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Remodeling of tobacco thylakoids by over-expression of maize plastidial transglutaminase

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ABSTRACT

Transglutaminases (TGases, EC 2.3.2.13) are intra- and extra-cellular enzymes that catalyze post-translational modification of proteins by establishing ε-(γ-glutamyl) links and covalent conjugation of polyamines. In chloroplast it is well established that TGases specifically polyaminylate the light-harvesting antenna of Photosystem (PS) II (LHCII, CP29, CP26, CP24) and therefore a role in photosynthesis has been hypothesised (Della Mea et al. [23] and refs therein). However, the role of TGases in chloroplast is not yet fully understood. Here we report the effect of the over-expression of maize (*Zea mays*) chloroplast TGase in tobacco (*Nicotiana tabacum* var. Petit Havana) chloroplasts. The transglutaminase activity in over-expressers was increased 4 times in comparison to the wild-type tobacco plants, which in turn increased the thylakoid associated polyamines about 90%. Functional comparison between Wt tobacco and *tgz* over-expressers is shown in terms of fast fluorescence induction kinetics, non-photochemical quenching of the singlet excited state of chlorophyll a and antenna heterogeneity of PSII. Both *in vivo* probing and electron microscopy studies verified thylakoid remodeling. PSII antenna heterogeneity *in vivo* changes in the over-expressers to a great extent, with an increase of the centers located in grana-appressed regions (PSIIα) at the expense of centers located mainly in stroma thylakoids (PSIIβ). A major increase in the granum size (i.e. increase of the number of stacked layers) with a concomitant decrease of stroma thylakoids is reported for the TGase over-expressers.

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1. Introduction

Chloroplasts of higher plants are bounded by two envelope membranes that surround an aqueous matrix, the stroma, and the internal photosynthetic membranes, the thylakoids [1]. Chloroplasts have an apparently periodic ultrastructure: cylindrical grana stacks of about 10–20 layers with a diameter of 300–600 nm, interconnected by lamellae of several hundred nm in length, but, although our understanding regarding architecture of thylakoids is advanced, many issues, such as self-assembly and structural flexibility, still remain to be explored [2].

The two photosystems are spatially separated in thylakoids *in vivo*: photosystem II and its main chlorophyll a/b light-harvesting complex, (LHCII), are found predominantly in the stacked membranes; this region is largely deficient in photosystem I (PSI), LHCI and ATPase, which are enriched in the stroma membranes [3]. Separation of the two pigment systems may be important in preventing unregulated excitation energy flow between the two photosystems [4] as, without this separation, the much faster action of PSI would disturb the balance of the energy distribution between the two photosystems [5]. PSII units are also heterogeneous in terms of antenna size with centers of large chlorophyll antenna, termed PSIIα (in grana) and of smaller antenna termed PSIIβ (in stroma lamellae) [6–9].

The abundance of LHCII in the granum suggests that these antenna complexes also have a structural role. LHCII has been shown to stabilize granum ultrastructure and to participate in the cation-

Abbreviations: PSII, photosystem II; RC, reaction center; LHCII, light-harvesting complex of PSII; Chl, chlorophyll; Put, putrescine; Spd, spermidine; Spm, spermine; F_V/F_M, maximum quantum efficiency of PSII; DCMU, (3-(3,4-dichlorophenyl1)-1,1-dimethylurea); PAs, polyamines; TGase, transglutaminase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; qE, energy dependent component of the non-photochemical quenching; NPQ, non-photochemical quenching of the singlet excited state of chla

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mediated stacking of the membranes [1,9–12]. These light-harvesting complexes have also been shown to be involved, via electrostatic and osmotic forces, in the lateral organization of the membranes [13]. Previous studies have shown that the strength of stacking is affected by the phosphorylation of LHCII and several other phosphoproteins [14]. LHCII is largely responsible for the organization of the plant photosynthetic system by maintaining tight appression of thylakoid membranes in chloroplast grana [15]. Here the stromal surface of the LHCII trimer has an important role, demonstrated by recent structural studies in higher plants to be mainly flat and negatively charged [16]. This complex collects excitation energy and transfers it to the reaction centres of PSII and PSI [17]. In addition, LHCII prevents damage to the photosynthetic system, by several different mechanisms, when there is too much light. Potentially harmful chlorophyll (Chl) triplets are quenched by carotenoids in the complex, while non-photochemical quenching (NPQ) has evolved in plants to dissipate excess energy as heat [18].

The balance between grana and stroma lamellae regions is of exceptional importance because it defines the available space for photosystems and the other supercomplexes of the photosynthetic apparatus such as ATPase. It is well established that sun and shade plants have differences in the organization of their thylakoid system, affecting the efficiency with which light is harvested and utilized [1].

With respect to thylakoid membrane biogenesis, Wang et al. [19] showed that the *Thf1* gene product plays a crucial role in a dynamic process of vesicle-mediated thylakoid membrane biogenesis in *Arabidopsis thaliana*. Recently, Chi et al. [20] have reported that a rice thioredoxin *m* isoform (*Ostrxm*) seems to be required for chloroplast biogenesis and differentiation. However, the factors that determine grana formation are not yet fully understood.

A rather overlooked post-translational modification of LHCII that might be important for stacking of thylakoids is its polyaminylation. Polyamines (Pas) are low molecular weight aliphatic amines that are almost fully protonated under normal pH values, so having a net charge of up to +4. The main polyamines putrescine (Put), spermidine (Spd) and spermine (Spm) are normally found in the LHCII of higher plants [21]. Plastidal transglutaminases covalently attach polyamines of all thylakoid proteins specifically in LHCII, CP29, CP26 and CP24 [22]. Recently, it was demonstrated that a plastidial transglutaminase activity in maize polyaminylates LHCII in a light dependent way [23]. The two terminal amino-groups of PAs conjugate to one or two glutamine residues giving rise either to mono-(c-glutamyl)-PAs (mono-PAs) or bis-(c-glutamyl)-PAs (bis-PAs). The additional positive charges inserted on proteins by the protein-bound PAs may induce conformational changes [23].

