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Transglutaminase is Involved in the Remodeling of Tobacco Thylakoids

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1. Introduction

Photosynthesis light reactions are among the more fast, complex and important processes in the ecosystem. They take place in specific membranes the so-called thylakoids and they produce O_2, energy (ATP) and reducing equivalents (NADPH). In this chapter we will discuss recent findings that shed light in important aspects of thylakoid architecture and functional organization. Key role for the remodeling of thylakoids plays a plastidal transglutaminase that was recently cloned from maize. 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. Transglutaminase (TGase) activity is present in chloroplasts of higher plants being PSII antenna proteins the enzyme's natural substrates. Although the functionality of this plastidial enzyme is not clear, a role in antenna regulation has been hypothesized. The isolation, for the first time in plants, of two related complementary maize DNA clones, *tgz15* and *tgz21,* encoding active maize (*Zea mays L*) chloroplastic TGase (chlTGZ) has contributed to deep on the role of this enzyme in plants (Torné et al. 2002; Villalobos et al 2004). In addition, the main polyamines, putrescine (Put), spermidine (Spd) and spermine (Spm) are normally produced and oxidized in chloroplasts. Thus, all types of post-translational modifications (i.e. mono-Put, mono-Spd, mono-Spm, bis-Put, bis-Spd and bis-Spm) are in theory probable for the target proteins. These modifications may alter charge and/or conformation of the target protein as well as their linking with other proteins (Kotzabasis et al. 1993; Del Duca et al. 1994; Della Mea et al. 2004).

A strong tool for a deeper study of gene functionality is the effect of its over-expression in an heterologous plant system. Here we will discuss in detail the information about the recent chlTGZ over-expression in tobacco (*Nicotiana tabacum* var. Petit Havana) chloroplasts (Ioannidis et al. 2009) and its characterization. After chloroplast transformation, transglutaminase activity in TGZ-over-expressers was up-regulated 4-fold with respect to the wild-type plants, which in turn rised its thylakoid-associated polyamine content about 90%. A major increase in the granum size (i.e. increase in the number of stacked layers)

accompanied by a concomitant decrease of stroma thylakoids in the TGase over-expressers was observed. Functional comparison between wild type tobacco and chlTGZ over-expressers was according to these observations, and illustrated 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 extensive electron microscopy studies indicated 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). Finally, late stages of plant development present alterations in the photosynthetic apparatus, chloroplast ultrastructure, and, particularly, oxidative and antioxidative metabolism pathways are induced (Ortigosa et al 2010). At the same time, the over-expressed TGZ protein, accumulated progressively in chloroplast inclusion bodies (Villar-Piqué et al. 2010). These results are discussed in line with chlTGZ involvement in chloroplast functionality.

2. Thylakoids and photosynthesis

The chloroplasts of higher plants are bounded by two envelope membranes that surround an aqueous matrix, the stroma, and the internal photosynthetic membranes, the thylakoids (Staehelin & van der Staay 1996). 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 (Mustardy & Garab 2003). Although our understanding regarding architecture of thylakoids is advanced, many issues such as self-assembly and structural flexibility, still remain to be explored (Mustardy & Garab 2003).

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 (Andersson & Andersson 1980). Separation of the two pigment systems is probably important in preventing unregulated excitation energy flow between the two photosystems (Andersson & Andersson 1988). Without this, PSI, which is much faster than PSII, would disturb the balance of the energy distribution between the two photosystems (Trissl & Wilhelm 1993). Also PSII exhibits a heterogeneity in terms of antenna size with centers of large chlorophyll antenna size termed PSIIα (occur in grana) and of smaller antenna termed PSIIβ (occur in stroma lamellae) (Melis & Homann 1976; Melis 1989; Kirschhoff et al 2007; Kaftan et al. 1999).

The abundance of LHCII in the granum suggests that these antenna complexes also play a structural role. Indeed, LHCII has been shown to stabilize the granum ultrastructure, and to participate in the cation-mediated stacking of the membranes (Staehelin & van der Staay 1996; Kirchhoff et al. 2007; Arnzten 1978; Duniec et al 1981; Barber 1982). These light-harvesting complexes have also been shown to be involved, via electrostatic and osmotic forces, in the lateral organization of the membranes (Garab et al. 1991). Previous studies showed that the strength of stacking is affected by the phosphorylation of LHCII and of several other phosphoproteins (Allen et al. 1981). LHCII is largely responsible for the organization of the plant photosynthetic system by maintaining the tight appression of thylakoid membranes in chloroplast grana (Allen & Forsberg 2001). An important role for

this effect plays the stromal surface of the LHCII trimer which is mainly flat and negatively charged as demonstrated by recent structural studies in higher plants (Standfuss et al. 2005). This complex collects excitation energy and transfers it to the reaction centres of PSII and PSI (van Amerongen & Dekker 2003). Also, 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 a special mechanism, referred to as nonphotochemical quenching (NPQ), has evolved in plants to dissipate excess energy as heat (Pascal et al. 2005).

The interplay 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 show distinct differences in the organization of their thylakoid system (Staehelin & van der Staay 1996). In turn, this affect the efficiency with which light is harvested and utilized.

