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Isolation and characterization of paracrystalline structures from transgenic P*ssu-ipt* tobacco

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Abstract

Distinct crystalloids were found in chloroplasts of transgenic P*ssu-ipt* tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) overproducing endogenous cytokinins. They were present both in rooted (T) and grafted (TC) transgenic plants contrary to control tobacco (C). The fractions enriched by crystalloids were isolated from chloroplasts using a continuous or a discontinuous *Percoll* gradient. Chlorophyll (Chl) fluorescence emission spectra at 77 K indicated the presence of aggregates of light-harvesting complex proteins (LHC2) that was not connected to reaction centres of photosystem 2 both in isolated chloroplasts and in the fraction of 80 % *Percoll* gradient from both types of transgenic tobacco. Further analyses, *i.e*. pigment contents, polypeptide composition by SDS-PAGE, and immunoblotting support our hypothesis that crystalloids inside chloroplasts of transgenic tobacco are formed by LHC2 aggregates. Treatment with two distinct detergents, chosen with respect to their effects (*i.e*. β-dodecyl maltoside or *Triton X-100*), resulted in different degree of disintegration of Chl *a/b* proteins in transgenic plants compared to the control. Electron microscopic observations and immunogold labelling with specific LHC2 antibodies carried on the resin embedded leaf sections or free suspensions of chloroplasts showed that gold particles were bound preferentially on the outer surface of crystalloids. Three-dimensional reconstruction of chloroplasts and crystalloids proved that paracrystalline structures varied moderately in their size and took up a significant portion of total chloroplast volume.

Additional key words: aggregates; chloroplast ultrastructure; cytokinins; fluorescence emission spectra; light-harvesting complex proteins; *Nicotiana*; three-dimensional reconstruction.

Introduction

Photosynthesis and growth are significantly affected in tobacco transformed with the *ipt* gene that results in an overproduction of endogenous cytokinins (CKs) (Synková *et al.* 1997, 1999). Although no significant differences in parameters of chloroplast ultrastructure such as length of chloroplasts, starch content, granum width, and number of thylakoids per granum were proved between chloroplasts from young mature leaves of control and transgenic tobacco throughout plant ontogeny, several anomalies were observed in the *ipt* transgenic

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Abbreviations: C, control rooted tobacco; Chl, chlorophyll; CK, cytokinins; 3D, three-dimensional; *ipt*, the gene for isopentenyl transferase; LHC, light-harvesting complex protein; P*ssu*, promoter sequence of the gene coding for a small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase; T, transgenic rooted plants; TC, transgenic grafts.
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plants (Synková *et al.* 2003). Among them distinct crystalloids were often found inside the chloroplasts of P*ssu-ipt* transgenic tobacco. Contrary to macrograna, which seem to be giant grana stacks (Knoth 1975, Hudák 1981, Ogawa *et al.* 2001), the structures observed in P*ssu-ipt* chloroplasts form larger or smaller crystalloids with a fine lamellar structure. Such aggregates are probably formed by an aggregation of light-harvesting complex of photosystem 2 (LHC2) proteins that can occur *in vivo* during degradation of the photosynthetic apparatus under CO_2 deficiency stress (Šiffel and Vácha 1998) or during senescence (Prakash *et al.* 2001). The major chlorophyll (Chl)-protein complex LHC2, which harvests photon energy and transfers it to reaction centres, is one of the most abundant photosynthetic proteins. The genes for LHC2 are located in the nucleus as a small multigene family (Jansson 1994). The apoproteins are synthesized by cytoplasmic ribosomes and imported into chloroplasts. These apoproteins bind Chls *a* and *b* in thylakoid membranes and assemble to PS2 core complexes. When the apoproteins do not bind Chl, they are soon degraded (Ohtsuka *et al.* 1997). CKs affect the expression of various genes and proteins encoded by plastidic DNA or by nucleus (McDaniel and Lightfoot 1997). This was proved also for LHC2 proteins, at least at mRNA level (Teyssendier de la Serve *et al.* 1985). Transcription of the genes encoding these polypeptides (*Lhcb*) is induced and regulated by light and also by CKs (Chory *et al.* 1994). Plants have developed strategies to delicately balanced LHC2 arrangements in the membrane, ensuring that highly quenching states are not populated and an efficient migration of excitons is still possible (Kirchhoff *et al.* 2003). Besides the intra-molecular organisation of pigments within the LHC2 complex, also the intermolecular packing of the LHCs in the thylakoid membrane has to be optimized, otherwise strong energy dissipation occurs. This is especially true for the grana-hosted LHC2, which has a strong tendency for aggregation (Kirchhoff *et al.* 2003). LHC2 forms stable trimers composed of *Lhcb1* and *Lhcb2* gene products. These trimers can further aggregate both laterally to sheets and three-dimensionally to stacks, which grow perpendicularly to the membrane. The former leads to paracrystalline hexagonal alignment, the latter is probably responsible for the formation of the grana stacks. The formation of two-dimensional LHC2 crystals was reported also in native thylakoid membranes under some conditions (Lyon and Miller 1985).

