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Unusual thylakoid structures appearing during degradation of the photosynthetic apparatus in chloroplasts

MERCEDES WRISCHER, TATJANA PREBEG, VOLKER MAGNUS, NIKOLA LJUBEŠIĆ*

Ruđer Bošković Institute, Department of Molecular Biology, PO Box 180,
HR-10002 Zagreb, Croatia

Two types of thylakoid degradation are described, both including leaf bleaching (i.e. chlorophyll degradation) and destruction of the photosynthetic membranes.

The first type of degradation is characterized by coiling of single thylakoids and by cup-shape stacking of grana thylakoids. This type of thylakoid degradation was observed in leaves of the *aurea* varieties (conditional mutants) of several trees and shrubs. Leaf bleaching and thylakoid damage required strong sun-illumination and was repairable by shading, although only in young, still growing leaves.

A structurally similar type of membrane damage, although not repairable, was detected in wheat and bean leaves treated with cadmium, followed by sun-exposure.

The formation of interthylakoidally located membrane coils was not always dependent on strong light; they were also found in plastids of senescing leaves of *Sophora japonica.*

A second type of thylakoid degradation appeared as the result of irreversible damage: leaf bleaching induced by treatment with amitrole, followed by sun-exposure. The damage began with tight stacking of long straight grana thylakoids. Later, on plane and perpendicularly oriented sections of these grana thylakoids, osmiophilic particles with diameters around 8 nm became visible. These were arranged in hexagonal arrays, with an approximate spacing of 12 nm. The possible origin of these regular structures is discussed.

Structurally similar substructures in tightly stacked grana thylakoids were also detected in plastids of the subepidermal cells of *Cucurbita pepo* var. *ovifera* and *pyriformis* during fruit ripening, i.e. during the chloroplast-chromoplast transition.

Keywords: thylakoid, degradation, chloroplast, ultrastructure

Introduction

Of all environmental factors light has the most important influence on plants and their leaves (BJÖRN 2008). The leaves of land plants adapt with exceptional efficiency to the light conditions at their habitats. They can adjust to the weak light inside a tree crown, and react promptly to changes in illumination during a sequence of sunny and cloudy days. Chloroplasts from shaded leaves contain more chlorophyll and have larger grana with more thylakoids than those from leaves exposed to full sunlight (KUTIK 1998). The adaptation of

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* Corresponding author, e-mail: ljubesic@irb.hr

leaves to increasing insolation is particularly efficient in young tissues and includes special degradational processes in the photosynthetic apparatus.

Some structural transformations of chloroplasts are, in fact, degradational processes in the thylakoid system. These occur, for instance, in flower buds or in ripening fruits during chloroplast-chromoplast transitions, when the chloroplasts lose their photosynthetic activity and start the synthesis of special chromoplast constituents (LJUBEŠIĆ et al. 1991).

Autumnal leaf senescence is a degradational process as well, during which the chloroplasts gradually loose their vital characteristics to end up as senescent plastids which no longer contain photosynthetic membranes, but numerous pastoglobules (KESKITALO et al. 2005). In the leaves of overwintering trees and shrubs, chloroplast senescence stops progressing, during fall and winter, and is completed during the following spring (LJUBEŠIĆ et al. 2003).

Many environmental agents, such as bacterial or viral infections, various antimetabolites (lead, cadmium) and numerous bleaching herbicides cause reversible or irreversible damage and degradation of the photosynthetic apparatus. The ultrastructural changes caused by these agents are so far incompletely known. Here we examine the time course of two unusual types of thylakoid degradation, on the ultrastructural level.