With respect to thylakoid membrane biogenesis, Wang et al. 2004 showed that the *Thf1* gene product played a crucial role in a dynamic process of vesicle-mediated thylakoid membrane biogenesis in *Arabidopsis.* Recently, Chi et al. 2008 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.

3. Transglutaminases and polyamines

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 (Lorand & Graham 2003). However, the role of TGases in chloroplast is not fully understood yet. Maize (*Zea mays L.)* TGase was 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 (Bernet 1997; Bernet et al 1999). Subcellular localization studies showed that, in adult plants, the enzyme was specifically localized in the chloroplast grana-appressed thylakoids and close to LHCII and its abundance depended on the degree of grana development (Villalobos et al 2001; Villalobos 2007; Santos et al. 2007). An important step for the elucidation of the plastidal TGase role in plants was the isolation for the first time in plants of two related complementary maize DNA clones, *tgz15* and *tgz21,* encoding active maize TGase (Torné et al. 2002; Villalobos et al. 2004). Interestingly, their expression is dependent on the duration of light exposure, indicating a role for adaptation in different light environmental conditions including natural habitats (Pintó-Marijuan et al 2007; Carvajal et al. 2007-2011). Proteomic studies indicates that plastidial maize TGase is a peripheral thylakoid protein forming part of a specific PSII protein complex which includes LHCII, ATPase and PsbS proteins, its expression pattern changing according to chloroplast developmental stage and light regime (Campos et al. 2010). Tacking into account all the described results, it has been hypothesized that TGases are implicated in the photosynthetic process (Villalobos et al 2004; Pintó-Marijuan et al 2007; Serafini-Fracassini & Del Duca, 2008).

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 and thus possess 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 (Kotzabasis et al. 1993a). Plastidal Transglutaminases might attach covalently polyamines of all thylakoid proteins specifically in LHCII, CP29, CP26 and CP24 (Del Duca et al 1994). Recently, it was demonstrated that a plastidial TGase activity in maize polyaminylates purified LHCII catalyzing the production of mono and bis glutamyl PAs in a light dependent way (Della Mea et al 2004). As commented elsewhere, authors indicated that the additional positive charges inserted on proteins by the protein-bound PAs might induce conformational changes by conjugation of the two terminal amino-groups of PAs to one or two glutamine residues of LHCII and they discussed if light sensitivity is due to the enzyme or to the substrate. In the work of Carvajal et al. (2011), when purified plastidial maize TGase (TGZ) was added to maize thylakoid protein extracts,TGase activity was significantly higher (in a light dependent manner) than that of the same extract without TGZ addition, indicating that thylakoid proteins are the specific substrate of TGZ. However, in the same work it is demonstrated that, if a non-plant protein was used as TGZ substrate, TGase activity was not light-dependent. These last results indicate that light dependence of plastidial TGase activity is probably related to its specific substrate (thylakoid proteins) and not to the enzyme itself.

First evidence for a role of plastidial TGase in the thylakoids 3D architecture comes from tobacco chloroplasts over expressing maize TGase (TGZ) (Ioannidis et al 2009). In that work, we hypothesized that TGase is implicated in the ratio regulation of grana to stroma thylakoids (Villalobos et al. 2004). A combination of genetic engineering and *in vivo* probing approach was used to test this hypothesis. Here we discuss in detail the information about the effect of maize *tgz* gene over-expression and its characterization in tobacco chloroplasts via plastid transformation, where the transgene is integrated in the plastid genome by homologous recombination (Maliga 2004; Fernández-San Millán et al 2007, 2008).

4. Maize transglutaminase over-expressed in tobacco chloroplasts

4.1 Vector construction, chloroplast transformation and plant regeneration

To introduce the *tgz13* gene into tobacco Wt chloroplasts, the *tgz* gene was PCR amplified, fused to the promoter and 5'untranslated region of the *psbA* gene and finally introduced into the multiple cloning site of the pAF vector, rendering 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 (Fernández-San Millán et al 2008). The regulatory sequences of the *psbA* gene were chosen due to the high levels of heterologous gene expression they confer in transplastomic plants (Fernández-San Millán et al 2003; Molina et al 2004). After that, leaves of tobacco Wt plants were bombarded with gold microprojectils coated with plasmid DNA containing the *tgz* gene and plants were regenerated in the selective spectinomycin medium. Southern blot analysis performed on shoots developed after the second round of selection with spectinomycin revealed some plants that were homoplasmic for the *tgz* gene (Fig. 1) (Ioannidis et al. 2009). These experiments were carried out in J. Veramendi laboratory (Public Univ. Navarra. Spain).

