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Thylakoid System Disassembly during Bleaching of Aurea Mutants of Maple *Acer negundo* Hassk. var. *Odessanum*

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Biogenesis of chloroplast photosynthetic membranes is a still not very well understood series of molecular events. Apart from performing photosynthesis reactions, these specialized biomembranes possess the ability of rearrangement to adapt to the ever-changing light environment. In this work, urea mutants of wild-growing maple trees were used to study events that lead to damage and disassembly of thylakoid membranes during the vegetation season. Plastid ultrastructural changes, changes in the composition of photosynthetic pigments and proteins, as well as the efficiency of photosystem II were monitored. It was concluded that strong photoinhibitory processes, coupled with the degradation of proteins, are responsible for the relatively fast reduction of photosynthetic membranes in aurea-type chloroplasts. It is evident that lipid constituents of thylakoid membranes are less susceptible to degradation processes. Once severely damaged, photosynthetic membranes of aurea-type leaves are no longer able to reconstitute the intertwined and functionally intact internal membrane system.

Keywords
• carotenoids
• light harvesting complex
• photoinhibition
• pulse amplitude modulated fluorimetry

INTRODUCTION

The photosynthetic machinery of vascular plants is located on a special internal membrane system of chloroplasts, the thylakoids. In addition to the dual genetic origin, these specialized biomembranes possess enormous physiological versatility, notably the ability to manage short- and long-term changes in the light environment.[1] Ultrastructural, biochemical and genetic analyses have resulted in our relatively well established knowledge about the thylakoid membrane system; however, the molecular processes connected with the synthesis, maintenance and adaptation of the thylakoids remain poorly investigated.[2] Two aspects of photosynthetic membranes found in modern chloroplasts remain most elusive. These are the evolution of an internal membrane system disconnected from the cell membrane and the exact mechanism by which this membrane is formed.[2]

Mutants are a powerful tool to study specific cellular processes. A large number of different mutants display deficiencies in thylakoid formation and restructuring. They include aurea, "golden", varieties of some vascular plants.[3,4,5] Leaves of these plants are extremely sensitive

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to high-light intensities and can remain normally green only in a deep shade. Prolonged exposure of aurea-type leaves to strong sunlight leads to their bleaching. When shaded, or during prolonged periods of low-light illumination, yellow and bleached leaves of aurea plants can regain green coloration. It has been shown that this type of regreening is associated with reassembly of the thylakoid system.[6] Conditional aurea mutants are especially suitable for ultrastructural studies of chloroplast transformations,[6] structure and function of photosystems I and II (PSI and PSII),[7,8] plastid signaling,[9] as well as the influence of aurea mutation on expression of nuclear *cab* genes.[4] In addition, some conditional mutants have considerably lower biosynthesis of carotenoids, which in high-light leads to photooxidative damage, destruction of chloroplast ultrastructure and termination of mRNA biosynthesis of some photosynthetic genes.[10]

In this work, we have used an aurea mutant of a wild-growing maple tree *Acer negundo* Hassk. var. *Odessanum* to study the mechanisms of thylakoid reduction and disassembly. Using electron microscopy and protein analyses, we observed that protein components of the thylakoid membrane are most susceptible to photooxidative damage. Photosynthetic pigments followed a similar degradation trend as observed with proteins, with carotenoids being most resistant to degradation. Measurements of the photosystem II efficiency indicated that this complex rapidly loses its activity in aurea leaves.

EXPERIMENTAL

Plant Material

Leaves of a wild-type maple *Acer negundo* Hassk. and an aurea-type mutant *Acer negundo* Hassk. var. *Odessanum* were collected from trees growing in the Zagreb Botanical Gardens. The samples were collected from mid-May to mid-October. Green aurea-type leaves were sampled from shaded portions of tree crowns where light exposure did not exceed 300 μmol m^{-2} s^{-1}. The unit μmol m^{-2} s^{-1} refers to the photon content of light. Most plant journals prefer the term "photosynthetically active photon flux density (PPFD)". Some journals prefer the term "photon irradiance" that is equivalent to PPFD. Yellow aurea-type leaves were sampled from sun exposed portions of the tree crown where light exposure did not exceed 1200 μmol m^{-2} s^{-1}. Maximal light intensity to which plants were exposed during sunny periods was 2500 μmol m^{-2} s^{-1}. Samples were collected in at least 3 replicates from 3 different trees. To prevent changes caused by the diurnal rhythm, samples were always collected at 9:00 a.m.