Materials and methods

Aurea varieties sensitive to sunlight of the following six plant species were investigated: box elder (*Acer negundo* L. var. *Odessanum* (H. Rothe)), European ash (*Fraxinus excelsior* L. var. *aurea* (Willd.)), privet (*Ligustrum ovalifolium* Hassk. var. *aureum*), wintercreeper (*Euonymus fortunei* (Turcz.) Hand.-Mazz. var. *radicans* (Miq) Rehd.)), Japanese winter-tree (*Euonymus japonicus* Thunb. *aureo-marginatus*), and zelkova (*Zelkova serrata* (Thunb.) Mak. *»aurea«*)**.** The leaves of these plants, growing under different light conditions, were used for light- and electron-microscopic studies and for pigment analyses. The leaves of young seedlings of bean (*Phaseolus vulgaris* L. cv. *Starozagorski*), wheat (*Triticum aestivum* L., *»superzlatna«*), and maize (*Zea mays* L. cv. L-131), and those of the tips of pondweed (*Elodea canadensis* L.) were examined several days after treatment with a 1 mM or 0.1 mM solution of amitrole (3-amino-1*H*-1,2,4-triazole), or with a 0.1 or 1 mM solution of $CdCl_2$.

The outer cell layers (subepidermis) of green, ripening pumpkin fruit (*Cucurbita pepo* var. *pyriformis* and *Cucurbita pepo* L. ssp. *ovifera* (L.) D. S. Decker), were studied by light and electron microscopies and by pigment analyses. Senescent leaves (in late fall) of sophora (pagoda-tree) (*Sophora japonica* L.) were subjected to electron microscopy.

Small pieces of leaf or fruit tissues were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2), at 2 °C, for 1 h. After washing in buffer, the material was postfixed in 1% OsO_4. Cytochemical analyses of photosynthetic activity using the photooxidation of diaminobenzidine (DAB – photosystem I) and the photoreduction of thiocarbamyl nitrotetrazolium blue chloride (TCNBT – photosystem II) were performed according to WRISCHER (1989). After dehydration, the samples were embedded in araldite or in Spurr's medium. The sections were stained with uranyl acetate and lead citrate and subjected to transmission electron microscopy.

Pigments, extracted in 80% acetone, were measured spectrophotometrically (LICHTENTHALER 1987). Fresh tissue slices or pondweed leaves were used for chlorophyll detection by fluorescent microscopy.

Results

Thylakoid coiling – an indicator of peculiar thylakoid degradation

Thylakoid coiling is connected to a particular type of leaf bleaching, which is – as in *aurea* conditional mutants – dependent on high insolation. In the chloroplasts of sun-illuminated, bleaching leaves, there appeared unusual coils of double membranes, which obviously represent deformed single thylakoids, and cup-shaped compressed membrane stacks to be interpreted as deformed grana (the peripheral thylakoids were usually dilated) (Figs. 1a–c). Some membrane coils were sometimes located interthylakoidally, i.e.they were sandwiched in between two thylakoids. In these inclusions, the membranes could still be discernible, but they could also be just dark osmiophilic blots (Fig. 1d).

Membrane coils and cup-shaped membrane stacks were found in bleaching leaves of quite different plants. Most striking were those in the *aurea* varieties of trees and shrubs (WRISCHER et al. 1975 a, b, WRISCHER et al. 1976). Leaves that were situated at the periphery of the crown, and thus exposed to direct sunlight, turned yellow and, after prolonged insolation, they bleached and could even abscise. In these leaves, the levels of pigments, in particular those of the chlorophylls, were very low: in yellow leaves of privet and European ash, chlorophyll levels were 4.8% and 5.9% of those found in green control leaves. The thylakoid system of their plastids consisted only of single thylakoids and some vesicles, derived obviously from dilated thylakoids. In the plastids of strongly bleached (yellow-white) leaves, thylakoids did not exist at all, and the stroma only contained a few empty vesicles. On the other hand, leaves of the same tree or shrub, which grew in deep shade inside the crown, were dark green and contained normally developed grana. The plastids of light green leaves, positioned in half-shade and thus receiving somewhat more sunlight contained, in addition to some grana, also membrane coils and cup-shaped membrane stacks. The type and quantity of these membrane structures was not only species-, but even variety-dependent. Abnormal membranes were particularly numerous in yellow-green leaves (developing in half-shade) of the *aurea* varieties of privet, European ash and wintercreeper, while they were rarely found in similarly bleached leaves of box elder (WRISCHER et al. 1975 a, b, WRISCHER et al. 1976). During the above profound structural changes in the plastids, other organelles in the leaf cells, such as the mitochondria, remained structurally unchanged.