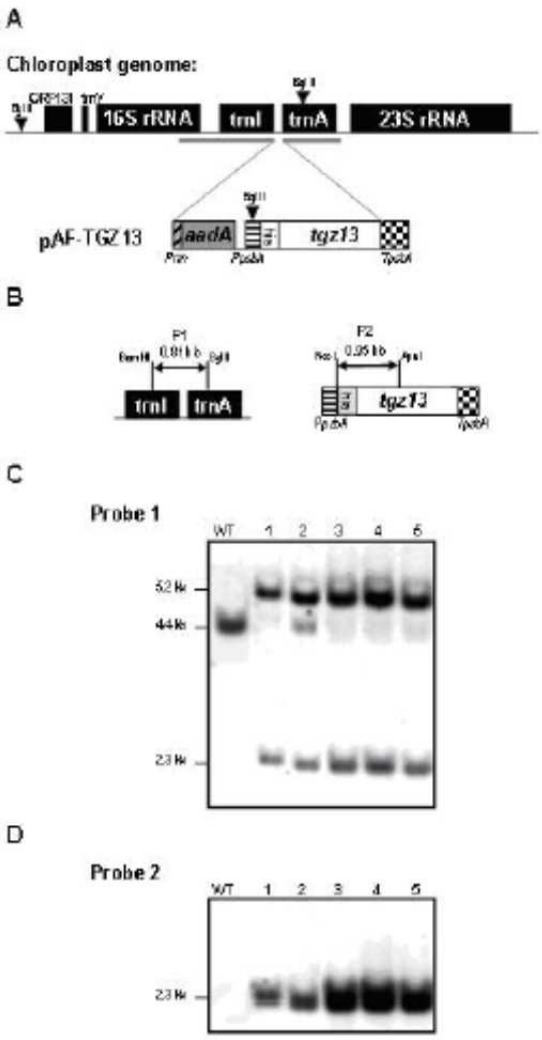

Fig. 1. Schematic representation of tobacco plastid genome transformation using the maize transglutaminase *tgz* gene. A, map of the wild-type and *tgz*-transformed genomes. Regions for homologous recombination are underlined in the native chloroplast genome; B, the 0.81 kb fragment (P1) of the targeting 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 is shown. 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. Phenotype of typical leaves used for this study from plants of TGZ-transplastomic tobacco (PG) and wild-type tobacco (WT). From Ioannidis et al. 2009.

4.2 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 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). By a sensitive HPLC method we have estimated the amount of associated polyamines in thylakoids (Kotzabasis et al 1993b). Plants over-expressing TGZ showed a total increase of 90% in the titer of thylakoid associated polyamines (Put, Spd and Spm) on a Chl basis (Fig. 2). Bound Put was increased about 3 times and the higher polyamines about 60% in comparison to the Wt.

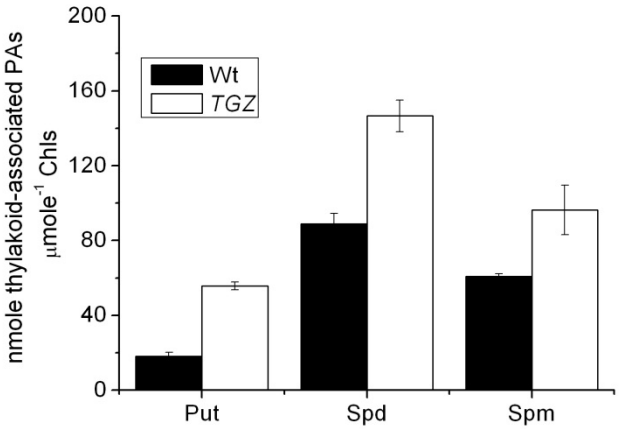

Fig. 2. Thylakoid associated polyamines of *Nicotiana tabacum* Wt and tobacco over-expressing *tgz* from maize. Data are presented on a chlorophyll basis because protein titer was substantially higher in transformed tobacco due to the over-expression of *tgz*. Vertical bars denote standard deviation (n=3). From Ioannidis et al. 2009.

4.3 Thylakoid ultrastructure and pigment content

Transmission electronic microscopy revealed important differences between Wt and TGZ over-expressing chloroplasts. Wt chloroplasts exhibit a normal thylakoid network architecture (Fig. 3, A and C), exhibiting grana and stroma lamellae in a normal proportion. Over_expression of *tgz* resulted in a severe depletion of chloroplast stroma lamellae and, interestingly, a grana dominance (Fig. 3, B and D), the granum size (number of stacked layers) being increased up to nearly 1000 nm, the double that of the Wt granum size (Table 1 and Fig. 3D). Furthermore, a reduction in the total Chl content was evident from 1.86 (mg·g^{-1}FW) in Wt to 0.6 (mg·g^{-1}FW) in over-*tgz* with a parallel decrease of the Chla/Chlb ratio (Table 1). The total carotenoid titer was also reduced (Table 1). In fact, as commented in the next paragraph, at later stages of development TGZ-plants are severely chlorotic (Ortigosa et al. 2010).

