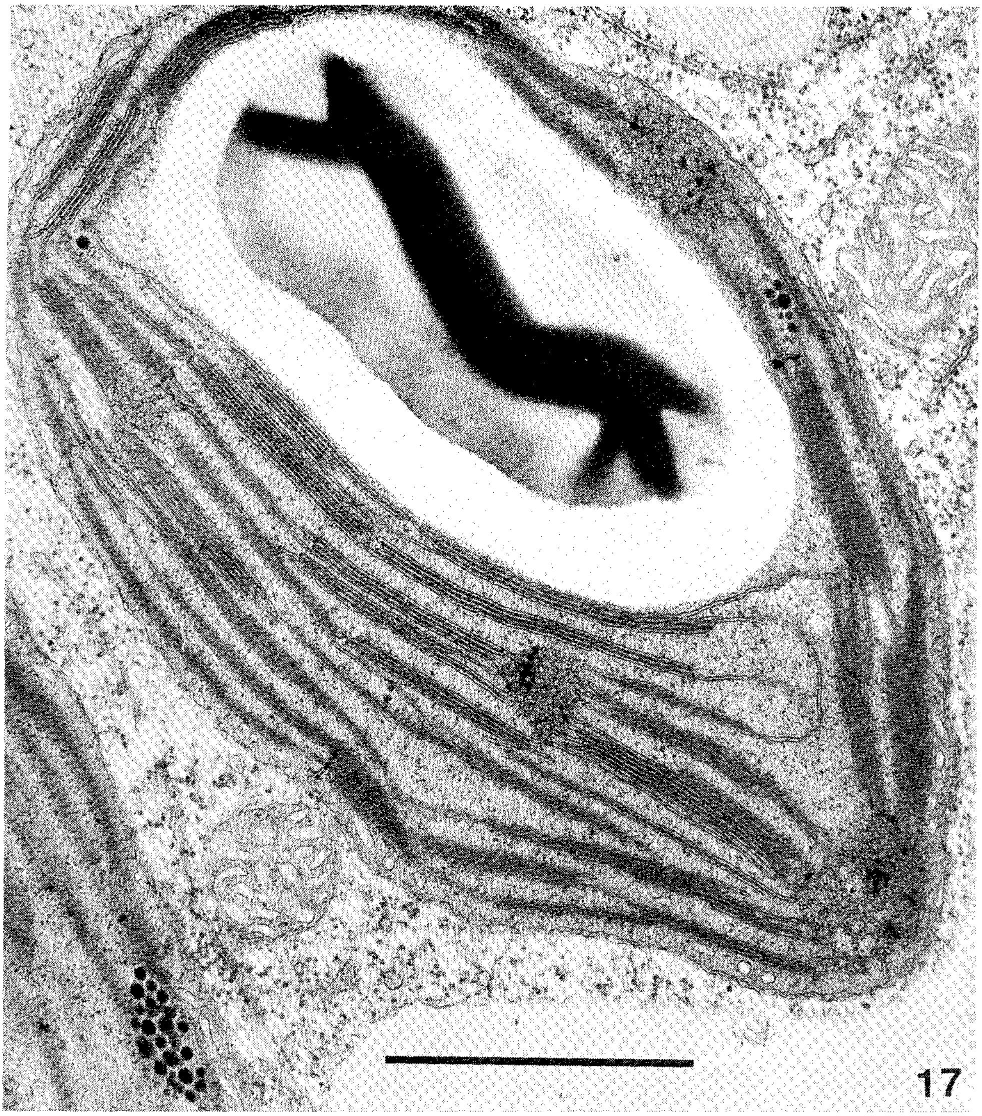
Figures 10—11. Plastids of dark-grown seedlings of pine, 13 days (Fig. 10) and 15 days (Fig. 11) old. The grana are elongated and formed by 2—4 discs. In Fig. 10 two groups of plastoglobuli occur. Note also the presence of plastid ribosomes and elongated starch grains.

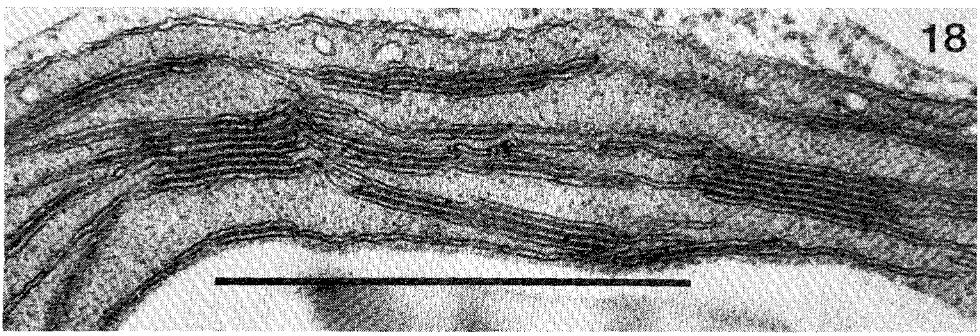


Figures 12—13. Pine chloroplasts from seedlings illuminated 24 hours (Fig. 12) and 48 hours (Fig. 13). These chloroplasts are of a plano-convex shape, their flat side facing the cell wall. The thylakoid system is well differentiated. No starch grains are present.

