RESEARCH PAPER

Characterisation of *Zea mays* L. plastidial transglutaminase: interactions with thylakoid membrane proteins

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INTRODUCTION

Transglutaminases (TGases, EC 2.3.2.13) are intracellular and extracellular enzymes that catalyse post-translational modification of proteins by establishing ε-(γ-glutamyl) links and covalent conjugation of polyamines. Detected for the first time in animals, these enzymes modify structural proteins and are widely distributed in bacteria, animals and plants (Folk et al. 1980). Coagulation factor XIII was the first TGase primary structure established (Ichinose et al. 1990). TGases have a catalytic triad of cysteine, histidine and aspartate, with the catalytic reaction proceeding via an intermediate product linked to the cysteine. In recent years, many studies on TGase have been carried out on tissues from humans, lower vertebrates, bacteria, algae and yeasts. Cloning of TGases has allowed a better understanding of their involvement in cellular differentiation processes, tissue stabilisation and apoptosis (Lorand & Graham 2003). In general, their sequence homology is moderate and, in some cases, there is no

ABSTRACT

Chloroplast transglutaminase (chlTGase) activity is considered to play a significant role in response to a light stimulus and photo-adaptation of plants, but its precise function in the chloroplast is unclear. The characterisation, at the proteomic level, of the chlTGase interaction with thylakoid proteins and demonstration of its association with photosystem II (PSII) protein complexes was accomplished with experiments using maize thylakoid protein extracts. By means of a specific antibody designed against the C-terminal sequence of the maize TGase gene product, different chlTGase forms were immunodetected in thylakoid membrane extracts from three different stages of maize chloroplast differentiation. These bands co-localised with those of lhcb 1, 2 and 3 antenna proteins. The most significant, a 58 kDa form present in mature chloroplasts, was characterised using biochemical and proteomic approaches. Sequential fractionation of thylakoid proteins from light-induced mature chloroplasts showed that the 58 kDa form was associated with the thylakoid membrane, behaving as a soluble or peripheral membrane protein. Two-dimensional gel electrophoresis discriminated, for the first time, the 58-kDa band in two different forms, probably corresponding to the two different TGase cDNAs previously cloned. Electrophoretic separation of thylakoid proteins in native gels, followed by LC-MS mass spectrometry identification of protein complexes indicated that maize chlTGase forms part of a specific PSII protein complex, which includes LHCII, ATPase and pSbS proteins. The results are discussed in relation to the interaction between these proteins and the suggested role of the enzyme in thylakoid membrane organisation and photoprotection.
structural or functional similarity. Protein cross-linking catalysed by TGases is the reaction that has attracted the greatest attention, although the significance of TGase-mediated post-translational modifications of proteins by amine incorporation (like polyamines) is also well recognised (Lorand & Graham 2003).

In contrast to the mammalian TGase superfamily, there has been little research on plant TGases. TGase-like activity in plants was first observed in pea seedlings (Icekson & Apelbaum 1987). Using an animal anti-TGase antibody, TGase activity was previously immunodetected by our group in total protein extracts of maize (Zea mays L.) meristematic calli and their isolated chloroplasts, identifying a unique 58-kDa band. In this material, TGase activity was shown to be light sensitive, affected by hormone deprivation and has a light/dark rhythm (Bernet et al. 1999). In further studies using a polyclonal antibody obtained from Helianthus tuberosus TGase, the enzyme was localised in the chloroplast grana-appressed thylakoids of mature maize leaves (Villalobos et al. 2001). Two related complementary maize DNA clones, tgz15 and tgz21, encoding active maize transglutaminase and exhibiting the biochemical characteristics described for other TGases, with their expression levels dependent on the length of light exposure, have been isolated from plants (Torné et al. 2002; Villalobos et al. 2004). Variants of the tgz cDNA have been sub-cloned into a pET28 vector and over-expressed in Escherichia coli cells to produce pure and active maize TGase (TGZ) for functional and structural studies. These TGZ proteins were shown to be active and to have the biochemical characteristics described for plant TGases (Carvajal-Vallejos et al. 2007).