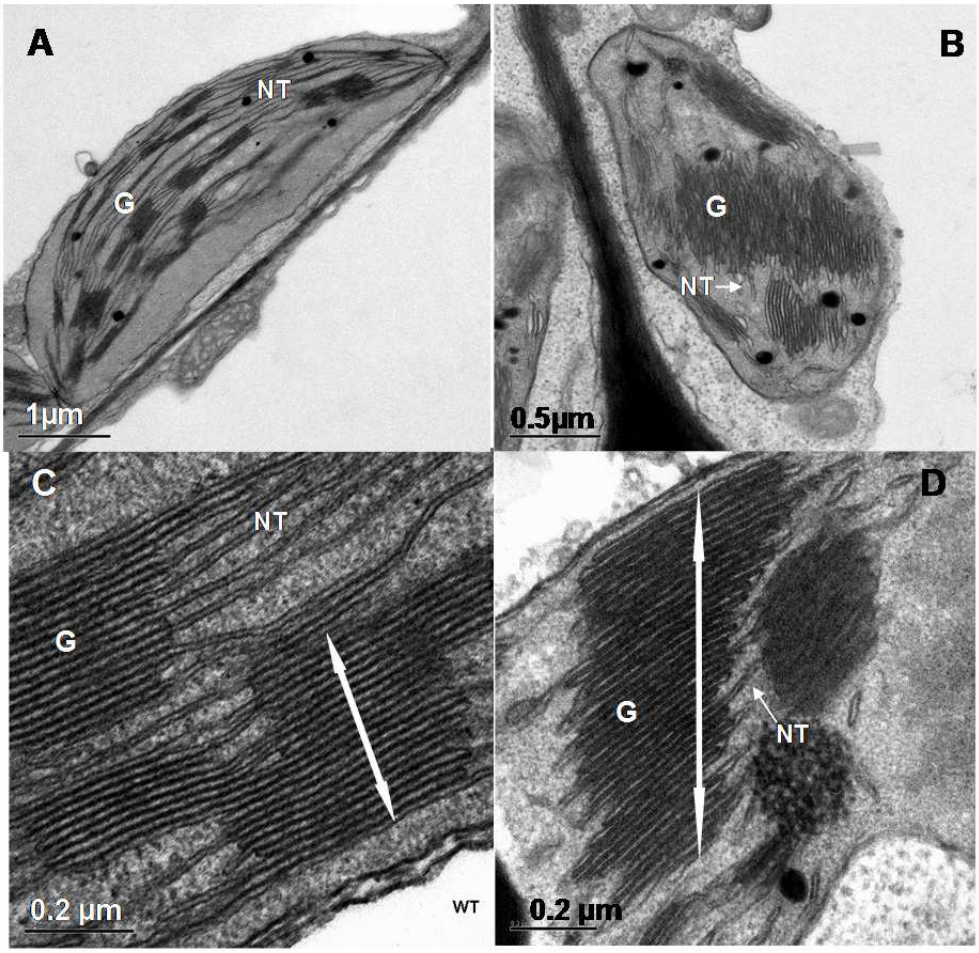

Fig. 3. Ultrastructure of chloroplasts from tobacco Wt and over-expressing TGZ. Transformed tobacco (B, D) shows an increased stacking of thylakoids and a reduced stroma thylakoid network. Large grana appear with a size many hundreds nm bigger than Wt plants (A, C). G= grana; NT= non-appressed thylakoids; p= plastoglobuli; pbl= prolamellar body lattice. Arrow in C= 0.35 µm approx.; arrow in D= 0.9 µm approx. From Ioannidis et al. 2009.

4.4 Fluorescence induction kinetics

Over-expression of maize *tgz* in tobacco has a small effect (about 13% decrease) in the structure and functionality of PSII, as judged by the F_V/F_M values. Maximum quantum efficiency of PSII in the transformants is about 0.7, whereas Wt tobacco exhibits optimal values of about 0.81 (Table 1).

	Wt tobacco	over TGZ	Oldest over TGZ
F_V/F_M	0.812 (0.031)	0.702 (0.070)	0,122 (0.02)
qE	0.16 (0.02)	1.008 (0.08)	-ND
$t_{1/2DCMU}$ (ms)	166 (9)	110 (12)	-ND
Chl a (mg ·g^{-1}FW)	1.38 (0.11)	0.42 (0.08)	0.1 (0.01)
Chl b (mg ·g^{-1}FW)	0.48 (0.04)	0.18 (0.05)	0.06 (0.01).
Total Chls (mg ·g^{-1}FW)	1.86 (0.15)	0.60 (0.13)	0.16 (0.02)
Chla/Chlb	2.87 (0.06)	2.33 (0.17)	1.73 (0.25)
Carotenoids (mg ·g^{-1}FW)	0.29 (0.02)	0.13 (0.02)	0.03
Maximum granum size (nm)	400 (*)	1000 (*)	----
Transglutaminase activity pmol Put ·mg protein ·h^{-1}	758.9 (89.2)	3067.3 (661)	1636.6 (172.4)

*measured from 50 chloroplasts

Table 1**.** Comparison of fluorescence parameters, pigment content, maximum granum size and transglutaminase activity in *Nicotiana tabacum* Wt and overexpressing *tgz* (over TGZ and oldest TGZ) leaves. 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 $p<0.05$. From Ioannidis et al. 2009.