Photosynthetic activity (the activity of photosystem I expressed as photooxidation of DAB) was detected in bleaching plastids as long as there existed portions of thylakoids with discernible lumina. In cup-shaped compressed stacks, as well as in vesicles of completely bleached (yellow-white) leaves, the DAB-reaction was negative (Fig. 1e).

The formation and disappearance of the abnormally formed thylakoids could be experimentally induced by appropriate illumination or shading. When green leaves of the *aurea* variety of privet, previously growing in the shade, were exposed to sunlight, they started to yellow and, within a few days, membrane coils and cup shaped stacks of membranes appeared in their plastids. On the other hand, yellow *aurea* leaves from sun-exposed locations regreened, and the deformed thylakoids disappeared from the plastids, within two or three

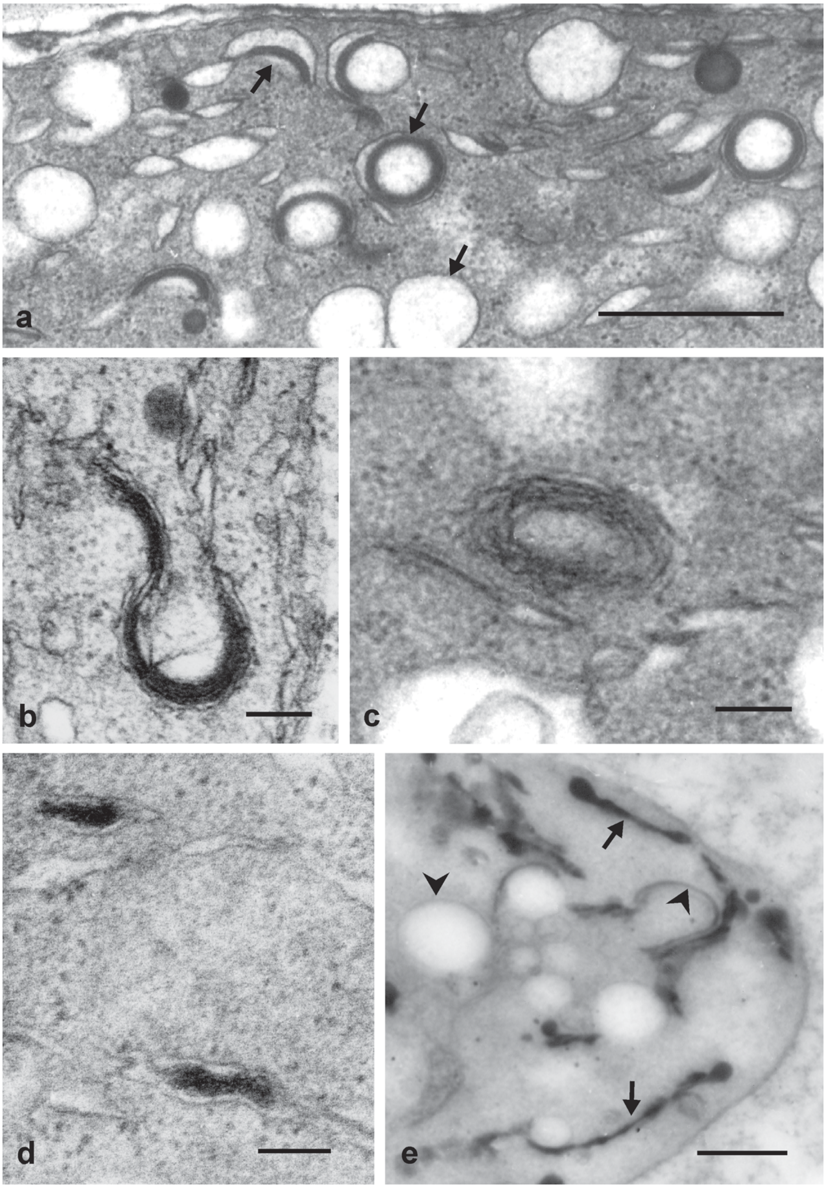

Fig. 1. Coiling of thylakoids in plastids of bleached (sun-exposed) leaves of *aurea* varieties.

a – *Ligustrum ovalifolium*. Plastid with membrane coils, cup-shape stacked membranes and vesicles (arrows). Bar = 1 µm.

b – *Ligustrum ovalifolium*. Part of plastid with cup-shape stacked membranes. Bar = 0.1 µm.

c – *Fraxinus excelsior*. Part of plastid with coil of membranes. Bar = 0.1 µm.

d – *Ligustrum ovalifolium*. Part of plastid with two interthylakoidal membrane inclusions. Bar = 0.1 µm.

e – *Ligustrum ovalifolium*. Plastid showing a positive DAB reaction (confirming photosynthetic activity) in the single thylakoids (arrows). Some membrane coils and vesicles are DAB negative (arrowheads). Bar = 0.5 µm.

days of shading. This reversal of the bleaching response was possible only in young, still growing leaves. After prolonged insolation, all thylakoids disappeared from the plastids and only large vesicles and some plastoglobules remained in the empty stroma (KUNST and WRISCHER 1984).