In this paper we attempted to isolate and further characterize crystalloids from P*ssu-ipt* tobacco chloroplasts. Although their composition and physiological function is still unknown, we suggest that crystalloids are formed by aggregates of LHC2 proteins. We performed structural, spectroscopical, and biochemical analyses to find more about the nature of those structures.

Materials and methods

Plants and growth conditions: Control tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was grown as rooted plants (C) from seeds. Transgenic tobacco containing a supplementary *ipt*-gene under a control of the promoter for the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase (P*ssu-ipt*) was generated by means of the *Agrobacterium tumefaciens* transformation system and grown *in vitro* as shoots unable to form roots. The transgenic shoots were grafted on C rootstock and grown as grafts (TC) as described by Beinsberger *et al.* (1992). P*ssu-ipt* transgenic plants (T), *i.e.* the autogamic progeny of the transgenic grafts, which are able to form a small root system, were grown from seeds, selected on agar medium with kanamycin (*in vitro*), and then transferred into soil.

All plants were grown after *in vitro* pre-cultivation in pots with soil substrate in a greenhouse from January till September under 25/18 °C day/night, and relative humidity of 60 %. Day irradiance [overall integrated mid-values estimated as *ca.* 500 μmol(quantum) m^{-2} s^{-1}] was prolonged by additional irradiation [*AgroSon T* and *HT9* lamps, *ca.* 200 μmol(quantum) m^{-2} s^{-1}] to 16 h.

Plants in the vegetative stage, at the onset of flowering, during flowering, and forming seeds were used for crystalloid isolation.

Chloroplast and crystalloid isolation: To prepare a crude chloroplast fraction, tobacco leaves (200 g) were homogenized in the extraction medium (0.3 M mannitol, 20 mM pyrophosphate buffer (pH 7.5), 1 mM EDTA, 0.1 % bovine serum albumin, BSA), and after a filtration centrifuged at 1 000×*g* for 15 min. The pellet was twice washed, re-suspended, and re-centrifuged at 10 000×*g*. The resulting pellet was re-suspended in 2 cm^3 of the washing medium (WM; extraction medium without BSA) and layered on top of *Percoll* discontinuous (20, 38, and 80 %) or continuous (0–100 %) gradient. After centrifugation at 40 000×*g* for 30 min in an *SS34* rotor, usually four main bands were distinguished. The fraction obtained from the 80 and 38 % *Percoll* interface was enriched in crystalloids. The fractions were diluted with twice as much of WM and centrifuged at 4 000×*g* for 15 min. All the procedures were done at 4 °C in dim light. Fluorescence spectra of isolated chloroplasts and separated fractions were measured immediately after the preparation. The suspensions for an electron microscopic evaluation were stored at –20 °C until use. At least 2–3 isolations for each plant type were done from plants of four independent series.

Chl determination and pigment analysis: Chl was extracted into 80 % acetone. The absorbance of the clear extract was measured after centrifugation (500×*g*, 5 min) at 645 and 663 nm and the Chl *a*+*b* content was calculated

according to Lichtenthaler and Wellburn (1983). Pigments were also analysed by HPLC method according to Šiffel and Vácha (1998) with slight modification on *Zorbax ODS* column 4.6×250.0 mm, 5 μm, non-end-capped (*Aglient Technologies*, USA) using a gradient elution with a mixture of acetonitrile : methanol : water (68 : 12 : 6) and methanol : hexane (4 :1).

Detergent treatment: Isolated chloroplast suspensions were treated with β-dodecyl maltoside and *Triton X-100*. Both detergents were added to the suspension to the final concentrations 1 and 3 % (v/v), respectively. After the incubation for 10 min at 4 °C in darkness, the suspensions were centrifuged at 10 000×*g* for 10 min. Both supernatants and pellets were used for fluorescence emission spectra analysis.

SDS-PAGE and Western blotting: Polypeptide composition was analysed by polyacrylamide gel electrophoresis under denaturing conditions on 12–20 % polyacrylamide gel in buffer system according to Laemmli (1970).

For immunoblot analysis, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using methods of Towbin *et al.* (1979). Immunostaining was performed with primary polyclonal antibodies raised in mouse against pea LHC2 proteins isolated according to Kühlbrandt *et al.* (1994). Goat anti-mouse secondary antibodies conjugated with alkaline phosphatase and BCP-NBT substrate were used for visualization according to Bumba *et al*. (2004).

Spectrofluorometric analysis: Suspensions of isolated chloroplasts and different fractions separated on *Percoll* gradient were analysed by Chl fluorescence emission spectra as in Šiffel and Braunová (1999). In order to quantify the yield of Chl fluorescence, spectra were recorded in the presence of an internal standard 10 mM rhodamine B using a *Fluorolog* spectrofluorometer (*SPEX*, USA). Prior to the calculation of the final spectra ratios of the transformants to control samples, the Chl emission spectra were first normalized to the fluorescence intensity of the internal standard at 571 nm (rhodamine B) to correct different intensities of the excitation radiation on various samples and then per Chl content. All Chl fluorescence emission spectra were measured at 77 K with excitation wavelength of 470 nm.

