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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Investigations into the »inverse contrast« of chloroplast thylakoids

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We detected an »inverse contrast« (i. e. dense lumen and lightly staining membrane) of thylakoids in some species or organs where it has not previously been described. We probed the chemical character of the dense lumenal substance by *in situ* extraction on ultrathin sections, and found that it was less hydrophilic in *Perilla frutescens,* than in *Helianthus annuus,* where it could be neither lipid nor protein. Its disappearance from the lumen upon prolonged illumination was accompanied by a drop in the plastidial polyphenol oxidase activity, so it may be a phenolic compound. Growing *Helianthus* seedlings at 30 °C (rather than at 25 °C) resulted in the density appearing in the stroma (rather than in the lumen), but still preventing the strong staining of the thylakoid membrane. Upon the removal of the dense substance from the stroma by plastid isolation the staining pattern reverted to its usual appearance in the plastids. This excludes the explanation that light membrane staining is produced by some compositional deficiency. This conclusion was supported also by gel electrophoretograms of polypeptides from such membranes. We think that the dense substance on either side of the membrane protects lipid molecules from a conformational change during solvent dehydration, which normally leads to the strong staining of the membrane.

Key words: chloroplast, thylakoid, dense lumenal substance, electron microscopy, polyphenol oxidase

Abbreviatons: DLS = dense (densely staining) lumenal substance, TLS = transparent lumenal substance, LSM = lightly staining membrane, SSM = strongly staining membrane, PPO = polyphenol oxidase

Introduction

Since the late sixties an increasing number of reports have appeared demonstrating an unusual density pattern of chloroplast thylakoids in certain species (SRIVASTAVA 1966,

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ISRAEL and STEWARD 1967, BLACKWELL et al. 1969, LJUBEŠIĆ 1970, LEDBETTER and PORTER 1970, SALEMA et al. 1972, DAMSZ and MIKULSKA 1976, PLATT-ALOIA and THOMSON 1977, CASADORO and RASCIO 1978a, KASHINA et al. 1982, VARKEY and NADAKAVUKAREN 1982). At a low magnification these thylakoids seemed to show a negative contrast, inasmuch as the membranes were lightly stained, while the lumens were electron-dense. At a higher resolution it turned out, however, that the membranes were still trilaminar, just the dense bands were very thin, and the transparent zone was wide. So the contrast was not really negative for the membranes themselves (CASADORO and RASCIO 1978b).

In order to elucidate the chemical nature of the dense lumenal substance (DLS), *in situ* enzymatic digestions were carried out. According to the results, the presence of lipoproteins seemed probable in some species (SALEMA et al. 1972, DAMSZ and MIKULSKA 1976), while in other species this finding could not be confirmed (VAN STEVENINCK and VAN STEVENINCK 1980b). We have attempted fixation experiments and extraction probes in two species (*Helianthus annuus* and *Perilla frutescens*) which have not been used, to the best of our knowledge, for such studies.

Another, but related problem is the interpretation of the lightly staining membranes (LSM). The early suggestions for a lipoproteinaceous character of DLS led to the conclusion, that these membranes might be incomplete, the missing building blocks being in the lumen. According to our present knowledge about membrane synthesis this view is highly improbable, especially in species where DLS has a different chemical character. Moreover, in certain chloroplasts with LSM, starch grains were demonstrated (CASADORO and RASCIO 1978b), which shows the photosynthetic competence of such thylakoid membranes. We isolated chloroplasts and thylakoids with or without DLS to see if there are any compositional differences.

Materials and methods

Cotyledons of *Helianthus annuus* L. cv. Iregi szürke csíkos, fully expanded leaves of *Achillea filipendulina* Lam., *Fragaria vesca* L. and *Perilla frutescens* Britt., as well as peel of freshly harvested fruits of *Pyrus domestica* Medik. cv. Hardenpont were examined. *Helianthus* was grown in the laboratory, *Achillea* and *Perilla* samples were taken from gardens, *Pyrus* was grown in an orchard, *Fragaria* leaves were collected from a forest.

The fruit wall of *Helianthus* achenes was removed and the seeds were germinated on wet filter paper in Petri-dishes generally at 25 °C or exceptionally at 30 °C. After 4 days of etiolation, cotyledons were sampled after 0, 8, 12, 16, 20, 24, 30 and 48 hours of 20 μmol m^{-2} s^{-1} illumination by white fluorescent tubes.