Electron Microscopy

Samples for electron microscopy analyses were collected in late June, when green, yellow and bleached aurea-type leaves were present on the same tree crown. For ultrastructural analyses, small pieces of tissue were fixed for 30 min with 1 % (vol. ratio) glutaraldehyde in 0.05 mol dm^{-3} cacodylate buffer (pH = 7.2) at 2 °C. Upon rinsing with the cacodylate buffer, the material was postfixed for 1 h with 1 % (vol. ratio) osmium tetroxide in the same buffer at 2 °C. The material was dehydrated through an ethanol series and embedded in Araldite resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Samples were observed using a Zeiss EM 10A electron microscope operated at an accelerating voltage of 60 kV.

Image Analyses and Computer Assisted Morphometry

For morphometric evaluations, micrographs were taken randomly from ultrathin sections of wild-type, green aurea-type and yellow aurea-type leaves at constant direct magnification. Quantitative morphometric analyses were conducted on 50 micrographs per sample according to Weibel and Elias.[11] Micrographs were digitized using a flat bed scanner, resolution 120 × 120 dpi. Images in TIFF format were imported into Adobe Photoshop 7.0 and subjected to image analysis using the indexed color method.

Pigment Analyses

For pigment extraction, fresh leaf tissue was mixed with a small amount of $BaCO_3$ and quartz sand and ground in a mortar in the presence of anhydrous acetone. Pigments (chlorophylls and carotenoids) were quantified spectrophotometrically according to Lichtenthaler.[12]

Measurements of PSII Efficiency

Measurement of chlorophyll *a* fluorescence was performed using a pulse amplitude modulated fluorimeter (PAM 100, Walz, Effeltrich, Germany). Plant material was dark-adapted for 30 min before measurements. Minimal (F_0) and maximal (F_m) fluorescence yields were measured using dark-adapted material. Maximum quantum yield (F_v/F_m) of PSII was calculated according to Schreiber: $F_v/F_m = (F_m - F_0)/F_m$.[13]

Extraction and Gel Electrophoresis of Proteins

Ten grams of fresh leaves were homogenized in extraction buffer (0.05 mol dm^{-3} Tris-HCl pH = 7.8, 0.4 mol dm^{-3} sucrose, 0.01 mol dm^{-3} NaCl and 1 % BSA) for 20 seconds on 4 °C. Homogenate was filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem) and centrifuged for 3 min on 200 *g*. Supernatant was further centrifuged for 10 min on 1500 *g*. Chloroplasts found in the pellet were lysed in hypotonic solution containing 0.01 mol dm^{-3} Tris-HCl pH = 7.8 and 0.01 mol dm^{-3} NaCl and centrifuged for 60 min on 34000 *g* in an ultracentrifuge. Pellet and supernatant were separated and protein concentration was determined according to Bradford.[14]

Different protein fractions were resuspended in gel loading buffer[15] and boiled for 2 min prior to loading onto discontinuous SDS-containing 10 % (T) polyacrylamide gels.[15] Upon completion of the run, gels were stained either with silver nitrate or with Coomassie Brilliant Blue R250.