For detection of Chl *b* emission, fluorescence emission spectra were measured using excitation wavelength of the Chl *b* Soret band at 465 nm and a reference wavelength at 490 nm. The resulting spectra were calculated by subtraction of the reference emission spectra excited at 490 nm from the Chl *b* emission spectra excited at 465 nm.

Transmission electron microscopy (TEM): Leaf samples were taken from the central part of the young fully developed leaf. Small pieces of tissue or suspensions of isolated chloroplasts were stained by osmium tetraoxide and aqueous uranyl acetate after overnight fixation in 3 % glutaraldehyde in 50 mM PIPES buffer (pH 7.5) at 4 °C. After several washes and dehydration through alcohol series, the samples were embedded in Spurr‘s resin. Ultrathin sections of samples embedded in Spurr‘s resin were cut on ultramicrotome (*Reichert*), stained by uranyl acetate and lead citrate, and examined in transmission electron microscope *JEM 1010* (*Jeol*, Japan) equipped by CCD camera.

The leaf samples were taken from eight independent series of plants. Four pieces from random leaf sample were embedded in resin, cut for ultrathin sections, and examined for each plant type.

Immunogold staining: Leaf samples or suspensions of isolated chloroplasts were fixed in 0.25 % (v/v) glutaraldehyde and 3 % (m/v) p-formaldehyde in PBS buffer (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM K_2HPO_4, pH 7.2) and after dehydration embedded in L.R. White´s resin (*Polysciences*, USA). Ultrathin sections were picked up on nickel grids. Immunogold staining was carried out with primary polyclonal antibodies raised in mouse against pea LHC2 proteins prepared according to Kühlbrandt *et al.* (1994). Goat anti-mouse secondary antibodies conjugated with 10 nm gold particles were used for visualization in TEM as described in Pechová *et al.* (2003).

3D reconstruction of chloroplasts and crystalloids: Digital images taken from serial sections of Spurr‘s embedded leaf segments were used for 3D reconstruction. This was done by *IMOD 2.42* software. The average number of sections used for the reconstruction of one chloroplast was 30.

Results

Isolation of crystalloids: Suspensions of chloroplasts isolated from older C plants and both types of transgenic tobacco contained large amounts of starch, therefore we were not able to obtain intact chloroplasts as a starting point. Both the discontinuous and continuous *Percoll* gradient centrifugation resulted in two (C) and three (T and TC) bands and pellet (Fig. 1) that were used for analysis. Two upper fractions were found at the 20–38 % layer of *Percoll* gradient (*i.e.* Z1, Z2 in Fig. 1), the third band was found at around 80 % of *Percoll* gradient (Z3) and it was present in both T and TC. In C, the Z3 zone was often missing. The pellets (Z4) contained usually large amount of starch. The original suspensions of broken chloroplasts from C and both T and TC plants (TM), the fraction 1 of *Percoll* gradient (Z1), the fraction 2 (Z2), the fraction 3 (Z3), and the pellet (Z4) were further investigated namely

for their Chl fluorescence emission spectra.

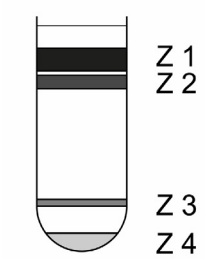

Fig. 1. Separation of chloroplast fractions using a continuous *Percoll* gradient (0–100 %). Three zones (Z1–3) and a pellet (Z4) were formed within a gradient. Z1 and Z2 were found at 20–40 % *Percoll*, Z3 was at the 80–90 % *Percoll* layer.

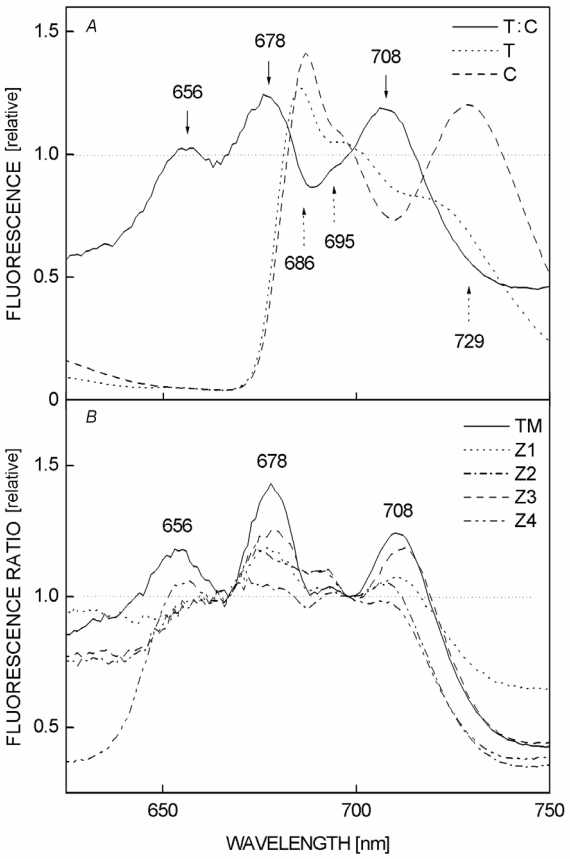

Fig. 2. Original chlorophyll fluorescence emission spectra at 77 K of chloroplasts isolated from control (C) and transgenic rooted (T) tobacco and their calculated ratio (T : C) (*A*). (*B*) The calculated fluorescence ratios for individual fractions of T separated on *Percoll* gradient. The ratios were calculated from fluorescence emission spectra of transgenic and control preparations. TM = suspension of broken chloroplasts, Z1–Z4 are identical to zones from the continuous *Percoll* gradient.