Transglutaminases (TGases) are intracellular and extra-cellular enzymes that catalyse post-translational modification of proteins by establishing ε-(γ-glutamyl) links and covalent conjugation of polyamines [24]. However the role of TGases in chloroplast is not fully understood yet. A maize (*Zea mays L.*) TGase has been immunodetected in meristematic calli and their isolated chloroplasts, as a unique 58 kDa band. The activity was shown to be light sensitive, affected by hormone deprivation and with a light/dark rhythm [25,26]. Subcellular localization studies have shown that, the enzyme is specifically localized in the chloroplast grana-appressed thylakoids, close to LHCII [27–29]. The abundance depended on the degree of grana development, and the enzyme activity was light dependent [27]. An important step in elucidating the role of plastidal transglutaminase was the isolation for the first time in plants of two related complementary maize DNA clones, *tgz15* and *tgz21*, encoding active maize TGase [30,31]. Their expression is dependent on length of light exposure, indicating a role in adaptation to different light environmental conditions including natural habitats [32].

Recently, we hypothesized that TGase may be implicated in the regulation of the ratio of grana thylakoids to stroma thylakoids [31]. A combination of genetic engineering and *in vivo* probing was used to test this hypothesis. Here we report the effect of the over-expression of maize (*Zea mays*) *tgz* gene in tobacco (*Nicotiana tabacum* var. Petit Havana) chloroplasts by plastid transformation, where the transgene is integrated in the plastid genome via homologous recombination [33–35]. The increase in transglutaminase activity, induced in the transformed tobacco plants resulted in enhanced polyaminylation of thylakoid proteins and in increased thylakoid appression. A functional comparison between Wt tobacco and *tgz* over-expressers is also shown in terms of fast fluorescence induction kinetics, non-photochemical quenching of the singlet excited state of chlorophyll a and PSII antenna heterogeneity. The results are discussed in the light of the possible implication of plastidial TGase in the architecture of the thylakoid network.

2. Materials and methods

2.1. Construction of the chloroplast expression vector

For the *tgz13* gene preceded by a sequence corresponding to a histidine tag, 2 PCR amplified fragments were fused. The first fragment was designed to overlap 2 primers (linker 5′: CCA TGG GTC ACC ATC ACC ATC ACC ATG ACG ATG ACG ATA AGA TG and linker 3′: AAG CTT TTC CAC TTC TGT AGT TTG CAT CTT ATC GTC ATC GTC ATG) that contain the sequences for a NcoI site, 6 histidines, the target sequence for an enterokinase, and the first 27 nucleotides of the coding sequence of the *tgz13* gene with a final HindIII restriction site. The second fragment was amplified using the primers *tgz*-5 (GGA AGC TTA TCA CGG AGA ATC AGC) and *tgz*-3 (CCT CGA GTC ACC ATA TTT GTC TGC) and, as template, the *tgz13* gene without the putative signal peptide [31]. The *tgz13* gene was PCR amplified from the HindIII site to the stop codon of the coding sequence including an additional XhoI site for cloning purposes. The 2 fragments were joined at the HindIII site and introduced between the NcoI–XhoI sites of an intermediate vector with the promoter and 5′ untranslated region of the *psbA* gene, flanked by the EcoRI–NcoI restriction sites. Finally, the EcoRI–XhoI fragment was introduced into the multiple cloning site of pAF to give the final vector, pAF-*tgz13*.

2.2. Chloroplast transformation and plant regeneration

Leaves from *in vitro*-grown tobacco (*Nicotiana tabacum* var. Petit Havana) plants were bombarded with gold microprojectiles (0.6 μm) coated with plasmid DNA containing the maize transglutaminase sequence [31] without the putative signal peptide and a modification in the number of tandem repeats, which in this case was of 13 (*tgz13*). The biolistic device PDS1000/He (Bio-Rad) was used as previously described [36]. Bombarded leaves were subjected to two rounds of selection on regeneration medium: Murashige and Skoog (MS) containing salts and vitamins, 2% of sucrose, 3 g/l of phytagel and 500 μg/ml spectinomycin. The growth conditions of the culture chamber were 28 °C, photon flux density of 100 μmol [photons] m^{-2} s^{-1} and 16 h photoperiod. The regenerated *tgz*-transformed plants were not able to set seed and died after transplanting. Therefore both wild-type tobacco (Wt) and *tgz*-transformed plants were maintained in tissue culture by sub-culturing of apex and single nodal explants.

2.3. Southern blot analysis

Southern blot analysis was performed on shoots from the second round of selection with spectinomycin. Total plant DNA (10 μg) from *in vitro*-grown plants was digested with BglII, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. The chloroplast vector DNA digested with BglII and BamHI generated a 0.8 kb probe homologous to the flanking sequences. Hybridization was using the

chemiluminiscent AlkPhos direct labelling-detection system (GE Healthcare). The membrane was stripped and re-probed with a 0.95 kb NcoI/ApaI fragment of the *tgz13* sequence to verify integration of the *tgz* gene in the plastid genome (Fig. 1B).