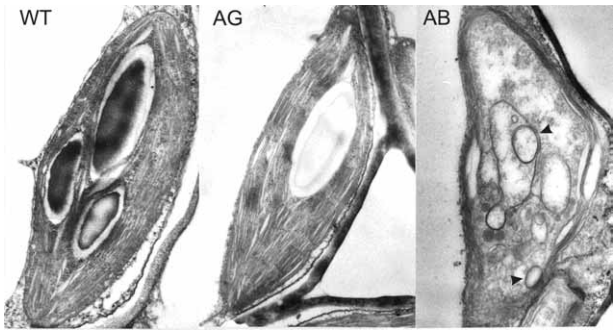

Figure 1. Transmission electron micrographs showing plastids in the wild-type (WT), green aurea-type (AG), and bleached aurea-type (AB) leaves. Concentric thylakoid leftovers are indicated by arrowheads. Magnification = 18 000 : 1.

RESULTS

Bleached Aurea Plastids Have Severely Altered Ultrastructure

Chloroplasts of wild-type *Acer negundo* have normally developed chloroplasts with regular grana stacks and stroma thylakoids (Figure 1). Stroma contains clearly visible ribosomes. Assimilation starch covers a large portion of stroma, while plastoglobules are present only in a very small number (Table I).

Chloroplasts of green aurea-type leaves are slightly smaller than the wild-type plastids (Figure 1). When quantified in cross sections, the thylakoid system is slightly less abundant than in the wild-type (Table I). Although grown in deep shade, aurea-type chloroplasts contain about equal amounts of assimilation starch as the wild-type ones. The number of plastoglobules is highly increased (Table I), indicating that these plastids were exposed to environmental stress.

Plastids found in yellow and bleached aurea-type leaves are enlarged (Table I). Their ultrastructure is highly altered, with the absence of assimilation starch and only very few ribosomes (Figure 1). Thylakoid system is severely damaged, completely lacking grana stacks. Stroma contains only a few unorganized single thylakoids and a large number of concentric membrane leftovers (Figure 1, indicated by arrowhead). Combined, these membranes occupy about 16 % of plastid cross sections (Table I). This value represents an almost seven-fold decrease in the amount of thylakoid structures compared to the chloroplasts of green aurea-type leaves. Due to the large reduction of plastid inner structures, as well as the swelling of plastids, the stromal space in the cross sections is highly increased (Table I). Plastoglobules are present in about the same amounts as in the chloroplasts of green aurea-type leaves.

Bleaching Is Accompanied by Disappearance of Photosynthetic Pigments

In the course of vegetation, photosynthetic pigments found in the wild-type leaves follow the usual seasonal changes in content. Fractions of chlorophyll and carotenoids (Car) increases until the late summer and is followed by a gradual decrease in the autumn (Figure 2). Chlorophyll *a* to chlorophyll *b* (Chl *a* / Chl *b*) ratio remains almost unchanged, from 1.55 to 1.80, until the mid-autumn. Total chlorophyll to carotenoids (Chl *a* + Chl *b*) / Car ratio follows the same trend and ranges between 5.49 and 5.90. Leaves collected in the late autumn have severely lower content of photosynthetic pigments. Chl *a* / Chl *b* ratio is lower than 1, while (Chl *a* + Chl *b*) / Car is lower than 2.

On average, green aurea-type leaves have 26 % less chlorophyll *a* and *b*, and about 20 % less carotenoids than the wild-type (Figure 2). Chlorophyll *b* deficiency is slightly more pronounced and averages 34 %. Chl *a* / Chl *b* ratio is increased to 2.2, while (Chl *a* + Chl *b*) / Car ratio averages 5.6.

Yellow aurea-type leaves have drastically decreased contents of all pigments, particularly of chlorophyll *a* and *b*, over 90 % (Figure 2). Fractions of chlorophylls change with light intensities. Lower light intensities increase the average fraction of chlorophylls while high-light rapidly

TABLE I. Total plastid area and particular ultrastructures in the ultrathin cross-sections of chloroplasts/plastids[a]

	Plastid area μm^2	Starch %	Thylakoids %	Thylakoid leftovers %	Plastoglobules %	Stroma %
Wild-type	10.8 (± 0.9)	14.6 (± 2.4)	72.9 (± 2.7)	0.0	0.3 (± 0.2)	12.2 (± 1.6)
Aurea-green	10.1 (± 1.4)	13.2 (± 2.9)	71.0 (± 2.2)	0.0	1.7 (± 0.5)	14.7 (± 1.3)
Aurea-yellow	11.9 (± 1.2)	0.0	11.4 (± 2.4)	4.8 (± 3.3)	1.9 (± 0.5)	82.0 (± 5.7)

[a]Results represent arithmetic mean values with standard errors in parentheses.