Fluorescence emission spectra at 77 K: Chl fluorescence spectra of isolated chloroplast suspension from control and transgenic tobacco plants were used for the calculation of the ratio of transformant to control fluorescence (Fig. 2*A*, *i.e.* T : C). The ratio T : C highlighted the differences detected between C and transgenic tobacco. There was no difference between both transgenic types, therefore only representative T spectra are shown (see Fig. 2 for T; TC is not shown). Three peaks were found in the ratio spectrum at 656, 678, and 708 nm. Small peak at 656 nm, not resolved in the original Chl fluorescence emission spectra, was detected in the ratio spectra and identified as an emission of Chl *b* by its fluorescence excitation spectrum (data not shown). The peak at 678 nm represents a different content of LHC2 proteins and the peak at 708 nm shows a presence of LHC2 aggregates in the transgenic compared to the C plants.

The Chl fluorescence ratio spectra of the original suspensions of thylakoid membranes (TM) and different *Percoll* gradient fractions (Z1–Z4) are shown in Fig. 2*B*. Compared to the broken chloroplast suspensions (TM) from transgenic tobacco plants, all fractions from *Percoll* gradient exhibited lower Chl fluorescence emission of LHC2 at 678 nm showing a distribution of the LHC2 complexes among the fractions Z1–Z4. However, only the fraction Z3 had almost the same intensity of Chl emission at 708 nm as TM that indicated the increased presence of aggregated LHC2 in the fraction Z3.

In order to further characterise the presence of fluorescence emission band of Chl *b* at 656 nm (Fig. 2), Chl fluorescence emission spectra excited at 465 and 490 nm were recorded and analysed. To detect small fluorescence differences in Chl *b* emission the reference spectra excited at 490 nm were subtracted from the spectra excited in Chl *b* at 465 nm. Fig. 3 shows the differences in the fluorescence emission intensity of Chl *b* between control and transgenic samples. In both transgenic plants (T and TC) a higher amount of Chl *b* molecules, which do not transfer the excitation energy to the Chl *a* molecules, was present.

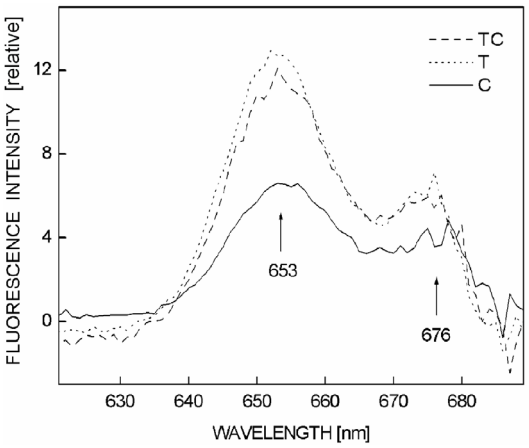

Fig. 3. Chlorophyll (Chl) fluorescence emission intensity of Chl *b* measured at 77 K in suspensions of chloroplasts isolated from control (C), transgenic rooted (T), and transgenic grafted (TC) tobacco. Excitation wavelengths were 465 and 490 nm. Reference spectra at 490 nm were subtracted from those at 465 nm.

SDS-PAGE and immunoblotting: Electrophoretic separation of proteins under denaturing conditions showed that suspensions of chloroplasts isolated from transgenic tobacco contained high molecular mass protein aggregates contrary to C (Fig. 4). After separation on the *Percoll* gradient, the aggregates were found also in Z3 fraction of both transgenic types (not shown). Besides LHC2 proteins detected around 29–21 kDa, LHC2 proteins were immunologically detected also in those aggregates (Fig. 4).

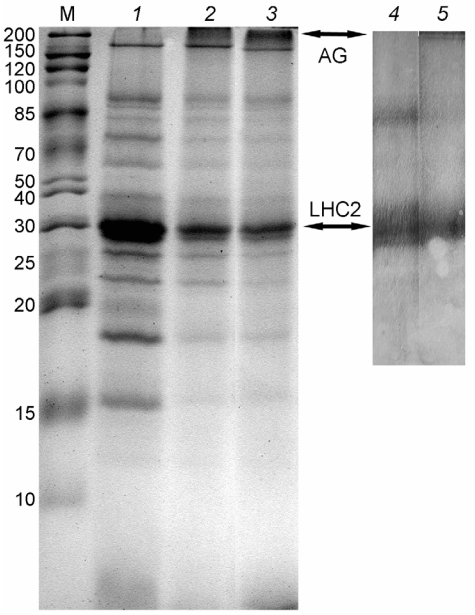

Fig. 4. Polypeptide composition of thylakoid membranes from control (*1*) and both types of transgenic tobacco (*2* = TC, *3* = T) and Western blotting with LHC2 polyclonal antibodies (*4* = C, *5* = T). M = marker, AG = high molecular mass aggregates of LHC2.