2.4. TGase activity

TGase activity in the presence of 0.6 mM cold Put and 185 kBq [1,4 (n)-^{3}H] Put (specific activity 962 GBq/mmol) was determined in protein extracts of *tgz*-transformed and wild-type tobacco leaves. The light conditions during the assays were the same as those during sample collection. The pH of the incubation mixture was adjusted to 8.0. The enzyme mixture was as described previously [27]. After 30 min of incubation at 30 °C, the reaction was blocked by adding 10% TCA containing 5 mM unlabelled Put. Samples were repeatedly precipitated and the radioactivity was measured in a scintillation counter (Beckman LS 6000 SC, Fullerton, CA) as previously described [27].

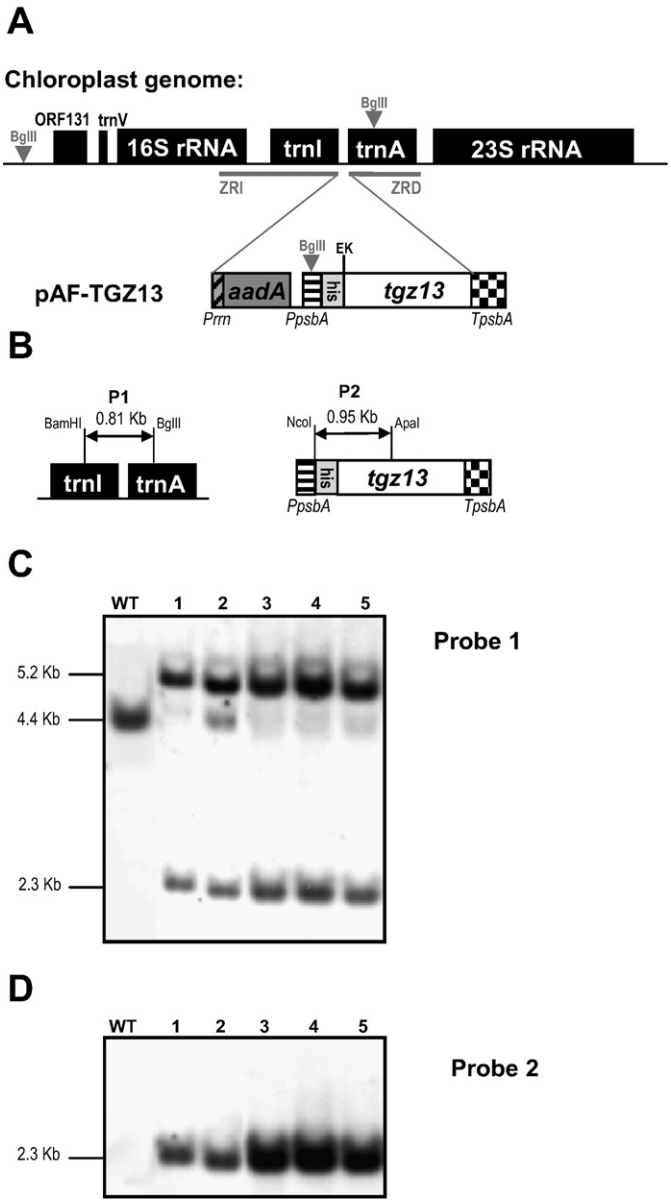

Fig. 1. Schematic representation of tobacco plastid genome transformation using the maize transglutaminase *tgz* gene. (A) map of the wild-type and *tgz*-transformed genomes. Regions for homologous recombination are underlined in the native chloroplast genome; (B) the 0.81 kb fragment (P1) of the targeting region for homologous recombination and the 0.95 kb *tgz* sequence (P2) were used as probes for Southern blot analysis; (C, D) Southern blot analysis of five independent transgenic lines. Blots were probed with P1 (C) and P2 (D). ORF131, trnV, 16S rRNA, *trnI*, *trnA*, 23S rRNA. Original sequences of the chloroplast genome; aadA: aminoglycoside 3′-adenylyltransferase; Prrn: 16S rRNA promoter; PpsbA: *psbA* promoter; TpsbA: terminator region of the *psbA* gene; WT: wild-type plant.

2.5. Thylakoid isolation

Thylakoids were isolated as previously described with minor modifications [37]. Leaves from tobacco plants were homogenized in 50 mM KCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 0.5 mM KH_2PO_4, 25 mM HEPES, pH 7.6, 330 mM sorbitol, 10 μM sodium ascorbate, and 0.2% (w/w) bovine serum albumin. The homogenate was filtered through four layers of cheesecloth, debris was removed by centrifugation at 300 ×*g* for 1 min, and the thylakoids were then pelleted by centrifugation at 4000 ×*g* for 10 min. These and all subsequent operations were carried out at 0–4 °C. The pellet was separated from starch, resuspended and washed in 7 mM $MgCl_2$, 10 mM KCl, 25 mM HEPES, pH 7.6 to break any intact chloroplasts and remove free polyamines. Finally thylakoids were pelleted and resuspended in a medium containing 7 mM $MgCl_2$, 50 mM KCl, 25 mM HEPES, pH 7.6 and 330 mM sorbitol for polyamine analysis.