Tissue pieces from all the materials were fixed in 5% glutaraldehyde (GA) in 0.035 M Na-K phosphate buffer, pH 7.2, for 2–24 hours, then postfixed in 1% OsO_4 for 2 hours. In *Helianthus*, alternative fixations included (1) 8 % formaldehyde (FA) then 1% OsO_4, (2) 5% GA alone, (3) 1% OsO_4 then 5% GA, and (4) 1% OsO_4 alone, using the above buffer. After dehydration in ethanol or acetone series, samples were embedded in Durcupan ACM or Spurr's resin, respectively. Ultrathin sections were made by a Porter-Blum ultramicrotome, stained with uranyl acetate and lead citrate, or viewed without staining in a Tesla BS-500 electron microscope.

For enzymatic digestion, sections from GA-OsO_4 fixed samples of *Helianthus* and *Perilla* were mounted on formvar coated Ni grids, then deosmicated with 10% H_2O_2 for 10 min at 37 °C. Alternatively, GA fixed samples were also used. Enzymatic digestion probes were carried out with 0.5% pepsin (Merck) in 0.1 M HCl at 37 °C for 2 hours, or 0.5% pronase E (Merck) at pH 7.4 (set with NaOH) at 37 °C for 24 hours, or 0.3% α-chymotrypsin (Sigma) at pH 7.8 (set with NaOH) at 25 °C for 24 hours. In the control groups the enzymes were omitted or distilled water was applied for 2–24 hours.

The chlorophyll content was measured in 80% acetone according to Porra et al. (1989). Protein concentration was determined with BRADFORD'S (1976) method.

Intact chloroplasts were isolated from *Helianthus* cotyledons with the method of Mills and Joy (1980), but using only 30% Percoll. Plastids were osmotically shocked in 10 mM Tricine-KOH (pH 7.9), 1 mM $MgCl_2$, and thylakoids were pelleted at 5000g for 7 min.

Denaturing gel electrophoresis of thylakoid proteins was performed according to LAEMMLI (1970) except that a 10–18% acrylamide gradient was used (14 cm long gels). Gels were stained with 0.025% Coomassie Brilliant Blue R-250 in 30% methanol and 10% acetic acid, destained in 30% methanol and 10% acetic acid, and stored in 7% acetic acid.

Polyphenol oxidase activity was measured as an absorbance change at 485 nm in 50 mM Na/K-phosphate buffer (pH 6.8) with 0.2 mM 3,4-dihydroxy-phenylalanine as a substrate, and the reaction was totally inhibited by 0.5 mM phenylhydrazine (LERNER et al., 1971). Supernatant of the organellum fraction (centrifugation of the homogenate at 20000g for 30 min), and that of the thylakoids isolated from the osmotically shocked chloroplasts were used as samples.

Chloroplast isolation, gel electrophoresis, and PPO activity measurements were performed three times (three different experiments).

Results

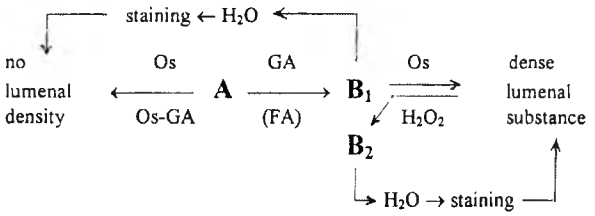
Occurrence of DLS

We found chloroplasts containing DLS in different organs of different species during our studies. In the inner cells of the peel of freshly harvested *Pyrus domestica* fruits, chloroplasts with DLS were encountered among the regular ones (Fig. 1). In *Fragaria vesca* chloroplasts with DLS appeared in the epidermis, and in shade plants also in the mesophyll (Fig. 2). In *Achillea filipendulina* these were found in the epidermal and subepidermal cells (Fig. 3), while in *Perilla frutescens* in the epidermis, the chlorenchyma and the vascular parenchyma (Fig. 4).

In *Helianthus* the occurrence of DLS was transient, depending on the duration of both the etiolation and the subsequent illumination (RASCIO et al. 1979). We have found that the seeds must be less than one year old, the illumination (at the intensity applied) may not exceed 24 hours (Figs. 5 and 6), and the temperature has to be kept below 30 °C (Fig. 13).