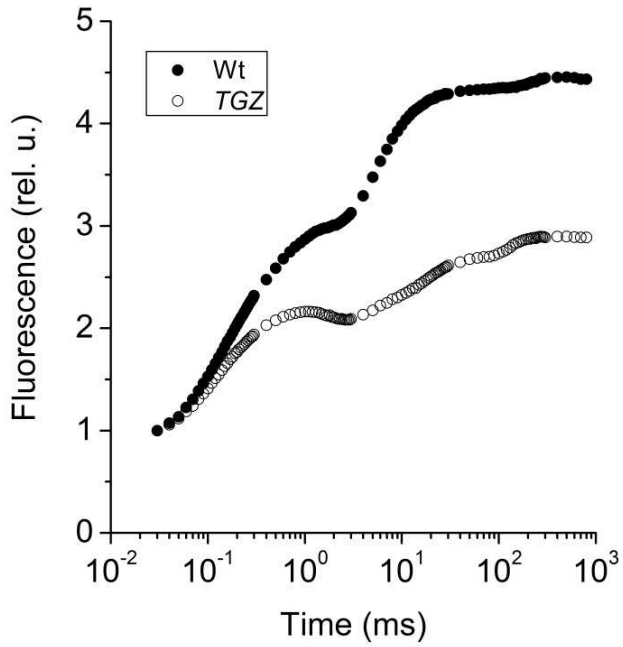

Fig. 4. Fluorescence induction curves of *Nicotiana tabacum* Wt and tobacco over-expressing *tgz* from maize (TGZ). Samples were dark adapted for 20 min and were illuminated with 3000 μmol [photons] m^{-2}s^{-1}. The time axis is semi-logarithmic for clarity and data are normalized to Fo.

More pronounced differences appear at later stages of development. A detailed transient kinetics of fluorescence induction shows that there is a major difference both in the shape and in the amplitude between Wt and over-TGZ tobacco plants (Fig. 4). The maximal difference in F_V during fluorescence induction is at 10 ms (about 70% higher values for Wt in comparison to the transformed) and there are also large differences in the F_M values (about 50% higher for the wild type). The effective PSII antenna size increased also in the over-expressers as indicated by the shortest closure time of their reaction centers (see Table 1 parameter $t_{1/2DCMU}$) in comparison to that of the Wt. The value of the energy-dependent component of the non-photochemical quenching (qE) in the case of the transformed tobacco is about 6 times higher than that of the Wt (Table 1).

4.5 Oxidative stress symptoms and leaf aging

The results obtained with later stages of leaf development revealed that photochemistry impairment and oxidative stress increased with transplastomic leaf age. These alterations included decrease in pigment levels, changes in the photosynthetic apparatus, in the

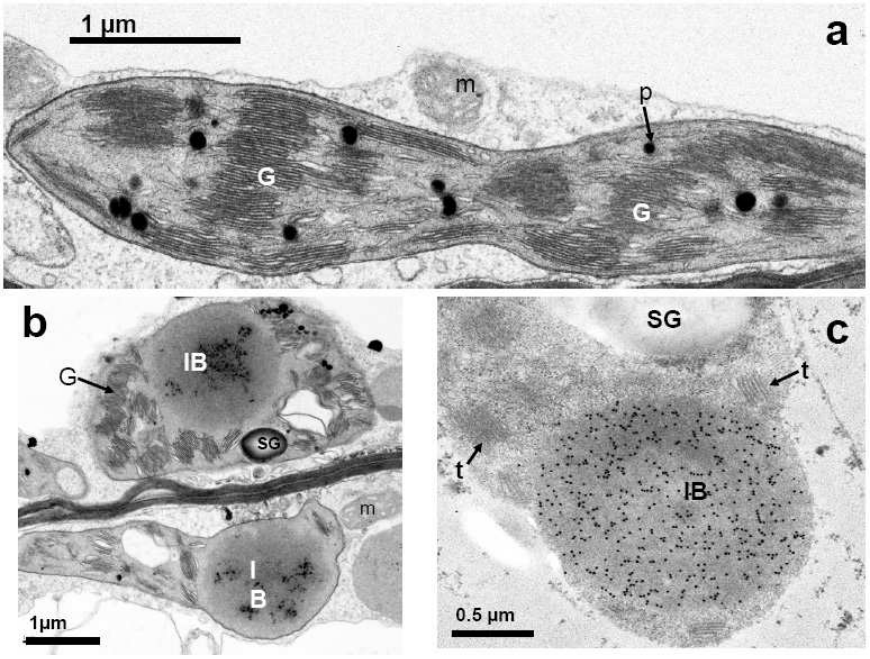

Fig. 5. Ultrastructure of tobacco chloroplasts over-expressing TGZ and TGZ immunolocalization. **a** A dividing chloroplast, showing increased grana appression and a reduced stroma thylakoid network. **b** Two oldest-leaf-chloroplasts containing large inclusion bodies. In the upper chloroplast an over-appressed granum is still visible. In the lower chloroplast the thylakoid network is disorganized. IBs larger than 1 µm are usually present. **c** Subcellular immunolocalization of TGZ-protein in the IB, using an anti-TGZ4 antibody. IB, inclusion body; G, grana; m, mitochondria; p, plastoglobuli; SG, starch grains; t, thylakoids. From Ortigosa et al. Planta 2010

chloroplast ultrastructure, and, particularly, the activation of oxidative and antioxidative metabolism pathways (see Tables 2 and 3). At the same time, the over-expressed TGZ protein accumulated progressively in chloroplast inclusion bodies. These traits were accompanied by thylakoid scattering, membrane degradation and reduction of thylakoid interconnections (Fig. 5). Consequently, the electron transport between photosystems decrease dramatically in the old leaves. In spite of these alterations, transplastomic plants can be maintained and reproduced *in vitro* (Ortigosa et al. 2010). These experiments were carried out in J. A. Hernandez laboratory (CEBAS-CSIC, Murcia, Spain).