2.6. Polyamine analysis by high performance liquid chromatography (HPLC)

Polyamines were extracted as previously described [38] and analyzed following the method of Kotzabasis et al. [39]. Briefly, for polyamine analysis isolated thylakoids were suspended in 1 N NaOH. A 0.2 ml volume from the hydrolysate was mixed with 36% HCl at a ratio of 1:1 (v/v) and incubated at 110 °C for 18 h. The hydrolysate was evaporated at 70–80 °C then re-dissolved in 0.2 ml of 5% (v/v) perchloric acid. To identify and estimate the polyamines, the samples were derivatized by benzoylation, as previously described [39]. For this purpose, 1 ml of 2 N NaOH and 10 μl benzoylchloride were added to 0.2 ml of the hydrolysate and the mixture vortexed for 30 s. After 20 min incubation at room temperature, 2 ml of saturated NaCl solution were added to stop the reaction. The benzoylpolyamines were extracted three times into 2–3 ml diethylether; all ether phases collected and evaporated to dryness. The remaining benzoylpolyamines were re-dissolved in 0.2 ml of 63% (v/v) methanol and 20 μl aliquots of this solution were injected into the HPLC system for the polyamine analysis, as described previously [39] using a Shimadzu Liquid Chromatography apparatus (LC-10AD) equipped with a SPD-M10A diode array detector (Shimadzu SPD-M10A) and a narrow-bore column (C18, 2.1×200 mm, 5 μm particle size Hypersyl, Hewlett-Packard, USA).

2.7. Photosynthetic pigments

Photosynthetic pigment content was determined according to Lichtenthaler [40].

2.8. Electron microscopy

Tobacco leaf thin sections (less than 0.5 mm) were fixed by vacuum infiltration with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. After washing, they were fixed in osmium tetroxide for 2 h in the same buffer, dehydrated through an acetone series and embedded in Spurr resin by infiltration. The blocks were polymerized for 48 h at 60 °C. Ultrathin sections were obtained with an Ultracut UCT ultramicrotome (Leica) using a diamond knife, and mounted on gold grids (200 mesh). To determinate the granum size of the Wt and the transformed chloroplasts, a minimum of 50 chloroplasts of each type of plant were examined.

Table 1
Comparison of fluorescence parameters, pigment content, granum size parameters and transglutaminase activity in Wt and *tgz* over-expressing (over TGZ) *Nicotiana tabacum* leaves.

	Wt tobacco	over TGZ
F_V/F_M	0.812 (0.031)	0.702 (0.070)
qE	0.16 (0.02)	1.008 (0.08)
$t_{1/2DCMU}$ (ms)	166 (9)	110 (12)
Chla (mg g^{-1}FW)	1.38 (0.11)	0.42 (0.08)
Chlb (mg g^{-1}FW)	0.48 (0.04)	0.18 (0.05)
Total Chls (mg g^{-1}FW)	1.86 (0.15)	0.60 (0.13)
Chla/Chlb	2.87 (0.06)	2.39 (0.17)
Carotenoids (mg g^{-1}FW)	0.29 (0.02)	0.13 (0.02)
Maximum granum height (nm)	400 (*)	1000 (*)
Number appressed thylakoids	15–20 (*)	40–60 (*)
Granum diameter (nm) (lateral extension)	400 (*)	500 (*)
Transglutaminase activity (pmol Put mg protein h^{-1})	758.9 (89.2)	3067.3 (661)

Numbers in parenthesis denote standard deviation ($n=3$). Transformed tobacco values (right column) were statistically different in comparison to the Wt values (left column) at $p<0.05$.
*Measured in 50 chloroplasts.

2.9. Fluorescence spectroscopy

The portable Plant Efficiency Analyser, Handy-PEA (Hansatech Instruments, Kings's Lynn, Norfolk, UK) was used for fluorescence induction measurements. This method is based on the measurement of a fast chla fluorescence transient OJIP with a maximum 10 μs data acquisition capacity (100 kHz) in a time span of 30 μs to 1 s [41,42]. Fluorescence was measured at 12 bit resolution and excited by three light-emitting diodes providing a light intensity of 3000 μmol photons m^{-2} s^{-1} of red (650 nm) light. To determine the maximum quantum efficiency of PSII charge separation, the following equation was used: $F_V/F_M=((F_M-F_0)/F_M))$. Leaves were dark-adapted for 20 min prior to the measurement. For the NPQ measurements leaves were continuously illuminated for 270 s with 500 μmol photons m^{-2} s^{-1} using the Handy-PEA (multi-hit mode). Every 30 s a saturating pulse of 3000 μmol photons m^{-2} s^{-1} (duration 0.8 s) was given for maximal fluorescence, F_M'. To calculate the NPQ at the end of the actinic light phase we followed the equation, $NPQ=F_M/F_M'-1$ [43]. For the calculation of ΔpH-dependent quenching of chla fluorescence (qE), the following equation was used: $qE=(F_M''-F_M')/F_M'$ were F_M'' is the maximum fluorescence after dark relaxation. Difference in energetic connectivity of PSII units between Wt and transformed tobacco was determined by the increase in fluorescence during the first 300 μs (L-band) [44]. The curves were normalized between F_0 (50 μs) and F_K (300 μs) expressed as W_{OK}($W_{OK}=(F_t-F_0)/(F_K-F_0)$ and then the difference ΔW_{OK} was calculated $\Delta W_{OK}=W_{OK(TGZ)}-W_{OK(Wt)}$ [44]. To estimate antenna heterogenicity, leaves were immersed in DCMU (50 μM) for 30 min in the dark. The half rise time of the F_V in the presence of DCMU was used as an estimate of effective antenna size of PSII [45]. The fraction of PSIIβ centers were calculated from the slope of ln[(Amx − At) / Amx] according to the method of Anderson and Melis with minor modifications [46,47]. Amx is the complementary area in the DCMU curves and At the area at time *t*. The data in Fig. 6 are plotted as a fraction of β centers per total number of PSII, [6,8,9].

2.10. Statistical analysis

All statistical procedures were done using SPSS for Windows (SPSS for Windows v. 14.0, SPSS Inc., Chicago, IL, USA). Analyses of variance (ANOVA) were used to test the main effects and interactions against appropriate error terms. Post-hoc Duncan's test was applied where appropriate. Statistical significance was set at $p<0.05$. The number of replicates is indicated in the table and figure legends.