A similar type of thylakoid degradation (cup-shaped, curved grana) was also detected in the plastids of some other, *non-aurea* plants, for instance in wheat and bean leaves treated with cadmium. In these cases, coiling of thylakoid membranes was found in the plastids of sun-exposed leaves and, much less frequently, in those growing in weak light. The bleaching of cadmium-treated leaves in strong light was, however, not reversible by shading (WRISCHER and KUNST 1981).

Interthylakoidally located osmiophilic inclusions not dependent on strong illumination, were found in the leaves of sophora examined in November during their prolonged senescence. At that time, grana thylakoids were still present in the chloroplasts, but numerous plastoglobules indicated that leaf senescence was in progress (WRISCHER 1994).

Tightly stacked long, straight grana thylakoids

A special type of thylakoid degradation was the appearance of tightly stacked long, straight grana thylakoids, towards the end of leaf bleaching. While pigment levels, in particular those of the chlorophylls, dropped considerably, most grana thylakoids disappeared from the chloroplasts, and only a few long and straight grana remained in the completely empty stroma (Fig. 2a). At the beginning of this stacking process, the cytochemical reactions, indicating photosynthetic activities of photosystems I and II (DAB-oxidation and TCNBT-reduction), were positive only in those parts of the grana with still discernible lumina in the thylakoids. When a tightly stacked granum was cross sectioned, a regular, perpendicularly oriented, striation of osmiophilic structures, with a period of about 12 nm, became visible (Fig. 2b). In ideally plane-sectioned membrane stacks, we observed osmiophilic particles, about 8 nm in diameter, that were arranged in a regular hexagonal array, with an approximate spacing of 12 nm (Fig. 2c). In suitable unfixed objects, such as the thin leaves of pondweed, some faint red fluorescence was visible in the tightly stacked grana, indicating the presence of at least some chlorophyll, or its degradation products.