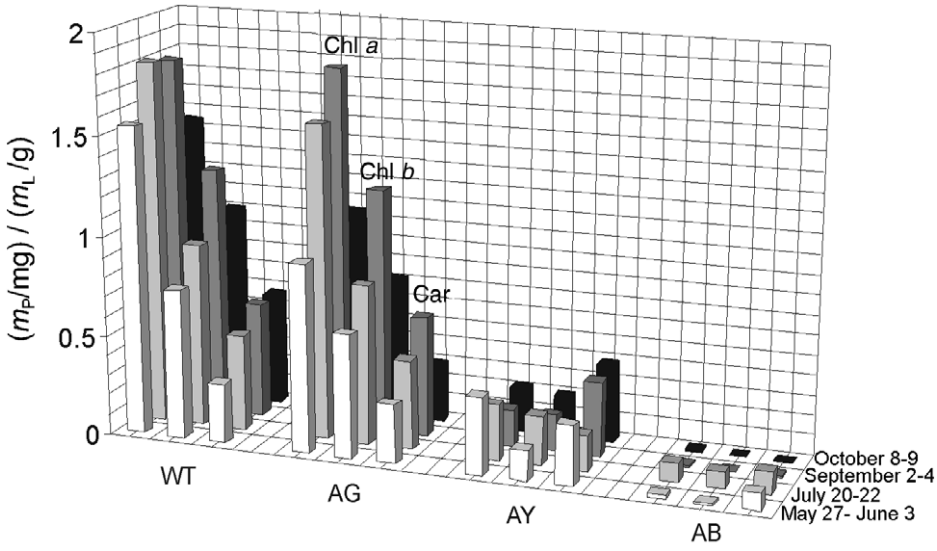

Figure 2. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid (Car) contents of the wild-type (WT), green aurea-type (AG), yellow aurea-type (AY), and bleached aurea-type (AB) leaves at various stages (indicated on the right) of the vegetation season. The results represent arithmetic means of four replications. Symbols: m_P – mass of pigments; m_L – mass of fresh leaves.

lowers it. Decrease in carotenoids is less pronounced, around 32 % on average. Chl *a* / Chl *b* ratio ranges between 0.83 to 2.4, while (Chl *a* + Chl *b*) / Car ratio is almost four fold decreased and ranges between 0.74 and 1.98. In the autumn, yellow aurea-type leaves have Chl *a* / Chl *b* and (Chl *a* + Chl *b*) / Car ratios similar to those in wild-type leaves.

Bleached aurea-type leaves have only 2–3 % of photosynthetic pigments left (Figure 2). Interestingly, Chl *a* / Chl *b* ratio is rather normal and ranges between 1.31 and 1.69. (Chl *a* + Chl *b*) / Car ratio is elevated in favor of carotenoids and averages 0.42.

Proteins of Photosynthetic Machinery are Degraded in Photoinhibited Aurea-type Leaves

Electrophoretic analysis of thylakoid membrane proteins (Figure 3) found in the wild-type chloroplasts revealed three distinct protein bands in the molecular weight range from 25–29 kDa (Figure 3, indicated by arrowheads), most likely corresponding to the LHCII apoproteins of the photosystem II antenna. Additional bands around 32 kDa and 34 kDa (Figure 3, indicated by arrowhead) correspond to the D1 and D2 core proteins of the photosystem II reaction center. Protein band at about 55 kDa corresponds to the large subunit of Rubisco and represents contamination of the membrane fraction. Plastids of green aurea-type leaves have a very similar electrophoretic picture, with clearly accumulated LHCII, D1, and D2 components (Figure 3).