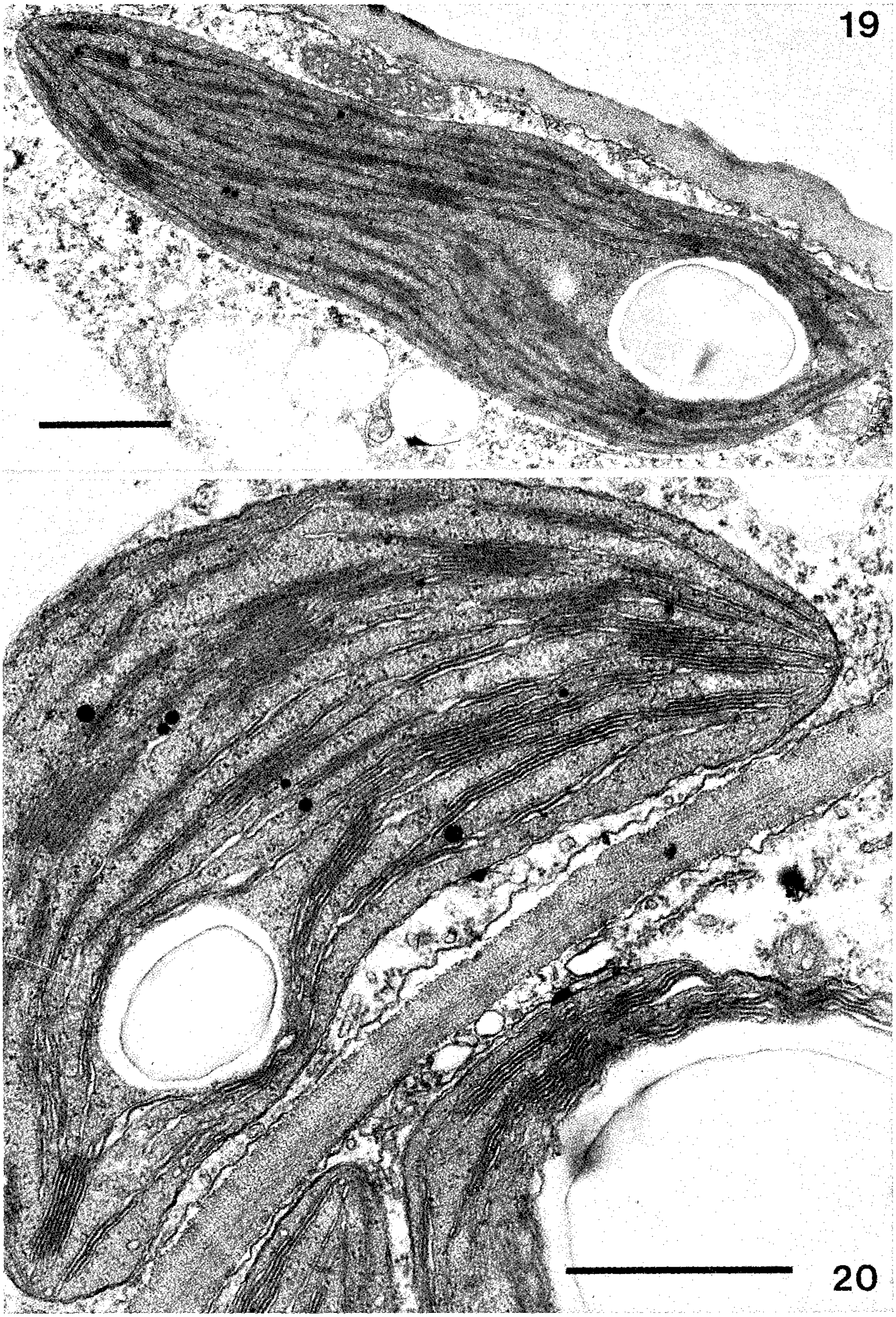


Figures 14—16. Chloroplasts in 11 days old dark-grown seedlings of spruce. In this conifer the prolamellar bodies are smaller but their number per plastid is higher than in larch and pine. The spacing of these bodies is narrow or wide. Sometimes both types of spacing occur in a single body (Figs. 14 (arrow) and 16). In spruce there are numerous prominent starch grains which are more or less rounded in shape.





Figures 17—18. Chloroplasts in 13 days old spruce seedlings (dark-grown). — Fig. 17. The thylakoid system contains many small prolamellar bodies and large grana formed by several discs. Fig. 18. Detail of the thylakoid system with typical grana. These two Figures demonstrate the remarkable ability of spruce seedlings to synthesize a differentiated thylakoid system in the absence of light. Compare with corresponding chloroplasts of larch (Fig. 2) and pine (Figs. 10—11)!



Figures 19—20. Spruce chloroplasts from seedlings illuminated 24 hours (Fig. 19) and 48 hours (Fig. 20). In contrast to corresponding organelles in larch and pine these chloroplasts contain starch grains. The plano-convex shape of the mature chloroplast is demonstrated in Fig. 20.