5. Current and future developments

The over-expression of a heterologous gene could be a valuable tool for the understanding of the corresponding protein functionality. As mentioned above, the over-expression of TGZ resulted in a 4-fold increase of plastidial TGase activity, causing a significant increase in grana size and about 90% increase in thylakoid-associated polyamines (Ioannidis et al 2009). Interestingly, transformed plants exhibit increased ability to induce NPQ, a small decrease in maximal quantum yield of PSII and about 6 times higher qE, in comparison to the Wt. These results are in line with recent studies showing that elevation of Spd and Spm titers could lead to an increase in NPQ in tobacco (Ioannidis et al. 2007). Also, the effect of TGZ over PSII antenna is showed in the decrease of Chl a/Chl b ratio, which is an indicator for changes in the stoichiometry of the photosystems (in particular their LHCs) (Table 1) and the effective PSII antenna size increase. These results are in line with accumulating data showing that a plastidial TGase activity specifically polyaminylate PSII antenna proteins such as LHCII, CP29, CP26 and CP24 (Del Duca et al. 1994; Della Mea et al 2004). Chl b is found in LHCII and, consequently, a decrease in the Chl a/Chl b ratio suggests an increase in the abundance of LHCs of PSII relative to PSI similar to that suggested for a hyperstacking mutant of *Arabidopsis* (Häussler et al. 2009). Also noteworthy is the fact that OJIP transients indicate an increase in the connectivity of PSII centers in the transformed tobacco. *In vitro* investigations such us microscopy of thylakoids at high resolution hopefully will shed light on this matter in the near future.

	H_2O_2 nmol g^{-1} FW	**TBARS nmol g^{-1} FW**
Wt		
Upper leaves	2.57c	0.108bc
Middle leaves	2.30 c	0.331b
Transformed Plants		
PG leaves	4.37a	1.11a
Y leaves	3.42 b	0.97a

Table 2. Effect of TGZ over-expression on H_2O_2 content (nmol g^{-1} FW) and lipid peroxidation (TBARS) (nmol g^{-1} FW) in tobacco plant leaves. Different letters indicate significant differences according to Tukey's test ($P \leq 0.05$). From Ortigosa et al. 2010.

Regarding the apparent PSII antenna increase, two possible explanations can be hypothesized: either both PSIIα and PSIIβ increase their antenna size or the portion of the large antenna centers (PSIIα) is increased. By using a non destructive method (Andersson

& Melis 1983), we have *in vivo* estimated the poise between PSIIα and PSIIβ centers. The results indicate that PSIIα centers are accumulating in over-TGZ plants and, simultaneously, the number of PSIIβ centers is declining. PSIIα centers are of large antenna size and are considered to occur in grana regions (Melis 1989; Kirchhoff et al. 2007). PSIIβ centers possess a smaller antenna size and are considered to occur in stroma lamellae (Melis 1989; Kirchhoff et al. 2007). As the phenotype of the transformed plants is getting more intense, the portion of PSIIα increases at the expense of PSIIβ centers approaching 100% (Fig. 6). Furthermore, the remarkable increase in PSIIα/PSIIβ ratio indicates diminishing of stroma thylakoids. In order to crosscheck this hypothesis we studied the ultra-thin structure of the chloroplast. Transmission electron microscopy revealed that *tgz* over-expression resulted in an increase of grana stacking and a decrease of stroma lamellae. Remarkably, the size of the granum (number of stacked layers) in TGZ- chloroplasts is up to 1000 nm, whereas in Wt chloroplasts it was not larger than 400 nm. On the ground that in higher plants granum diameter is up to 600 nm (Mustardy and Garab 2003) the over-expression of *tgz* caused a significant and relative uncommon increase in granum size.

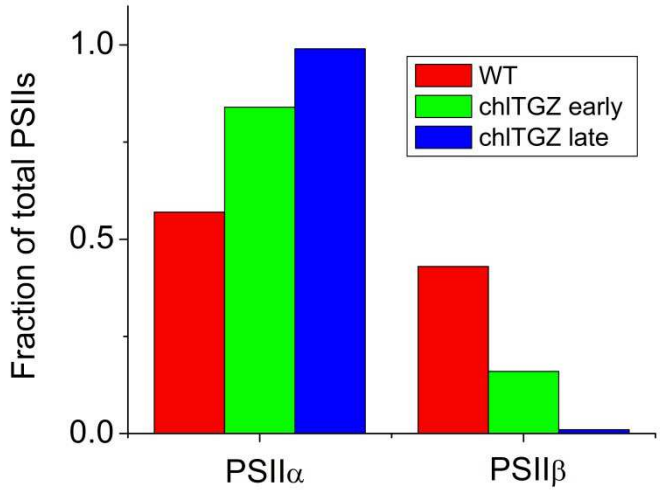

Fig. 6. The comparison of PSII antenna heterogeneity of *Nicotiana tabacum* Wt and tobacco overexpressing *tgz* from maize, as estimated by the fraction of PSIIα (white) and PSIIβ (black). Relative amount of PSIIα and PSIIβ for early and later stages of development of TGZ-transplastomic tobacco. From Ioannidis et al. 2009.