During bleaching, clear degradation of LHCII components can be observed, while three new protein bands appear at about 17 kDa, 24 kDa and 31 kDa (Figure 3, indicated by asterisk). These bands are most likely proteolytic fragments of the LHCII components and the D1 protein, respectively. When entirely photo-bleached, chloroplasts of aurea-type leaves contain no detectable amounts

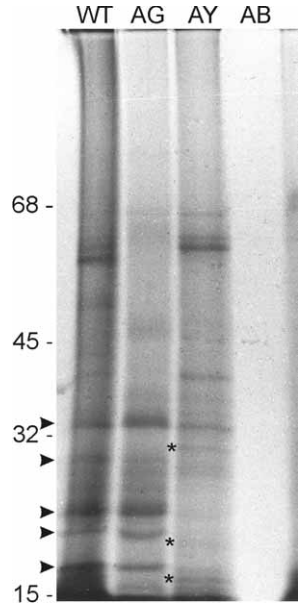

Figure 3. Polypeptide composition of plastid membrane fractions isolated from the wild-type (WT), green aurea-type (AG), yellow aurea-type (AY), and bleached aurea-type (AB) plastids. Left: positions of molecular weight standard in kDa. Equal amounts of proteins were loaded in each lane. Proteins were resolved in SDS-containing 10 % polyacrylamide gel and stained with silver nitrate. Arrowheads indicate major protein constituents, asterisks indicate polypeptides most likely arising from proteolytic degradation.

of photosynthetic proteins (Figure 3, lane AB), which indicates that membrane-like structures observed in electron-micrographs are devoid of proteinaceus components.

Aurea-type Leaves Have a More Efficient Photosystem II in Moderate Light Conditions

Measurements of *in situ* chlorophyll fluorescence were used to assess the PSII efficiency in wild- and aurea-type leaves. Values for the maximum quantum yield (F_v/F_m)

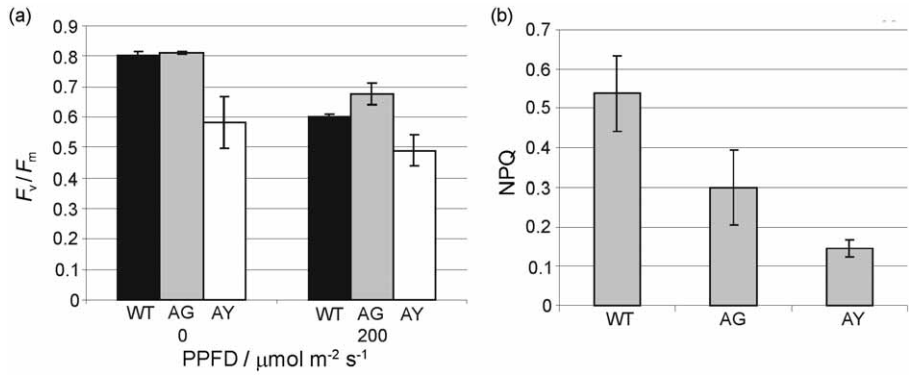

Figure 4. (a) Maximum quantum yield (F_v/F_m) of PSII at PPFD (photosynthetically active photon flux density) of 0 and 200 μmol m^{-2} s^{-1} in the wild-type (WT), green aurea-type (AG), and yellow aurea-type (AY) leaves. (b) Non-photochemical quenching (NPQ) in the wild-type and the two stages of aurea-type leaves at PPFD of 200 μmol m^{-2} s^{-1}.

of PSII are shown in Figure 4a. Wild-type and green aurea-type leaves have about the same F_v/F_m value, while the same parameter in yellow-aurea leaves is drastically lower. Under PPFD of 200 μmol m^{-2} s^{-1}, the highest PSII efficiency is recorded in green aurea-type leaves, while yellow aurea-type leaves perform poorly under conditions of moderate light intensity (Figure 4a). Apparently, in yellow aurea-type leaves, components of PSII are damaged and cannot sustain efficient photosynthesis under such conditions.