Apart from the developmental and environmental parameters, the way of fixation is also decisive (Scheme 1). The lumenal substance in question (A) is not electron-dense in itself but is stainable with Os and/or other heavy metals. It can be retained in the lumen, however, by aldehyde prefixation only (especially by GA, the FA being less effective). The

aldehyde may modify it chemically to substance B_1, which can be stained with OsO_4, or directly with uranyl- and Pb-salts (Fig. 7). OsO_4 as a first fixative does not retain it in the lumen, not even if osmication is followed by GA fixation. Presumably substance A diffuses out from the lumen during this procedure (Fig. 8). After deosmication of GA-OsO_4 fixed samples, we get B_2 in the lumen, which differs from B_1 in water solubility.



Scheme 1 – Effects of fixatives on lumenal staining ability

Extraction of DLS

We attempted *in situ* digestion of the DLS with pepsin (Fig. 9), chymotrypsin and pronase in both *Helianthus* and *Perilla*. In *Helianthus,* when using deosmicated (H_2O_2 treated) sections from GA-OsO_4 fixed samples, no digestion was seen. Using GA fixed samples, however, DLS could be extracted by distilled water (Fig. 10). In GA-OsO_4 fixed *Perilla,* already H_2O_2 removed DLS from the grana, while leaving behind DLS in the stroma thylakoids (Fig. 11). When GA fixed samples were probed with distilled water, DLS was not solved out even during 24 hours (Fig. 12).

Polyphenol oxidase (PPO) activity

The activity of this enzyme decreased in *Helianthus* cotyledons with a lengthening of the illumination. The decrease was slight in the cytoplasm fraction, but it was marked in the plastids, between 24 and 48 hours (Tab. 1).

Tab. 1. Polyphenol oxidase activity measured as absorbance increase/mg protein at 485 nm in different compartments of *Helianthus* cotyledons after different time of illumination. (Values are given as mean ± S.D.)

Illumination time (h)	Polyphenol oxidase activity (ΔA_{485}/mg protein)	
	cytoplasm	chloroplast stroma
24	0.428 ± 0.031	0.122 ± 0.035
48	0.374 ± 0.053	0.046 ± 0.009

Examination of LSM

As mentioned earlier, growing *Helianthus* seedlings at 30 °C, even if illuminated for less than 24 hours, precluded the appearence of DLS. The stroma, however, was unusually dense, and the thylakoid membranes were lightly stained (Fig. 13). When we removed the

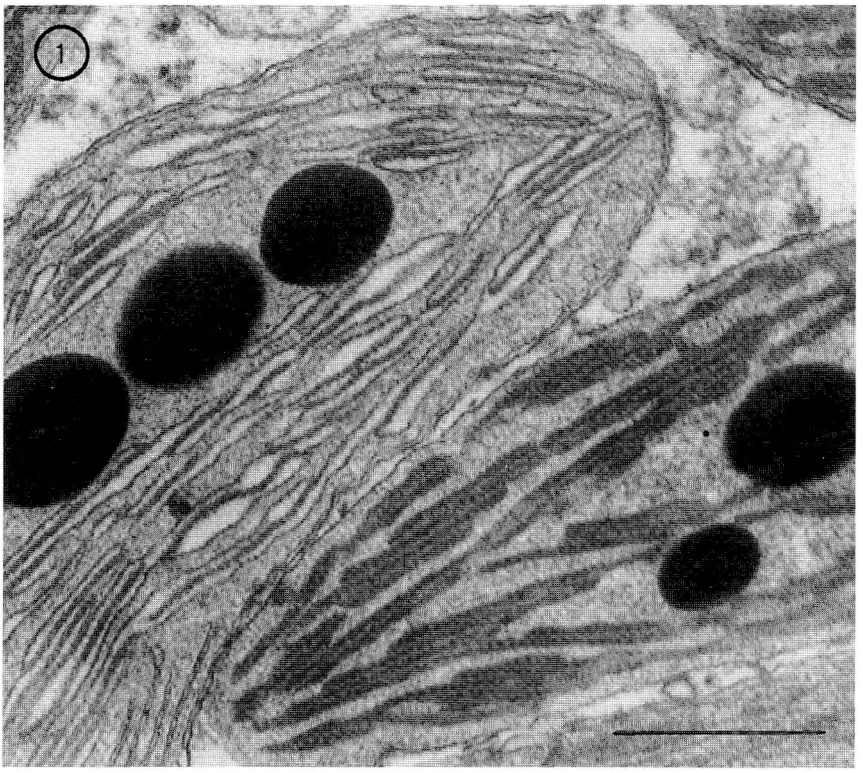


Fig. 1. Plastids of normal and »inverse« contrast in the same cell from the peel of freshly harvested ripe fruit of *Pyrus domestica*. Bar = 0.5 μm.