Effects of detergents: Two detergents differing in the effect on thylakoid membranes were used to study their ability to solubilize pigment-protein complexes of transgenic tobacco. The treatment by stronger detergent, *Triton X-100*, showed that only chloroplast suspensions isolated from T were strongly affected compared to C and TC (Fig. 5*A*). This was indicated by the presence of Chl fluorescence emission band at 654 nm corresponding to free Chl *b* in supernatants. Pellets contained both uncoupled Chl *b* and free LHC2 (678 nm) that was not connected to photosystems.

Suspensions of thylakoid membranes treated with β-dodecyl maltoside were only moderately affected by the detergent and no significant changes in fluorescence emission spectra were found both in C and transgenic types (Fig. 5*B*).

Pigment analysis: Chl contents of the leaves used for the isolation of chloroplasts were significantly higher in C and T compared to TC (Table 1). Total pigment contents in suspensions of isolated chloroplasts and fractions from *Percoll* gradient were highly variable and dependent on plant type. The pigment yield from C was usually significantly higher than that from both transgenic types, therefore we compared solely a relative representation of individual pigments. The ratio of Chl *a/b* was significantly highest in C, while the highest content of Chl *b* was found in T (Table 1). However, we did not observe any significant increase in Chl *b* content in *Percoll* separated fractions (not shown). The differences in contents of individual carotenoids between C and both transgenic types were negligible and this was not changed by separation of several fractions on the *Percoll* gradient (Fig. 6).

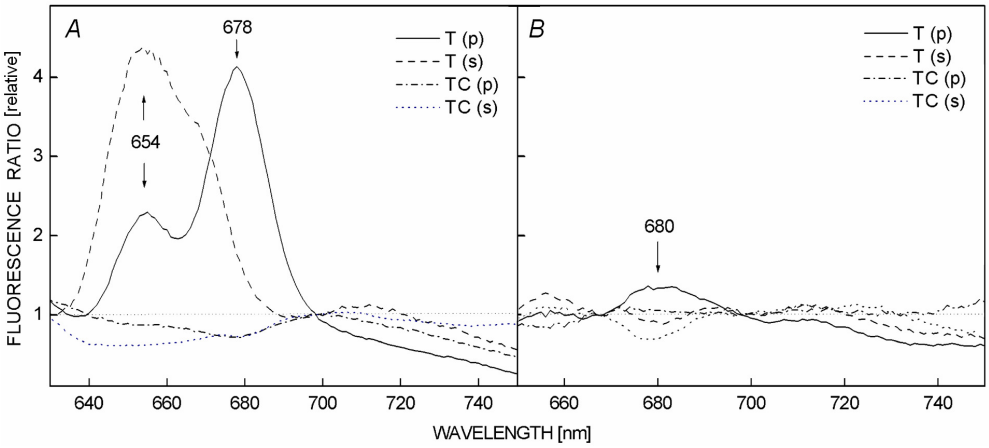

Fig. 5. The effect of treatment of isolated chloroplasts by 3 % *Triton X-100* (*A*) and 1 % β-dodecyl maltoside (*B*) on chlorophyll (Chl) fluorescence emission spectra ratio at 77 K of transgenic rooted (T) and transgenic grafted (TC) tobacco. Suspensions of chloroplasts were centrifuged after detergent treatment and both supernatants [*i.e.* T(s) and TC(s)] and pellets [T(p) and TC(p)] were examined. The Chl fluorescence ratio was calculated from the original spectra of transgenic and control samples.

Table 1. Total chlorophyll (Chl) *a+b* content [mg m^{-2}] in leaves of control (C) and transgenic tobacco (rooted = T, grafted = TC) and Chl *a/b* ratio in chloroplasts isolated from C and both types of transgenic P*ssu-ipt* tobacco. Chloroplasts were isolated from mature leaves of the plants in the developmental stage characterized by the appearance of first flower buds. Means ± S.E. Statistically significant differences at $p = 0.05$ are marked by different letters.