3. Results

3.1. Vector construction. Determination of chloroplast integration and homoplasmy

The *tgz13* gene was PCR amplified, fused to the promoter and 5′ untranslated region of the *psbA* gene and then introduced into the pAF multiple cloning site to give the final vector, pAF-*tgz13* (Fig. 1A). The pAF vector was specifically constructed for tobacco plastid transformation and includes the *trnI* and *trnA* border sequences, homologous to the inverted repeat regions of the tobacco plastid genome [35]. The regulatory sequences of the *psbA* gene were chosen due to the high levels of heterologous gene expression they confer in transplastomic plants [48,49].

Southern blot analysis was performed on shoots developed after the second round of selection with spectinomycin. Total plant DNA was digested with BglII. The 0.81-kb probe (Fig. 1B) was used to verify site-specific integration and to check homoplasmy. DNA from the wild-type plant produced a 4.4-kb fragment (Fig. 1C) and transformed plants two fragments of 5.2 and 2.3-kb. Plants #1, #3, #4 and #5 were homoplasmic (Fig. 1C). Plant #2 was heteroplasmic (with both wild-type and transformed plastid genomes) and was discarded. The same membrane was hybridized with the P2 probe (Fig. 1B) to confirm the presence of the *tgz* gene. As expected, hybridization was observed in the five transgenic lines, being absent in the wild-type control plant (Fig. 1D).

3.2. Transglutaminase activity and thylakoid associated polyamines

The TGase activity in tobacco leaves over-expressing maize *TGZ* was nearly four times higher than that of the tobacco Wt plants (Table 1). This result was corroborated by the presence of TGZ protein in the over-expressers, detected by western blot and analyzed by mass spectrometry (data not presented). A sensitive HPLC method was used to estimate the amount of associated polyamines in thylakoids [39]. Plants over-expressing *tgz* showed a total increase of 90% in the titre of thylakoid associated polyamines (Put, Spd and Spm) on a chl basis (Fig. 2). Bound Put increased about 3 fold and the higher polyamines about 1.6, in comparison to the Wt.

3.3. Ultrastructure of thylakoids and pigment content

Electron microscopy revealed major differences between the Wt and the *TGZ* line. In our experimental conditions, Wt exhibited the normal architecture of the thylakoid network (Fig. 3A and C) arranging in grana and stroma lamellae in a normal proportion: the granum diameter was about 400 nm and grana stacks

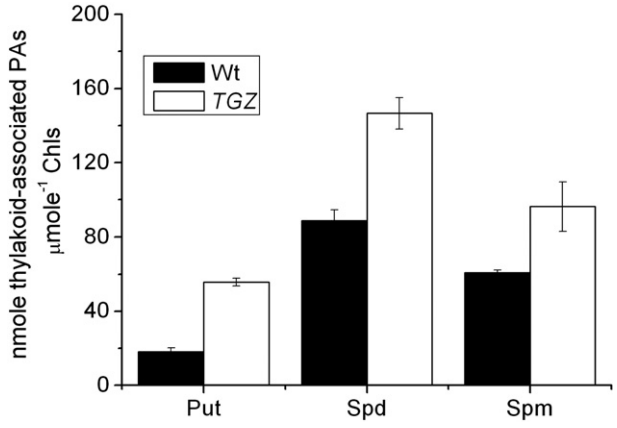

Fig. 2. Thylakoid associated polyamines of *Nicotiana tabacum* Wt and *tgz* over-expressing plants. Data are presented on a chlorophyll basis because protein titer was substantially higher in transformed tobacco due to *tgz* over-expression. Vertical bars denote standard deviation ($n=3$).