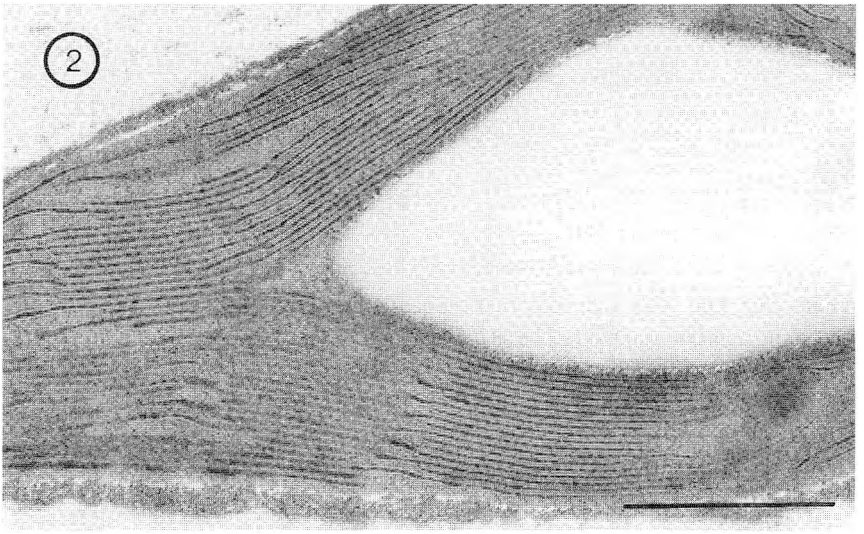
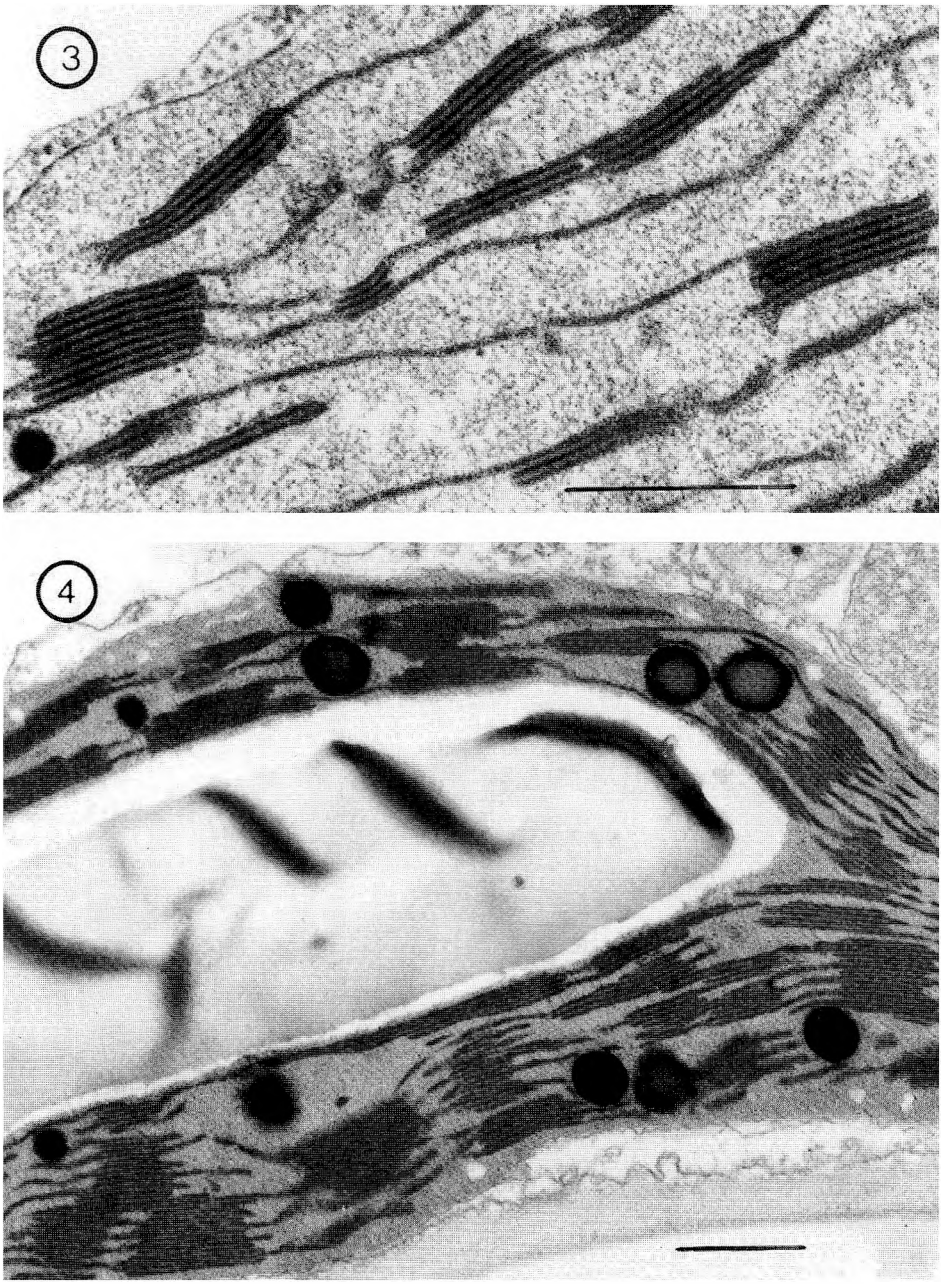