Non-photochemical Quenching in Aurea-type Leaves Is Low

To assess the ability of leaves to dissipate excess light energy, we have measured non-photochemical quenching (NPQ) in the wild-type and the two stages of aurea-type leaves (Figure 4b). Under PPFD of 200 μmol m^{-2} s^{-1} green aurea-type leaves have NPQ = 0.27, which is only half of the value recorded in the wild-type. In yellow aurea-type leaves, NPQ decreases even further and is about five times lower than in the wild-type. It is possible that low NPQ is the main cause of the photoinhibitory processes that take place in the aurea mutant.

DISCUSSION

The ability of the cell to build and alter the thylakoid membrane system is essential for efficient photosynthesis. The biogenesis of photosynthetic membranes is a series of well orchestrated molecular events, which must include biosynthesis of lipids, proteins and pigments.[2] The assembly process is initiated by formation of long lamellae, which are later upgraded by disc-shaped structures associating into the so-called grana stacks. As a result of these processes, mature plant chloroplasts contain a complex internal membrane system, which is responsible for driving high-efficiency photosynthesis.[16]

In this work, we have addressed the processes that lead to disassembly of the thylakoid system. Conditional aurea mutants are a valuable tool to study these processes, since they possess the ability of regreening, or more precisely, the ability to reassemble functional photosynthetic machinery. Aurea-type leaves develop normally in low-light conditions. Their chloroplasts contain the same amount of normally developed thylakoid membranes as chloroplasts from the wild-type leaves. Prolonged exposure to high-light intensities leads to bleaching of leaves and drastic changes in the chloroplast ultrastructure. Plastids are enlarged and starch biosynthesis is minimized. The thylakoid system undergoes major restructuring and reduction. Similar changes have been observed in aurea-type mutants of other plants.[17] It has been reported earlier that plastids with impaired carotenoid biosynthesis display similar ultrastructural changes.[18] However, lack of carotenoids *per se* does not influence chloroplast development, and it has no influence on the expression of genes important to chloroplast biogenesis.[19] Changes of the ultrastructure can be ascribed to photooxidative processes caused by the accumulation of non-photochemical energy within the photosystems. This type of energy, which cannot be utilized for photosynthetic reactions, accumulates in a special state of chlorophyll, the trilpet state.[20] Plants with inhibited chlorophyll biosynthesis exhibit no changes characteristic of photooxidative processes.

Aurea-type plastids have the same maximal efficiency of PSII as the wild-type, but their efficiency in moderate light conditions is increased. Total chlorophyll and carotenoid content in green and yellow aurea-type leaves is approximately 20 % lower than in the wild-type. Chlorophyll *b* deficiency is slightly more pronounced, which also increases the chlorophyll *a* to *b* ratio. Similar phenotypes have been observed in other aurea plants.[21,8] Okabe and co-workers[21] correlate the chlorophyll content with the size of photosynthetic unit; lower chlorophyll content is equivalent to a smaller photosynthetic unit. It has been proposed that a smaller photosynthetic unit enables more

efficient conversion of light energy under the elevated light conditions. Kunst and Wrischer[3] demonstrated that the photosynthetic efficiency of yellow aurea-type leaves of *Ligustrum* is almost five times higher than in green leaves. It is noteworthy that yellow leaves are deficient in chlorophyll *b*. Chlorophyll *b* molecules are mostly bound to proteins located on grana thylakoids and therefore their decrease can be linked to photooxidative damage of these structures.[7]

Green and yellow aurea-type leaves have drastically lower NPQ when measured at PPFD of 200 μmol m^{-2} s^{-1} than the wild-type. This might be a result of the more efficient electron transport rate, but it could be also attributed to the lower carotenoid pool. We have noticed that pigments of the violaxanthin cycle[22] are drastically lowered (data not shown), indicating that dissipation of excess light energy through this biochemical pathway might be impaired. This disability is most likely the trigger of photooxidative damage of photosynthetic proteins. The effect is mostly observed in bleached leaves, which have almost undetectable levels of β carotene. Consequently, violaxanthin can no longer be converted to zeaxanthin.