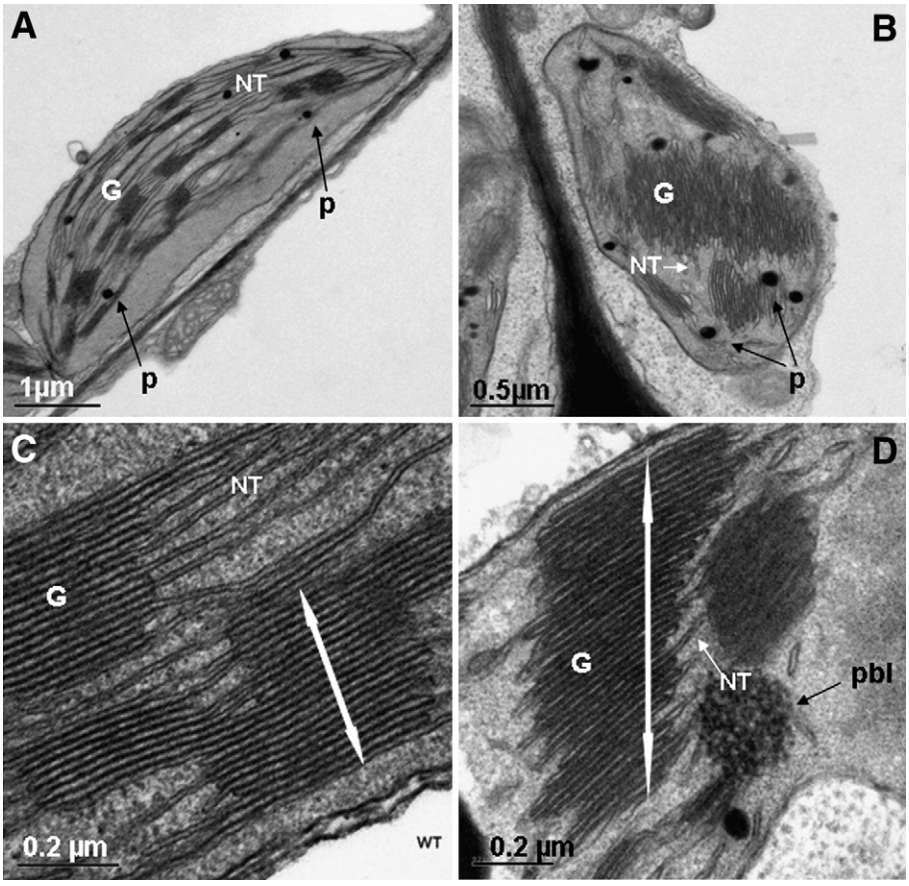

Fig. 3. Electron microscopy in chloroplasts of *Nicotiana tabacum* Wt and *tgz* over-expressers. Transformed tobacco (B, D) shows an increased grana appression and a reduced stroma thylakoid network with respect to the Wt (A, C). The grana height (arrow) is about 400 nm in Wt plants (C) and about 1000 nm in the over-expressers (D) (see Results and Table 1). G = grana; NT = non-appressed thylakoids; *p* = plastoglobuli; pbl = prolamellar body lattice. Arrow in C = 0.35 μm.; arrow in D = 0.9 μm.

consisted of 15–20 tightly appressed thylakoid membranes interconnected by stroma thylakoids. Over-expression of *tgz* resulted in chloroplasts having mainly grana (Fig. 3B and D) with a granum diameter (lateral extension) of about 500 nm and the size (height) increased up to 1000 nm (Table 1 and Fig 3D). Grana stacks had 40–60 tightly appressed thylakoid membranes and only a few stroma thylakoids. In general, grana interconnections were rare and stroma thylakoids short and swollen. Prolamellar body lattices (pbl,

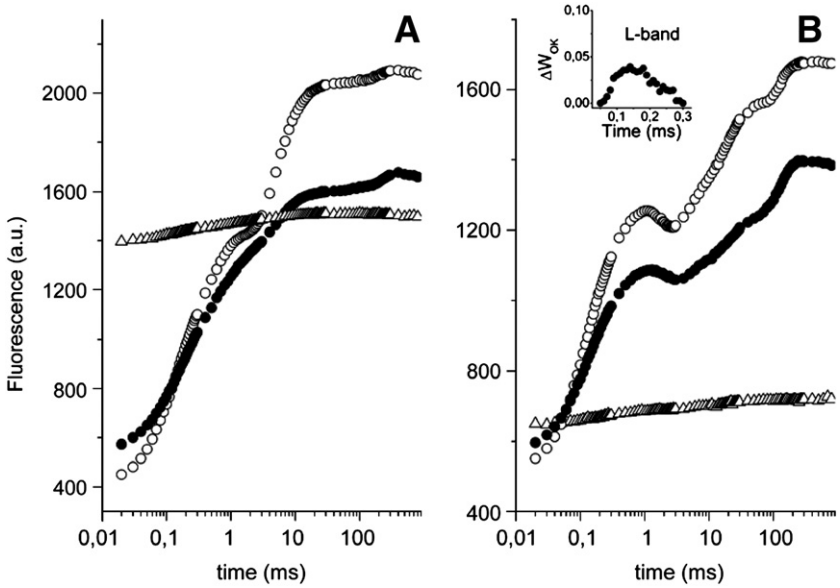

Fig. 4. Fluorescence induction curves of *Nicotiana tabacum* (A) Wt and (B) *tgz* over-expressing plants. Open circles, dark-adapted sample; open triangles, end of actinic illumination (270 s of 500 μmol [photons] m^{-2} s^{-1}); closed circles, end of relaxation phase at 570 s. Samples were dark-adapted for 20 min then illuminated with 3000 μmol [photons] m^{-2} s^{-1}. The time axis is semi-logarithmic for clarity. Insert: Change in the shape of the chlorophyll a transient fluorescence curves normalized between F_0 and F_K, expressed as W_{OK}($W_{OK} = (F_t - F_0)/(F_K - F_0)$. $\Delta W_{OK} = W_{OK(TGZ)} - W_{OK(Wt)}$.

Fig 3D), a remnant of membrane growth in darkness prior to EM studies, were sometimes present in Wt as well as in transplastomic chloroplasts.

Furthermore, there was a reduction in the total Chl content from 1.86 (mg g^{-1} FW) in Wt to 0.6 (mg g^{-1} FW) in plants over-expressing *tgz*, with a parallel decrease of the chla/chlb ratio (Table 1). The total carotenoid titre was also reduced (Table 1), to the point where plants over-expressing *tgz* were severely chlorotic at later stages of development (Ortigosa et al., in preparation). These later stages were not analysed here.

3.4. Fluorescence induction kinetics

Over-expression of maize *tgz* in tobacco has a minor effect (about 13% decrease) in the structure and functionality of PSII as judged by the F_V/F_M values. For transformants, maximum quantum efficiency of PSII is about 0.7, whereas Wt tobacco exhibits optimal values of about 0.81 (Table 1). More pronounced differences appear at later stages of development, but, as mentioned above, in this study we have worked solely with plants at an early stage, before any major decline of F_V/F_M. (For the typical phenotype of the leaves studied see supplemental Fig. 1.) Detailed transient kinetics of fluorescence induction showed that there are major differences both in the shape and amplitude, between the Wt (Fig. 4A open circles) and *tgz* over-expressers (Fig. 4B open circles). The maximal difference in F_V during fluorescence induction is at 10 ms (about 70% higher values for Wt in comparison to the transformed tobacco) and there is a large difference in the F_M values (about 50% more for the wild-type). The fluorescence rise during the first 300 μs (L-band) provides information on the energetic connectivity between PSII units (insert Fig. 4) [44]. The appearance of a positive L-band in transformed tobacco indicates a decrease in energetic connectivity between PSII units in comparison to Wt. In addition, the effective antenna size of PSII increased in over-expressers as indicated by the shortest closure time of their reaction centers (see Table 1 parameter $t_{1/2DCMU}$) in comparison to the Wt (for more detailed analysis of antenna size estimation see ref. [45] and refs therein).