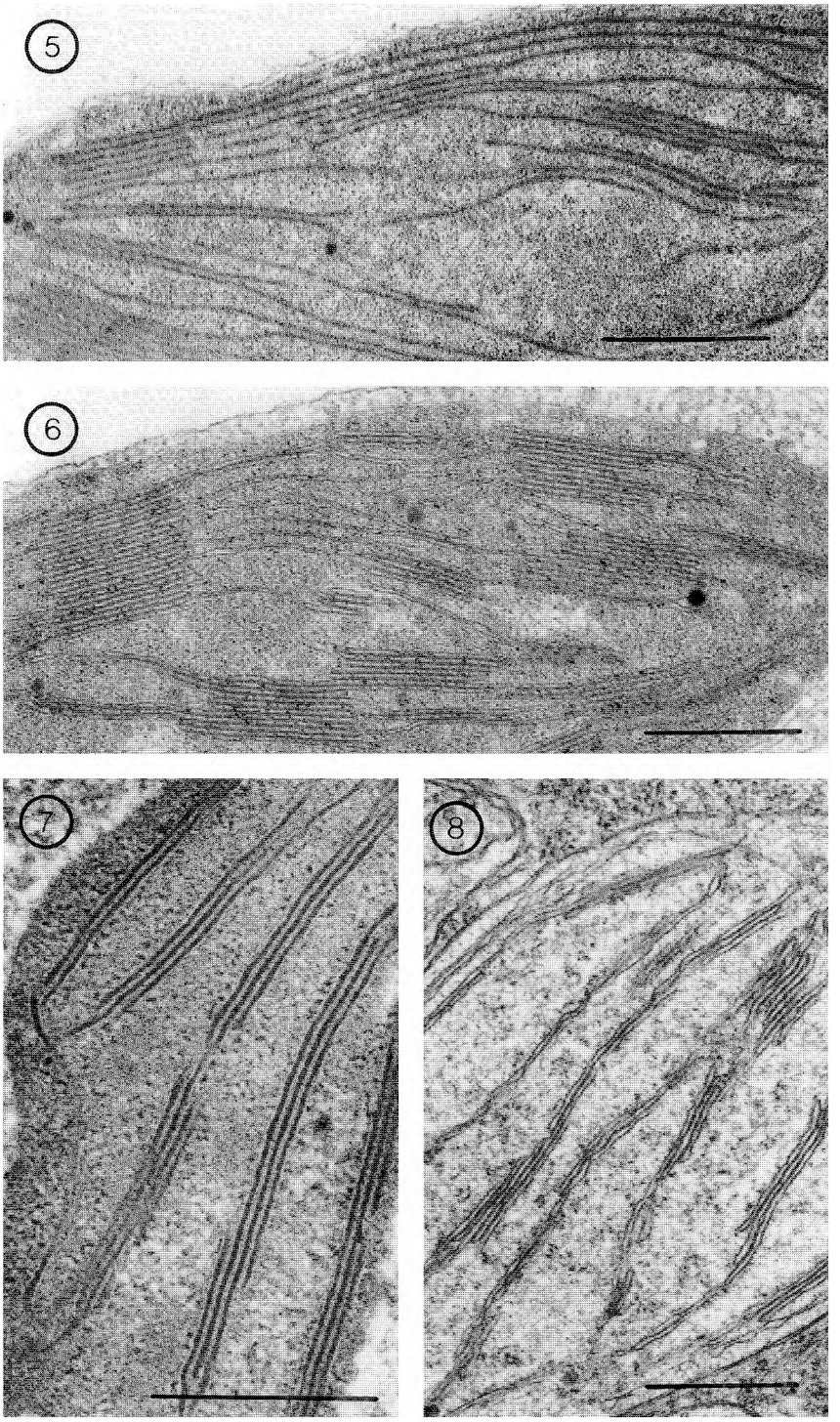