LHCII apoproteins are major constituents of thylakoid membranes. These proteins are pivotal for light energy harvesting and for simultaneous protection of reaction centers from photooxidation.[23] Our electrophoretic analyses indicate that chloroplasts of green aurea-type leaves accumulate normal levels of LHCII. In yellow aurea-type leaves, LHCII proteins are almost undetectable. The same degradation can be observed for proteins in the range of 32–34 kDa. This molecular mass range consists mainly of reaction center proteins, D1 and D2. Similar degradation of photosynthetic proteins has been observed in several high-light susceptible vascular plant mutants.[24,25] It is reasonable to assume that reaction center and antenna proteins are most affected by photoinhibitory processes, which lead to initial disassembly of the photosynthetic apparatus. Proteins that are photodamaged are prone to fast proteolytic degradation.[26] It seems likely that systems of controlled proteolytic degradation are highly active in aurea-type chloroplasts.

It is evident that lipid constituents of thylakoid membranes are least susceptible to photooxidative damage. Lipid vesicles and thylakoid leftovers can be found in severely photobleached plastids. Lipids apparently aggregate into concentrically arranged filaments, which remain intact even under conditions of prolonged high-light exposure. Also, lipids seem to be essential for the reassembly of the functional photosynthetic apparatus which can be found in regreened aurea leaves (data not shown). It is probable that the lipid backbone is required for integration of integral membrane proteins, which are in turn necessary for the assembly of the photosynthetic apparatus. One of the problems that have to be overcome during the re-assembly is the reconstitution of the lumenal space. Translocation of proteins into the thylakoid lumen must be re-established and processing peptidases have to work properly.[27] It would be valuable to explore which proteins of the photosynthetic apparatus are first integrated into lipid bilayers to catalyze the integration and the assembly of functional photosystems.

To conclude, aurea mutants represent a valuable tool to study the series of events that lead to disassembly of thylakoid membranes. By studying individual steps of this process, as well as by monitoring the mechanisms of reassembly occurring in aurea mutants, we aim to gain a better insight into the biogenesis of photosynthetic membranes of vascular plants.

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SAŽETAK

Razgradnja tilakoidnog sustava tijekom izbjeljivanja aurea mutante javora *Acer negundo* Hassk. var. *Odessanum*

Hrvoje Fulgosi, Snježana Jurić, Hrvoje Lepeduš, Kroata Hazler-Pilepić, Tatjana Prebeg i Nikola Ljubešić

Biogeneza fotosintetskih membrana kloroplasta još uvijek je slabo istražen niz molekularnih procesa. Osim što provode reakcije fotosinteze, ove specijalizirane biološke membrane posjeduju veliku sposobnost rearanžmana i prilagodbe stalnim izmjenama u intenzitetu svjetlosti. U ovom smo radu koristili aurea mutante javora kako bi istražili procese koji dovode do oštećivanja i raspadanja tilakoidnih membrana tijekom sezone vegetacije. Pratili smo promjene ultrastrukture plastida, sadržaja fotosintetskih pigmenata i proteina, kao i efikasnosti fotosustava II. Zaključili smo da su fotoinhibitorni procesi u kloroplastima aurea-tipa povezani s degradacijom proteina i brzom redukcijom fotosintetskih membrana. Razvidno je da su lipidne komponente tilakoidnih membrana najmanje osjetljive na procese degradacije. Uslijed velikih oštećenja uzrokovanih dugotrajnim izlaganjem svjetlosti visokog intenziteta kod listova tipa aurea nije više moguća rekonstitucija funkcionalno cjelovitog fotosintetskog sustava membrana.