This type of thylakoid degradation has so far been observed in bleached, sun-exposed leaves or leaf parts of bean, maize and pondweed treated with the herbicide, amitrole (WRISCHER and LJUBEŠIĆ 1992). The bleaching appeared only in young tissue portions of the leaves, which were subsequently sun-exposed. The ultrastructure of older leaves, or leaf segments (in Monocotyles), remained unchanged, i. e. there was no bleaching and no tight stacking of grana thylakoids. Amitrole-treated leaves, growing in deep shade did not bleach. Tightly stacked (lysed) grana thylakoids with detectable substructures were also repeatedly observed in the subepidermis of ripening pumpkin fruit during the transformation of their chloroplasts into chromoplasts (DEVIDÉ 1970, LJUBEŠIĆ 1972, LJUBEŠIĆ et al. 1991).

Discussion

In the two types of thylakoid degradation described in this paper, intense sun-illumination caused bleaching of the leaves, i.e. photodestruction of chlorophyll and degradation of the photosynthetic apparatus. In *aurea* plant varieties, the bleaching of chloroplasts and the

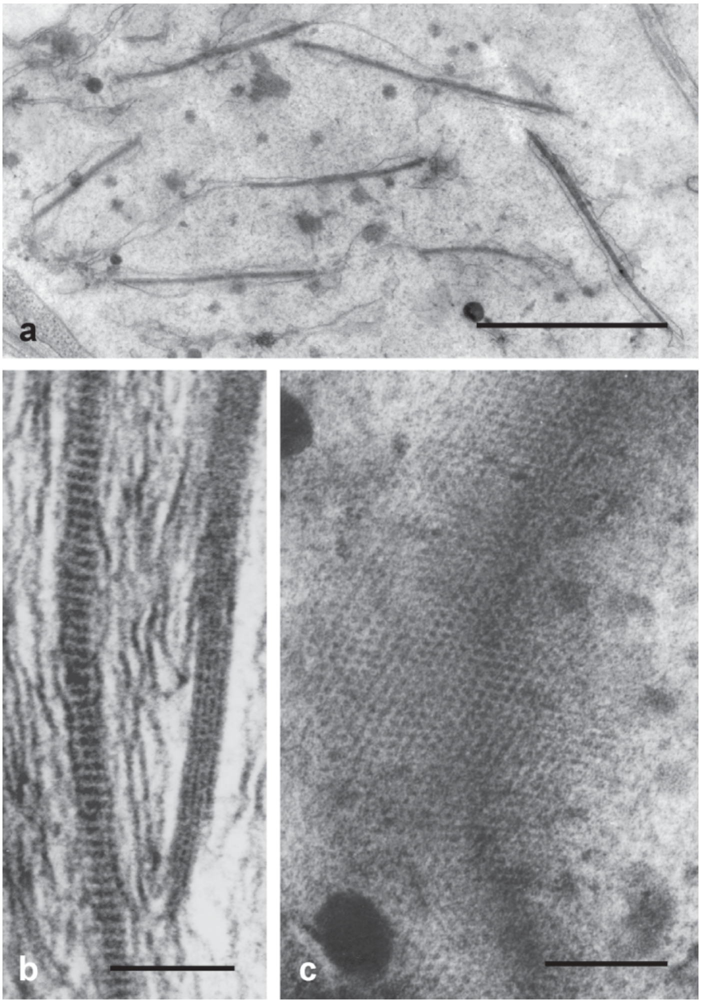

Fig. 2. Tight stacking of grana thylakoids in degrading chloroplasts.
a – *Phaseolus vulgaris.* Plastid from a bleached leaf treated with amitrole. The long grana consist of tightly stacked thylakoids. Bar = 1 µm.
b – *Cucurbita pepo* ssp. *ovifera.* Part of a plastid from a subepidermal cell of a yellow-green fruit. The tightly stacked thylakoids show perpendicularly oriented striation with a 12 nm period. Bar = 0.1 µm.
c – *Elodea canadensis*. Part of a plastid from a bleached leaf treated with amitrole; a plane section of a tightly stacked granum is presented. Particles of 8 nm diameter are arranged in a hexagonal pattern, with 12 nm spacing. Bar = 0.1 µm.