Fig. 2. DLS fills the lumen of thylakoids in mesophyll chloroplast in fully expanded leaves of *Fragaria vesca*. Bar = 0.5 μm.

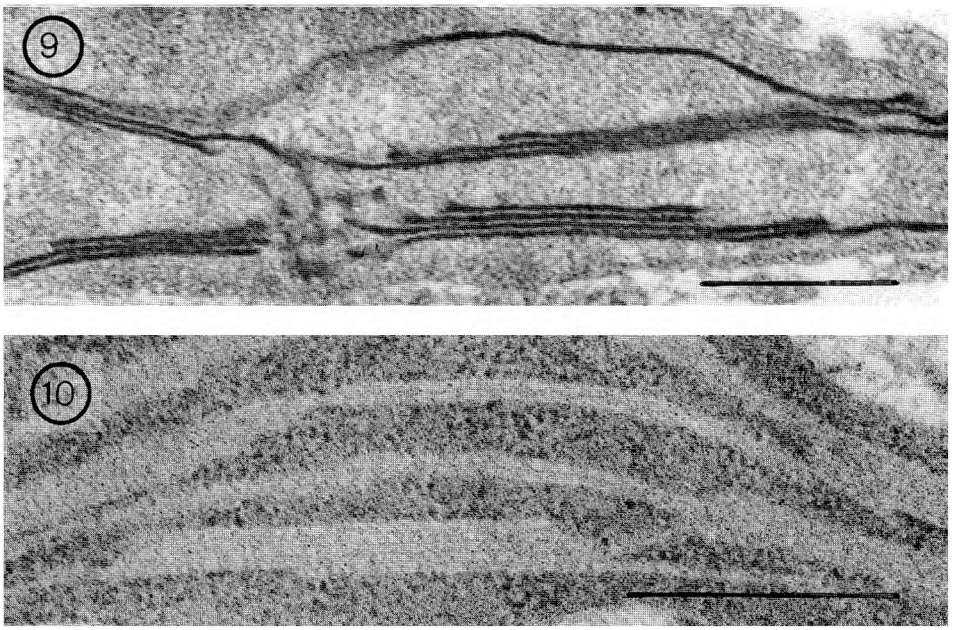
dense stroma by isolating and osmotically shocking such plastids, the thylakoid membranes became strongly stainable (Fig. 14).



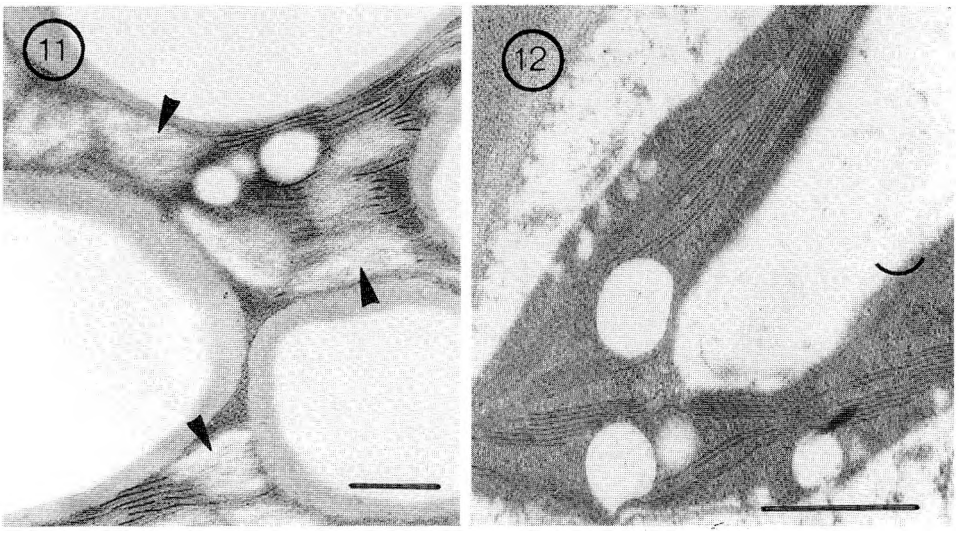
Figs. 3–4. »Inverse contrast« in mesophyll chloroplasts in fully expanded leaves of *Achillea filipendulina* (Fig. 3), and *Perilla frutescens* (Fig. 4). Bars = 0.5 μm.