3.5. NPQ induction and relaxation

Transformed tobacco illuminated with 500 μmol [photons] $m^{-2}s^{-1}$ for ~5 min gives NPQ values proximal to 1.4 whereas there is little activation of photoprotection in Wt, with an NPQ of ~0.4 (Fig. 5). Almost all NPQ in the transformed tobacco was found to relax rapidly within the first 30 s of dark period and no signs of significant photoinhibition were evident at the end of dark relaxation (Fig. 5). The value of energy dependent component of the non-photochemical quenching (qE) in the case of the transformed tobacco is about 6 times higher than Wt (Table 1).

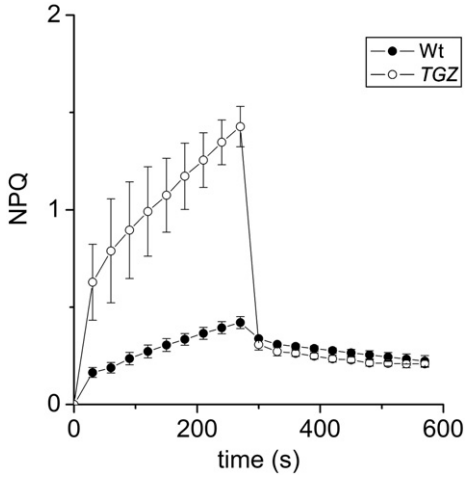

Fig. 5. NPQ induction and relaxation kinetics of *Nicotiana tabacum* Wt and *tgz* over-expressing plants. Plants were grown at 100 μmol [photons] m^{-2} s^{-1} and illuminated during the induction period with red light (500 μmol [photons] m^{-2} s^{-1}). Vertical bars denote standard deviation ($n=3$).

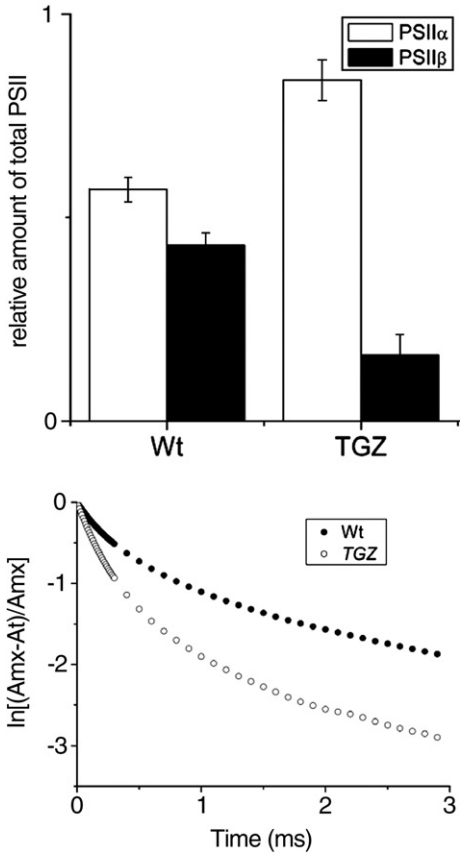

Fig. 6. Comparison of PSII antenna heterogeneity of *Nicotiana tabacum* Wt and *tgz* over-expressing plants, as estimated by the fraction of PSIIα (white) and PSIIβ (black) (for details see Materials and methods). Insert: Typical traces of ln[(Amx − At) / Amx] as a function of time of *Nicotiana tabacum* Wt (closed circles) and *tgz* over-expressing plants (open circles). Vertical bars denote standard deviation ($n=3$).

3.6. Heterogeneity of the antenna of PSII centers

The kinetic spectrophotometric method [46] was used to estimate the balance between PSIIs located in grana regions (PSIIα) and PSII located mainly in stroma lamelae (PSIIβ) *in vivo*. Wt tobacco has ~58% PSIIα centers and 42% PSIIβ centers, while the transformed tobacco exhibits 84% PSIIα and only ~16% PSIIβ centers (Fig. 6).

4. Discussion

We recently reported the cloning of a maize transglutaminase in plants [30,31]. The over-expression of a heterologous gene could be a valuable tool for understanding the role of the corresponding enzyme. In the present work over-expression of maize *tgz* in tobacco chloroplast resulted in a 4-fold increase of transglutaminase activity, causing a significant increase (about 90%) in the polyamines associated with tobacco thylakoids. Interestingly, these plants show a small decrease in maximal quantum yield of PSII (about 13%) in comparison to the Wt and they exhibit increased ability to induce NPQ. The transformed line has about 6 times higher qE in comparison to Wt. These results are in line with recent studies showing that elevation of the Spd and Spm levels can lead to an increase in NPQ in tobacco [47].

The ratio of chla/chlb was lower in transformed tobacco plants (Table 1). Chl b is found in LHCII so the decrease suggests an increase in the abundance of LHCs of PSII relative to PSI similar to that suggested for a hyperstacking mutant of *Arabidopsis* [50]. There was also a slight increase in the L-band ($\Delta W_{OK} = W_{OK(TGZ)} - W_{OK(Wt)}$) in transformed tobacco plants, indicating a decrease in energetic connectivity between PSII units in comparison to Wt [44].