	Plant		
	C	T	TC
Chl *a+b*	13.40±5.60^{a}	11.00±3.70^{a}	7.76±2.60^{b}
Chl *a/b*	2.38±0.10^{a}	2.09±0.07^{b}	2.29±0.09^{ab}

Electron microscopic observations and immunogold labelling: Chloroplasts containing crystalloids were found in leaves of TC throughout the plant ontogeny, in T particularly after the onset of flowering. The crystalloids showed often a regular structure (Fig. 7*C*), however, irregularities were also observed (Fig.7*B*). The isolated chloroplasts often lost their envelopes but maintained their shape and crystalloids (Fig. 7*F*). Immunogold labelling with specific LHC2 antibodies which was carried out on ultrathin sections of L.R.White resin embedded leaf samples, marked preferentially outer margins of crystalloids (Fig. 7*E*). Immunogold labelling of free suspensions of isolated organelles combined with negative staining

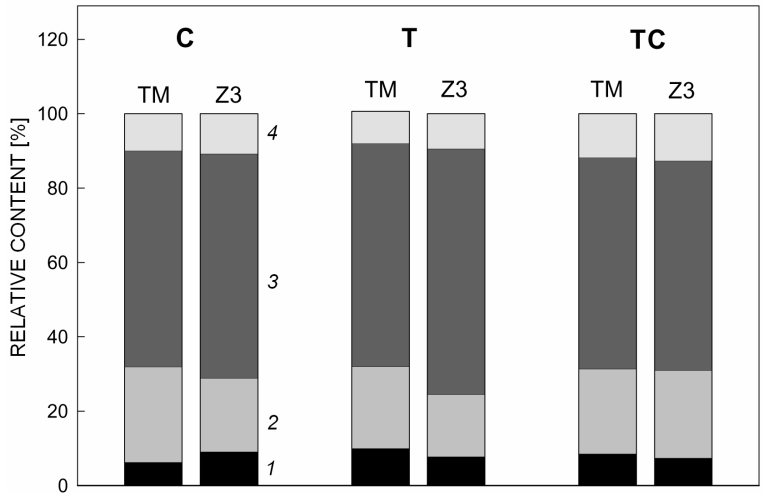

Fig. 6. Relative contents of individual carotenoids separated by HPLC method in suspensions of chloroplasts (TM) and Z3 *Percoll* fraction isolated from control (C), transgenic rooted (T), and grafted (TC) tobacco. *1* = neoxanthin, *2* = β-carotene, *3* = lutein and cis-lutein, *4* = violaxanthin and antheraxanthin. The values are the means, but no statistically significant differences were found at p=0.05.

with uranyl acetate confirmed the binding of gold particles on the outer surface of crystalloids (Fig. 7*G*, *H*).

3D reconstruction: The reconstruction of chloroplasts and crystalloids from transgenic tobacco enabled to estimate an absolute size and dimensions of crystalloids (Fig. 8). The size of crystalloids varied moderately, but there was no significant difference between T and TC. The mean value of parameter characterizing the thickness of the crystalloid was 437±46 nm, the minimum being 203 nm and maximum 845 nm. The mean value of the length of the crystalloid was 5 327±1 000 nm, varying between 2 904 and 15 000 nm. The width of the crystalloids, *i.e.* the parameter which was dependent on the number of sections used for the reconstruction, varied between 675 and 2 325 nm, with the average value of 1 663±147 nm.

Discussion

Isolation: We proved that suspensions of isolated chloroplasts from transgenic P*ssu-ipt* tobacco contained a fraction with fluorescence emission corresponding to LHC2 aggregates. We aimed to separate this fraction from "normal" thylakoid membranes. First we tried to separate the crystalloids using differential pelleting, but this approach was not successful. This technique was not highly effective as the crystalloids were found in several different fractions. Separation based on a density of particles using a *Percoll* density gradient turned up to be more effective. Using both the discontinuous and continuous *Percoll* gradients resulted in three main fractions and the pellet containing predominantly starch. The crystalloids were concentrated in one fraction (Fig. 1, Z3 zone at about 80 % *Percoll* layer). Chl fluorescence emission spectra, TEM examination, and negative staining of free

suspensions confirmed the presence of crystalloids (Figs. 2*B* and 7*F*,*G*). However, the purity of a preparation was hardly to estimate, as only Chl fluorescence emission proved to be a good marker for the LHC2 aggregates presence. It was shown earlier by parallel measurements of fluorescence spectra and electron microscopy and/or sedimentation that the aggregation of the isolated LHC2 is accompanied by the formation of a new band near 700 nm in emission spectrum (Mullet and Arntzen 1980, Ruban *et al.* 1994, Šiffel and Vácha 1998).

We could not find any other reliable "marker". LHC2 proteins belong to the most abundant plant proteins and it is very difficult to distinguish between "normal" present in thylakoids and "abnormal" LHC2 bound in crystalloids among proteins separated *e.g.* by SDS-PAGE.