coiling and cup-shaped stacking of membranes (deformed grana) could be induced by increasing or lowering sun-exposure (WRISCHER et al. 1975 a, b; WRISCHER et al. 1976). Regreening of bleached *aurea* leaves is possible, at least in young ones, and could be induced by appropriate shading (KUNST and WRISCHER 1984). The unusual membrane structures formed in sunlight obviously represent defective or incompletely assembled parts of the thylakoids arising by photodestruction of some important, probably protein, components of the photosynthetic apparatus (FULGOSI et al. 2008). Also, the curved shape of the thylakoids could be the result of changes in fatty acid composition (MILLAR et al. 1998). In chloroplasts of leaf tissue bleaching after treatment with $CdCl_2$, a similar type of thylakoid degradation, i.e. the formation of cup-shaped grana thylakoids, could also be induced by strong illumination (WRISCHER and KUNST 1981). It should be mentioned, that, for the formation of interthylakoidal membrane inclusions, sunlight appears not to be the only dominant agent, as such structures were also observed in senescing leaves of sophora, in late fall (WRISCHER 1994).

The appearance of »substructures« in the lysed grana of amitrole-treated bleached leaves is induced by sunlight as well (WRISCHER and LJUBEŠIĆ 1992). These »substructures« became visible at the end of the degradation process of the grana thylakoids. Amitrole is believed to inhibit the synthesis of β-carotene, thus causing noncyclic carotenes (probably lycopene) to crystallize in vesicles in the plastid stroma, instead of being incorporated into the thylakoids (WRISCHER and LJUBEŠIĆ 1992). This lack of β-carotene causes instability of the photosynthetic membranes and photooxidation of the unprotected chlorophylls (SANDMANN and ALBRECHT 1990).

The hexagonally arranged, 8 nm large, particles observed in cross-sections of the stacked grana thylakoids resembled, both in form and in dimension, isolated antennae (light-harvesting chlorophyll a/b protein complexes) of the photosynthetic apparatus, which were incorporated into artificial membranes (MCDONNEL and STAEHELIN 1980). It was reported that, in strong light, amitrole blocked the formation of the reaction centers of both photosystems I and II and, in part, also that of the antennae (BUSCHMANN and GRUMBACH 1982). Our detection of traces of chlorophyll, or its degradation products, by a faint red fluorescence, in the already damaged thylakoid membranes, is therefore interesting, and indicates that the regularly arranged 8 nm large particles may be parts of the photosynthetic antennae. Our data on the progressive loss of positive cytochemical reactions for the two photosystems, in the course of thylakoid stacking are also well in accord with the observed simultaneous disappearance of the thylakoid lumina. Tight stacking of grana thylakoids was found in plastids of leaf tissue damaged by other bleaching herbicides as well. Substructural details of the lysed grana were so far only detected after amitrole treatment (WRISCHER and LJUBEŠIĆ 1992).

The appearance of regular »substructures« in the degraded grana thylakoids of chloroplasts in the subepidermis of ripening pumpkin fruit is unlikely to be induced directly by strong sunlight (DEVIDÉ 1970, LJUBEŠIĆ 1972, LJUBEŠIĆ et al. 1991). Rather, light appears to induce the degradation of the photosynthetic apparatus, by so far unknown indirect mechanisms.

The two described types of thylakoid degradation have the following common characteristics: (1) bleaching as a consequence of chlorophyll destruction, and (2) structural damage of the grana thylakoids. Thylakoid coiling and cup-shaped stacking, characteristic for the first type of degradation described, is mostly induced by strong illumination. The process could be reversed, at least in young tissues of *aurea* plant varieties, by lowering the intensity of the light exposure. The second described type of thylakoid degradation represents an irreversible process, which is – in some cases – also dependent on strong illumination. This type of degradation begins with tight stacking of the thylakoids in the grana and ends with their complete lysis.

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