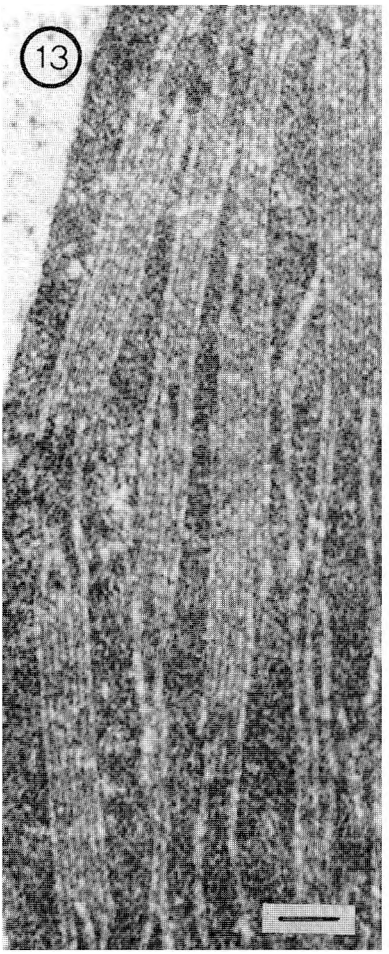
Figs. 5–8. Chloroplasts in *Helianthus annuus* cotyledons, after 24 hours (Fig. 5), and 48 hours (Fig. 6) of illumination, then GA – OsO_4 fixation. Fig. 7: GA fixation, Fig. 8: OsO_4 fixation, both after 8 hours of illumination. Bars = 0.5 μm.



Figs. 9, 10. Chloroplasts in *Helianthus annuus* cotyledons after GA – OsO_4 fixation, deosmication of sections with H_2O_2, then pepsin treatment (Fig. 9), or after GA fixation, then floating the section on distilled water for 2 hours (Fig. 10). Bars = 0.5 μm.



Figs. 11, 12. Chloroplasts in *Perilla frutescens* leaf, after GA–OsO_4 fixation, then deosmication of sections with H_2O_2 (Fig. 11, arrowheads point to remnants of grana), or after GA fixation, then floating the section on distilled water for 24 hours (Fig. 12). Bars = 0.5 μm.



Figs. 13–14. Chloroplasts of *Helianthus annuus* grown at 30 °C, and illuminated for 20 hours, in the cotyledon (Fig. 13), or after isolation (Fig. 14). Bars = 0.1 μm in Fig. 13, and 1 μm in Fig. 14.

Chlorophyll content and chlorophyll *a/b* ratio were normal in cotyledons illuminated for 24 hours, the former increased, the latter somewhat decreased by an extension of the illumination to 48 hours, as expected (Tab. 2). The decrease of the chlorophyll *a/b* ratio (in each experiment) was due to the later increase in the amount of light-harvesting chlorophyll *a/b* complexes of photosystem II, as was also seen in the polypeptide pattern of thylakoids (Fig. 15: 26 kDa band).

Comparing the thylakoids isolated from *Helianthus* cotyledons illuminated at 25 °C for 24 and for 48 hours, we could not find substantial differences between LSM and SSM in respect of polypeptide composition with polyacrylamide gel electrophoresis (Fig. 15). Both of them contained the > 60 kDa apoproteins of the P700 chlorophyll-protein, the characteristic large subunits of CF_1 (58 and 55 kDa) still attached to the membrane, the core

Tab. 2. Chlorophyll content and chlorophyll *a/b* ratios in differently illuminated *Helianthus* cotyledons. (Values are given as mean ± S.D.)