On the other hand, the reduction in the amount of stroma thylakoids leads to a number of problems regarding the functionality of the photosynthetic apparatus. Stroma lamellae are among others the major site of ATPase and a chloroplast with severely reduced stroma lamellae would not accommodate as many ATPases as Wt. Given that ATPases allow lumen protons to escape in stroma less "proton channels" means higher ΔpH between stroma and lumen during illumination (Kramer et al. 2003). Consistent with this view, the light induced energization of the thylakoid was higher (i.e. higher qE for *tgz*). At this point it should be noted that the high NPQ of the over-expressers is not fully understood at the moment. Perhaps the increased stacking (Goss et al. 2007) or the increased antenna of PSII (Pascal et al. 2005) are also contributing factors to the high NPQ values since total carotenoids in transplastomic plants are less than in Wt. First evidence i.e. the elevated qE (Table 1) and the F_M' value (end of light phase) that is close to F_0 value (Fig 4B open triangles Ioannidis et al. 2009 for transformed tobacco, indicate that the lumen-pH induced dissipative conformation of antenna and/or PSII reaction center is more efficiently formed in TGZ than in Wt. A possible interpretation -which still needs experimental verification- on the ground that LHCII, CP29, CP26 and CP24 are normal substrates of the plastidal TGase (Del Duca et al. 1994; Della Mea et al. 2004) and putative sites of qE (Pascal et al. 2005; Kovacs et al 2006 and refs therein) is that they are changing their conformation upon polyaminylation which in turn promotes dissipation. However, further research is on going for the elucidation of this phenomenon. In addition, structural and biochemical changes that appeared only sparsely in early phases (Fig 3D) and progressively appear more frequently in the latest phases of plant development are indicative of oxidative stress (Austin et al 2006; Ortigosa et al. 2010), due to impairment of photochemistry, as indicated also by the decreased F_V/F_M (Table 1).

On the contrary the underlying causes of increased stacking are better understood. Polyaminylation of proteins result in significant change in the charge of the target protein (Della Mea et al 2004). 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 (Standfuss et al. 2005). This kind of charge neutralization is feasible with monovalent or divalent inorganic cations (Kirchhoff et al. 2007; Barber 1982) or with organic cations such as polyamines (Ioannidis et al. 2007). Noteworthy, fluorescence transients of tobacco thylakoids indicate that the higher polyamines are much more efficient in stacking than Mg^{+2} (Ioannidis et al. 2007). Although the later works quantified the coulombic effects of non-covalently bound polyamines it seems that bound polyamines can also cause stacking (Ioannidis et al. 2009). The self-assembly of the thylakoids into grana was suggested to occur upon *in vitro* cation addition, and migration of minor LHCIIs from PSIIβ to PSIIα (Kirchhoff et al. 2007). Our *in vivo* results showing a PSIIβ reduction and a thylakoid-stacking increase in the *tgz*-transformants are in line with this view, Recent electron microscope tomography results and proposed models for the three-dimensional organisation of thylakoids are also in agreement with our results (Shimoni et al 2005). In addition, lower chlorophyll content and lower Chl a/Chl b ratio was also the case for a mutant of Arabidopsis (*adg1-1/tpt-1*) that exhibit increased stacking (Häusler et al. 2009). This phenomenon is also present in our tgz-transformants that presented less chlorophyll content per leaf basis than the Wt and, at later stages of development, this phenomenon is more intense (Ortigosa et al. 2010).

	Wt		Transformed plants	
Enzymatic activity	**Upper leaves**	**Middle leaves**	**PG leaves**	**Y leaves**
APX nmol min^{-1} mg^{-1} prot	1053b	961b	1557a	1479a
MDHAR nmol min^{-1} mg^{-1} prot	57.98c	60.18c	75.48b	93.62a
DHAR nmol min^{-1} mg^{-1} prot	15.11b	4.74c	26.67a	20.22b
GR nmol min^{-1} mg^{-1} prot	54.37a	57.73a	62.83a	57.25a
CATALASE µmol min^{-1} mg^{-1} prot	172.1a	178.2a	88.3b	74.9c
POX nmol min^{-1} mg^{-1} prot	118.6c	164.2c	423.0b	822.4a
NADH-POX nmol min^{-1} mg^{-1} prot	13.64c	28.02c	211.6b	330.3a
GST nmol min^{-1} mg^{-1} prot	6.17a	6.13a	5.47ab	4.25b
GPX nmol min^{-1} mg^{-1} prot	nd	nd	nd	nd
G6PDH nmol min^{-1} mg^{-1} prot	10.55b	6.12c	16.15a	16.74a
SOD U mg-1 prot	31.0b	39.7b	57.6a	54.9a

Table 3. Effect of chlTGZ over-expression on antioxidative enzyme activities in tobacco plant leaves. Different letters indicate statistical significance according to Tukey's test ($P \leq 0.05$); nd, not detectable. From Ortigosa et al. 2010.