Composition of crystalloids: As it was mentioned above, SDS-PAGE was used to characterize protein composition of isolated chloroplast suspensions (Fig. 4) and fractions from *Percoll* gradient (not shown). In comparison to controls, both transgenic preparations contained more aggregated proteins in the region of 200 kDa and less LHC2 in the region of 30 kDa. This was partly in contradiction to the results from Chl fluorescence emission analysis that showed the increased emission from free LHC2. The probable explanation is that in transgenic samples the fraction of LHC2 detached

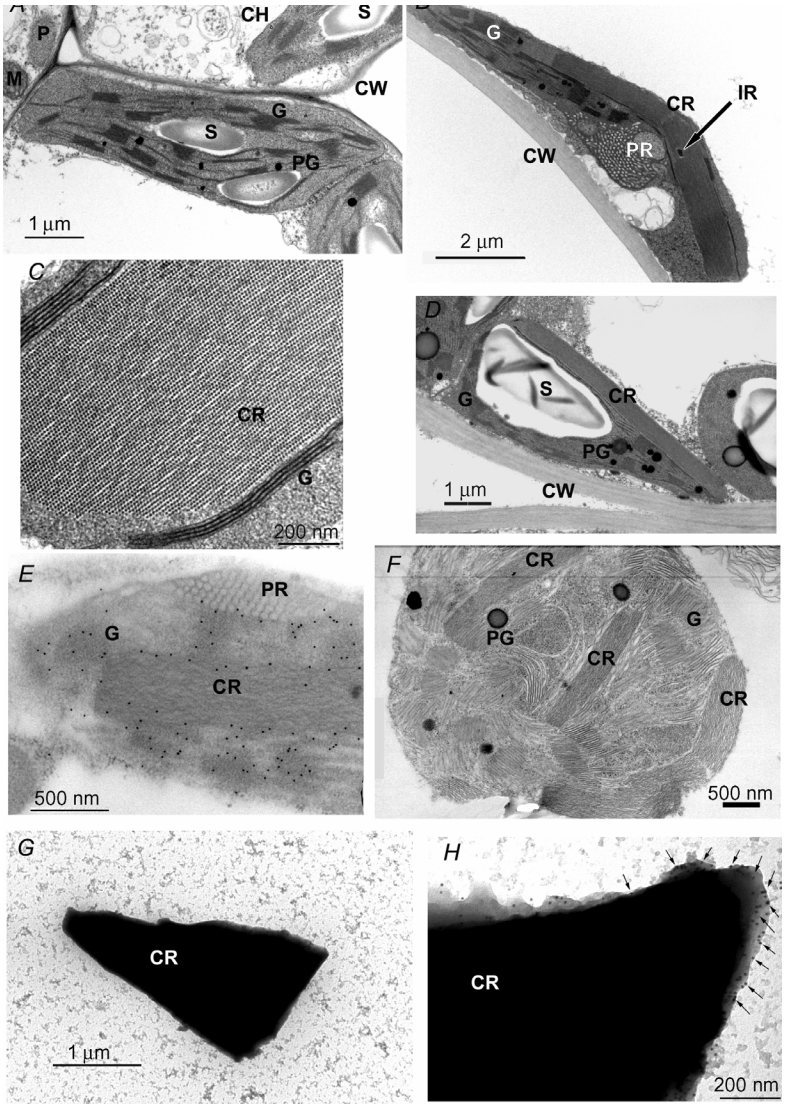

Fig. 7. TEM micrographs of control (*A*) and anomalous chloroplasts from P*ssu-ipt* tobacco (*B*, *C*, *D*) from Spurr´s embedded leaf samples. Immunogold staining of chloroplast from transgenic tobacco in L.R. White´s resin embedded leaf sample (*E*). (*F*) Suspension of chloroplasts isolated from transgenic plants embedded in Spurr´s resin, (*G*) crystalloid from Z3 zone of *Percoll* gradient after negative staining by uranyl acetate, (*H*) crystalloid from Z3 zone of *Percoll* gradient after negative staining by uranyl acetate and immunogold staining with LHC2 polyclonal antibodies (*arrows* mark gold particles). CH = chloroplast, CR = crystalloid, CW = cell wall, G = granum, IR = irregularity, M = mitochondrion, PR = peripheral reticulum, S = starch.

from PS2 exists. It does not transfer energy to the reaction centres, but it increases the fluorescence intensity. LHC2 release that precedes LHC2 aggregation out of photosystems is reflected by an increase of the emission at 678 nm (Šiffel and Braunová 1999). The fraction of LHC2 aggregates was higher both in Chl fluorescence emission spectra (708 nm band in Fig. 2) and in SDS-PAGE (Fig. 4) in P*ssu-ipt* transgenic tobacco compared to C plants. Accumulation of LHC2 aggregates that are detached from photosystems is accompanied by an increase of the Chl fluorescence yield at 708 nm because the excitation energy transfer from LHC2 to photosystems is interrupted. This was also observed in plants under stress (Šiffel and Vácha 1998, Šiffel and Braunová 1999).

Fluorescence emission of Chl *b* was also significantly higher in transgenic plants (Fig. 3), both in suspensions of isolated chloroplasts and Z3 fractions of *Percoll* gradient (Fig. 2*B*). Since there is a total energy transfer from Chl *b* to Chl *a*, the presence of Chl *b* emission in transgenic samples could be a result of LHC2 degradation and a release of Chl *b* or conversely by an enhanced synthesis of Chl *b* and its insufficient binding to apoproteins. Both these facts could lead to the increase of Chl *b* fluorescence emission that is negligible under normal conditions when all energy is transferred from Chl *b* to Chl *a* (Šiffel and Vavřinec 1980).