Illumination time (h)	Chlorophyll *a+b* (μg/g fresh mass)	Chlorophyll *a/b*
24	369.6 ± 70.5	4.07 ± 0.22
48	531.0 ± 97.7	3.89 ± 0.15

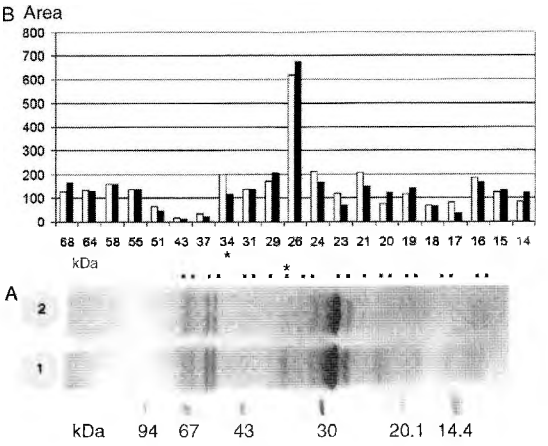


Fig. 15. Polypeptide patterns (A) and densitograms (B) of thylakoids isolated from *Helianthus* cotyledons greened for 24h (1, white) and 48h (2, black), respectively. Standard proteins (in kDa): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean tripsin inhibitor (20.1), α-lactalbumin (14.4). Polypeptides characterised by their molecular mass (in kDa) on B are marked by points in the same order next to the bands of the gels on A. Densitograms were normalised to the same amount of CF_1 polypeptides (58 and 56 kDa).

polypeptides of photosystem II (in the range of 51–32 kDa), and the apoproteins of light harvesting complexes of photosystem I and II (in the range of 29–21 kDa), as well as photosystem I core polypeptides of low molecular mass. The greatest difference between the thylakoids having DLS and those greened longer, during which DLS disappeared, was the higher amount of an unknown 34 kDa polypeptide in the former sample.

Discussion

The nature of DLS

In *Helianthus* the DLS can be solved out from the sections of GA fixed material by water. This is not the case with most proteins, which are crosslinked by dialdehydes. Also, proteinases failed to digest DLS after deosmication. The hydrophilic character precludes its lipid nature as well. These results are at variance with those by SALEMA et al. (1972) on *Hedera,* and DAMSZ and MIKULSKA (1976) on *Cattleya,* but are in accordance with the findings by VAN STEVENINCK and VAN STEVENINCK (1980b) on *Nymphoides.* They set up the

hypothesis that the DLS could be a phenol type substance of low molecular weight. Our finding that the PPO activity drops in *Helianthus* plastids when the DLS is developmentally lost also points in this direction. PPO activity has been demonstrated in the thylakoids, or precisely in the thylakoid lumen of some species, e.g. soybean (LAZAROVITS and SINGH 1986), tomato, pea, and maize (SOMMER et al. 1994, KOUSSEVITZKY et al. 1998). This renders probable the presence of phenolics in this compartment, even if PPO may be latent here normally (VAUGHN and DUKE 1984). There are also other lines of circumstantial evidence suggesting that phenol type substances may constitute a likely candidate for DLS. We observed that γ-irradiation induced the appearance of DLS in apple and pear fruit peel (KOVÁCS and KERESZTES 1989, KERESZTES and KOVÁCS 1991, KOVÁCS and KERESZTES, in press). It is known, that γ-irradiation specifically enhances phenol metabolism (RIOV et al. 1972).

An interesting question is the possible function of phenols in the thylakoid lumen. According to HUTCHESON et al. (1980), a phenol may be an electron donor to $NADP^+$ reduction.

In *Perilla* the DLS is either more hydrophobic, or just the degree of aldehyde cross linking may be higher, than in *Helianthus*. At present we are not able to interpret the different sensitivity of the DLS against H_2O_2 in the lumen of grana and stroma thylakoids.

Characterization of LSM

In the different species examined hitherto under natural or experimental conditions, the presence of DLS was always coupled to LSM. We have found an experimental regime (*Helianthus* cotyledons grown at 30 °C), which separates these two phenomena. In this way we can see a transparent lumen surrounded by a lightly staining membrane, although on the other side of the membrane (in the stroma) there is an increased density. We suppose that the dense substance was synthesised but not transferred into the lumen at the elevated temperature. However, its presence on either side of the membrane may protect lipid molecules from conformational changes during solvent dehydration, which normally leads to the usual »unit membrane«-staining pattern (THOMSON 1974, OLESEN 1978, VAN STEVENINCK and VAN STEVENINCK 1980a). A further support for this concept is provided by the images of these plastids after isolation: the dense substance seems to leak out from the stroma (the density decreases), and concomitantly the thylakoid membranes become strongly staining.

The fact that the very same membrane may stain differently according to the presence or absence of the dense substance precludes the possibility that these LS membranes might have an abnormal composition. With respect to membrane polypeptides, we have acquired experimental proof that this is not the case.

Acknowledgements

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