5.1 Implications of the work

Thylakoid architecture is a major factor which affects functionality and efficiency of the photosynthetic apparatus. Light conditions in terms of quality and intensity define thylakoid architecture, but the details of the molecular mechanism which is responsible for this regulation is largely unknown (Anderson 1999; Mullineaux 2005). We provide evidence that the remodeling of the grana could be feasible through over-expression of a single enzyme. Therefore, we suggest that *tgz* has an important functional role in the formation of the grana stacks. Moreover TGZ over-expression, due to the enormous and stable granum size, may provide a powerful tool for the study and understanding of grana function that has long been debated (Mullineaux 2005).

i. Insight into the role of thylakoid bound polyamines

Polyamines are ubiquitous molecules with an ill defined mode of action. Although thousands of papers appeared the last decades concerning their effects, their role remains obscure. The interest is still high because polyamines are essential for cell growth and important for plant tolerance to stress. The fact that, in plants, free, bound and phenolic-conjugated polyamine forms are present, make their role more puzzling. This work significantly improve our understanding by sheding light mainly on the role of bound polyamines that will facilitate to understand the implication of the other polyamine forms.

ii. Transglutaminases in thylakoids and photosynthetic implications

Transglutaminase activity depends on Ca^{2+}, GTP and light (Villalobos et al. 2004; Del Duca & Fracassini 2008), which are key factors for chloroplast energetics. Transglutaminase activity was shown to be light sensitive, affected by hormone deprivation and with a light/dark rhythm (Bernet 1997; Bernet et al. 1999). Subcellular localization studies showed that, the enzyme was specifically localized in the chloroplast grana-appressed thylakoids and close to LHCII (Villalobos et al. 2001; Villalobos 2007; Santos et al. 2007). Finally, proteomic studies indicates that maize chloroplastic TGase is a peripheral thylakoid protein forming part of a specific PSII protein complex which includes LHCII, ATPase and PsbS proteins (Campos et al. 2010). With the presented results, we give important *in vivo* and *in vitro* data that reinforce the idea that the role of TGase in thylakoids is the modification of LHCII antenna proteins by polyaminilation, giving new properties to the complex, in particular under low light or stress conditions.

Why the photosynthetic apparatus has enzymes with TGase action near the reaction centers of PSII? A plausible hypothesis is that biological glues such as transglutaminases have a "polymerizing" and/or a "stabilizing" role. More particularly, crosslink of LHCIIs could increase the absorption cross section of PSII which in turn will increase photon harvesting by PSII. The latter could account for the significant increase of PSIIα centers in TGZ over-expressers. On the other hand, attachment of polyamines increase the positive charge of the protein as well as the connections intra and inter molecularly, stabilizing more firmly loosely aggregated complexes. This stabilization may be of importance during stress conditions conferring tolerance to the photosynthetic apparatus (Lütz et al 2005; Navakoudis et al 2007; Demetriou et al 2007; Sfichi et al 2008). In consequence, a possible role for polyamines on LHCII could be the activation of the dissipative antenna conformation (Ioannidis et al 2011). Furthermore, although thylakoid localization of ADC (arginine decarboxylase, Put producer) was long ago reported (Borrell et al 1995) and its importance for stress tolerance acknowledged (Galston 2001), only recently it becomes apparent that Put, and higher polyamines derived from Put, could modulate the photosynthesis protonic circuit, which is central for plant life and stress tolerance (Ioannidis et al 2011 submitted). An enzyme as TGase, that modulates the poise between free and bound polyamine forms in the following equilibrium may have a key role for the fine tuning of these processes.

$$\text{Pas + LHCII} \underset{}{\overset{\text{TGase}}{\rightleftharpoons}} \text{Pas-LHCII} \quad (1)$$

5.2 Future experiments

This chapter summarized recent results showing that over-expression of TGZ in tobacco, dramatically alter the organization of the thylakoid network. TGZ acted as a grana making

enzyme and increased granum size more than 100%. PSIIα centers increased, and , concomitantly, stroma thylakoids were depleted. At the same time, thylakoid associated polyamines increased 90%.

On the grounds that TGases have LHCbs as a natural substrate it is plausible that polyamines increase in thylakoids were due to LHCII modification. In future works, we will test whether LHCII has a different profile of bound polyamines due to TGZ over-expression. If this is the case (if more polyamines are LHCII-attached), then, PSIIα centers increase could be the direct outcome of LHCII polyaminylation. First results show a 80% Spd and Spm increase in isolated LHCII antenna proteins from tobacco TGZ over-expressers (Ioannidis et al. in preparation). TGases may affect, not only the thylakoid structure, but also the architecture of the thylakoid network. This enzyme could alter the function of photosynthetic complexes and affect photosynthesis in multiple ways. Given that LHCII has a key role in light harvesting, photoprotective qE and state transitions, a highly polyaminylated LHCII *in vivo* should be tested for every one of these processes. First results show that antenna down regulation is much more sensitive under these conditions. Future experiments should also reveal the exact residue(s) of polyaminylation and increase further our understanding regarding the structure and plasticity of the thylakoid network. Last but not least, TGases may cross link the complexes of PSII outer antenna with the core. Newly engineered plants will help to elucidate these issues.

6. Conclusion

Overexpression of chlTGZ in tobacco increased the activity of plastidal transglutaminase, the thylakoid associated polyamines, the fraction of PSIIα centers and thylakoid stacking. We suggest that chlTGZ has an important role in the remodeling of the thylakoid network.

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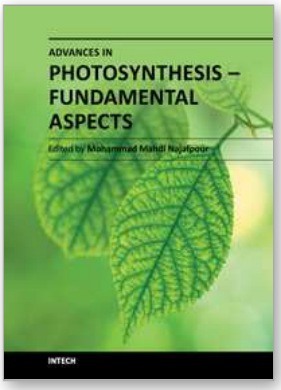

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