The effective antenna size of PSII increased in transformed tobacco, indicating an effect of transglutaminase in the antenna of PSII, in agreement with accumulating data showing that plastidial transglutaminases specifically polyaminylate PSII antenna proteins such as LHCII, CP29, CP26 and CP24 [22,23]. This may be due to an increase in both PSIIα and PSIIβ antenna size or an increase in the proportion of large antenna centres (PSIIα). A non-destructive method [46] was used to estimate the balance between PSIIα and PSIIβ centres *in vivo*. PSIIα centres accumulate in *tgz* over-expressers while the number of PSIIβ centres declines. PSIIα centres have large antenna size and are reported to occur in grana regions [6–9]. Antenna size in PSIIβ is smaller and they are reported to occur in stroma lamellae [7,9]. As the phenotype of the transformed plants becomes more accentuated the proportion of PSIIα increases at the expense of PSIIβ centres, approaching 100% (see Supplementary Fig. 2).

The major increase in the PSIIα/PSIIβ ratio indicates a reduction of stroma thylakoids. To crosscheck this we studied the ultrastructure of the chloroplast. Electron microscopy revealed that *tgz* over-expression resulted in an increase in grana stacking (i.e. increased number of layers in each granum) and a decrease in stroma lamellae. In *tgz*-transformed tobacco chloroplasts grana stacks of up to 1000 nm were observed (more than 40 layers on average), whereas in the Wt tobacco chloroplasts, the granum stacks were a maximum of 400 nm (less than 20 layers on average).

On the grounds that, in higher plants, 10–20 layers stack in each granum [2] the over-expression of *tgz* caused a significant and relative uncommon increase in the number of layers per granum. The average number of stacked thylakoids in the transformed plants doubled that the Wt plants, indicates that not only grana height but also the number of stacked membranes is increased in the *tgz* over-expressers. It is known that plants adapted to shade and low-light have many more grana per chloroplast with many more appressed relative to non-appressed thylakoids, compared to plants adapted to sun and high-light [51]. However, the increased grana size in tobacco transplastomic plants is not related to decreased light intensity but to *tgz* over-expression, since Wt tobacco plants growing under the same light conditions do not have these grana features.

The reduction in the amount of stroma thylakoids leads to a number of problems regarding the functionality of the photosynthetic apparatus. Stroma lamellae are one of the major site of ATPase and a chloroplast with severely reduced stroma lamellae would not accommodate as many ATPases. These proteins allow lumen protons to escape in stroma, thus, less ATPases implicates less "proton channels" and higher ΔpH between stroma and lumen during illumination [52]. Consistent with this view, the light induced energization of the thylakoid was higher (i.e. higher qE for *tgz*-transformants). The high NPQ of the over-expressers is not fully understood at the moment. It may be that the increased stacking [53] or the increased antenna size [18] are contributing factors to the high NPQ values, since total carotenoids in transplastomic plants are less than in Wt. The elevated qE (Table 1) and the F_M' value (end of light phase) close to F_0 value (Fig. 4B open triangles) for transformed tobacco, indicate that the lumen-pH induced dissipative conformation of antenna and/or PSII reaction center is more efficiently formed in transformed than in Wt plants. Considering that LHCII, CP29, CP26 and CP24 are normal substrates of the plastidal TGase [22,23] and putative sites of qE [18, 54 and refs therein], a possible interpretation, for which research is being carried out to verify, is that they change conformation upon polyaminylation, which in turn promotes dissipation. Structural and biochemical changes that appeared only rarely in early phases (Fig. 3D) and progressively more frequently in the latest phases of plant development (Ortigosa et al., in preparation), are indicative of oxidative stress [55] due to impairment of photochemistry as indicated also by the decreased F_V/F_M (Table 1).

In contrast, the underlying causes of increased thylakoid appression are better understood. Polyaminylation of proteins result in significant change in the charge of the target protein [23]. It is well established that negative charges of chlorophyll binding proteins must be neutralized by positive cations in order adjacent membranes to stack and in turn grana formation to occur [16]. This kind of charge neutralization is feasible with monovalent or divalent inorganic cations [9,12] or with organic cations such as polyamines [47]. Fluorescence transients of tobacco thylakoids indicate that the higher polyamines are much more efficient in stacking than Mg^{+2} [47]. While these authors quantified the short term coulombic effects of non-covalently bound polyamines, our results indicate that bound polyamines also increase in highly appressed membranes *in vivo* (in the case of transformed tobacco thylakoids). The self-assembly of the thylakoids into grana has been suggested to occur upon cation addition *in vitro* and migration of minor LHCIIs from PSIIβ to PSIIα [9]. In line with this view our *in vivo* results show a reduction of PSIIβ and increase of membrane appression in transformed tobacco plants.

Recent electron microscope tomography results and proposed models for the three-dimensional organisation and assembly of the thylakoid membrane system support our results [56] but there are no apparent axial grana linkages [57]. In addition, the over-expressers have less chlorophyll content per leaf basis than Wt and at later stages of development this phenomenon is more intense (Ortigosa et al. in preparation). Lower chlorophyll content and lower chla/chlb ratio has also been found in a mutant of *Arabidopsis* (*adg1-1*/*tpt-1*) with increased stacking [50].

The architecture of thylakoids is a major factor which affects functionality and efficiency of the photosynthetic apparatus. Light conditions in terms of light quality and intensity; define thylakoid architecture, but the details of the molecular mechanism responsible for this regulation are largely unknown. We provide evidence that remodeling of the grana may be possible through over-expression of a single enzyme, and suggest that *tgz* has an important functional role in the formation of the grana stacks. Future experiments will identify the exact residue(s) of polyaminylation and increase our understanding of the structure and plasticity of the thylakoid network.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2009.05.014.

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