Contrary to our results obtained by other methods, which did not show any significant differences between T and TC, the use of detergents (particularly *Triton X-100*) proved higher resistance of thylakoid membranes of TC to solubilization. *Triton X-100* caused the increase of fluorescence emission of Chl *b* in T. This was the result of uncoupling of energy transfer from Chl *b* to Chl *a* and/or a release of molecules of Chl *b* into micelles formed by detergent. However, no such effect was observed when milder detergent, *i.e.* β-dodecyl maltoside, was used.

Structure: Ultrastructure of chloroplasts from P*ssu-ipt* tobacco during plant ontogeny was studied in our previous paper (Synková *et al.* 2003) and no striking differences were found in "normal" population of chloroplasts between C and both transgenic types. Nevertheless, the portion of anomalous plastids containing the crystalloids increased with the increasing age of transgenic plants. 3D reconstruction helped estimate the total size of crystalloids and the part taken by this structure within a chloroplast. Although mostly only a part of the organelle was reconstructed, the portion occupied by crystalloids was similar as when almost the whole chloroplast model was obtained (*i.e.* 50 sections of 75 nm). In average, about 16 % of total chloroplast volume was taken by crystalloids (Synková *et al.* unpublished). However, there were some differences among individual crystalloids particularly in their length (Fig. 8*E-H*). This means that individual crystalloids can differ in their total size and this

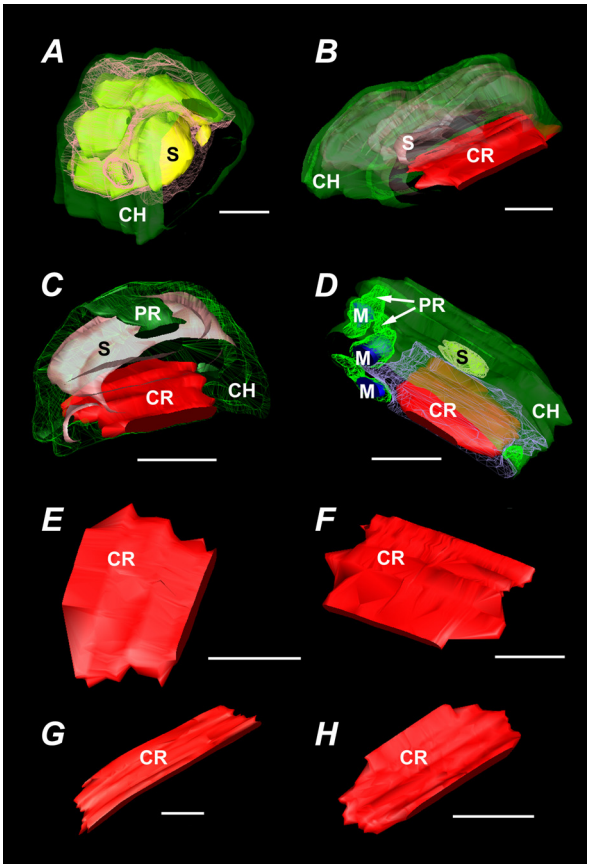

Fig. 8. Tree-dimensional reconstruction of chloroplasts from TEM images of control (*A*) and transgenic tobacco (*B, C, D*). Examples of 3D reconstruction of individual crystalloids (*E, F, G, H*) found in P*ssu-ipt* chloroplasts. Scale bars are 2 μm. CH = chloroplast envelope, CR = crystalloid, M = mitochondrion, PR = peripheral reticulum, S = starch.

can complicate the isolation and purification of those particles.

In previous experiments, the size of basic crystal unit cell of crystalloids was calculated using Fourier transformation. The average parameters were determined as a = 11.0 nm, b = 11.9, γ = 99.8° (Synková *et al.* unpublished). Although the structural studies revealed the structure of various photosynthetic protein complexes (Bumba and Vácha 2003) including the structure of plant light-harvesting complex (Kuhlbrandt *et al.* 1994), the parameters of basic crystal unit of LHC2 are still not available. However, the size that we found is small enough to fit into the known parameters reported for the PS2-LHC2 supercomplex, *i.e.* a = 25.6 nm, b = 21.4 nm, γ = 77° (Yakushevska *et al.* 2001).

Immunogold staining for LHC2 localization was not completely successful in case of ultrathin sections as gold particles were bound only along the outer margins of crystalloids (Fig. 7*E*). This could be the result of non-accessibility of antigenic determinants in densely packed protein units inside the crystalloid. However, on the outer surface of crystalloids obtained from the zone Z3 of

Percoll gradient, the gold particles were bound sufficiently (Fig. 7*H*) suggesting the LHC2 proteins to be the major constituent of the crystalloids.

Therefore we conclude that the large crystalloids inside the chloroplasts of *ipt*-transformed tobacco are aggregated LHC2 proteins. The question of the physiological importance of those structures could not be solved from our data. We can only speculate that if any imbalance in LHC2 apoprotein and Chl *a* and *b* accumulation caused by CKs exists, then this could be probably the mechanism to balance the optimal functioning of photosynthetic apparatus.

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