The contribution of polyamines (PAs), especially putrescine (Put), to protecting the photosynthetic apparatus has attracted considerable interest for understanding this complex phenomenon. Binding of Put to thylakoid membranes has been proposed to be a photo-adaptation response under controlled stress conditions (Besford et al. 1993; Navakoudis et al. 2007). Bound Put accumulation mediated by TGase activity has also recently been shown to increase during the photoprotective response to high-light stress in Quercus ilex leaves, in both natural forest and controlled conditions (Pinto-Marijuan et al. 2007). These results reinforce the hypothesis on the importance of this enzyme in photoprotection by polyamine conjugation to light-harvesting complex II (LHClI) proteins. The reported plasticity of the photosynthetic apparatus associated with the oligomeric state of LHClI, and the levels of putrescine and spermine bound to this antenna protein in the unicellular oxygenic alga Scenedesmus obliquus, as a mechanism of photo-adaptation and prevention of photo-damage in response to several types of stress, points to a similar process mediated by TGase (Navakoudis et al. 2007; Sfichi-Duke et al. 2008).

Despite the above-mentioned research, information regarding the presence of this enzyme during chloroplast development and its interaction with other plastidial proteins is necessary to understand its functionality. To the best of our knowledge, at the proteomic level there is no conclusive information on proteins or protein complexes implicated in this interaction. The aim of the present work was to study chlTGase expression during maize chloroplast development and its association with the PSII protein complexes in thylakoid membranes, using complementary biochemical and proteomic approaches. The results are discussed in light of possible involvement of this enzyme in photosynthesis-related processes.

**MATERIALS AND METHODS**

**Plant material**

Maize seedlings (G-5054) were germinated and grown in trays under the following conditions: (i) 9 days in a dark room at 23 °C (etiolated chloroplasts), (ii) 9 days in the same conditions followed by 3 days in a greenhouse with a light/dark period of 16 h/8 h at 600–800 μmol·m⁻²·s⁻¹ (differentiating chloroplasts), and (iii) germinated and grown for 20 days in a greenhouse in the conditions described (mature chloroplasts).

**Thylakoid extraction and protein quantification**

The upper parts of maize plants were collected, and plastid membrane extracts immediately obtained as described in Berthold et al. (1981) and stored at −80 °C. Protein was quantified by the Bradford (1976) method. None of the detergent concentrations used in all extraction processes interfere with the quantification assay.

**TGase activity assay**

Endogenous TGase activity from maize plastid membrane extracts was measured by incorporation of [³H] putrescine into plastid proteins (Villalobos et al. 2001). Assays were made using 400 μg or 600 μg protein over 30 min in the presence or absence of a 90–100 μmol·m⁻²·s⁻¹ white-light lamp. Control assays were made by precipitating the protein before adding the substrate with TCA solution (10%, w/v). Assays were made in triplicate, and statistically analysed with ANOVA and Student t-tests.

**Production of a polyclonal antibody from the C-terminal sequence of the maize TGase gene**

A peptide sequence of 20 amino acids, corresponding to the C-terminal sequence of the TGZ15p (Q6KF70) maize tgz gene product was synthesised. This sequence has no homology with other published sequences present in the DNA maize databases. The peptide was conjugated to the KLH crab protein (27 peptide mols:1 protein mol) and inoculated into rabbits. Antibody titration was realized with the DMP (dimethyl pimelimidate dihydrochloride) method (Peptide Synthesis service, Pompeu Fabra University, Barcelona, Spain). This antibody was named PAB/18QA.
SDS-PAGE and western blotting

Proteins were solubilised from plastid membrane extracts with 0.24 M Tris–HCl, 6% SDS (w/v) and 2.24 M β-mercaptoethanol, pH 6.8 (5X Laemmlı dye stock), or with the following buffers: (i) 50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100 (v/v), 0.5% sodium deoxycholate (NaD doc, w/v), 0.1% SDS (w/v) and 5 mM EDTA, pH 7.4, or (ii) 50 mM Tris–HCl, 150 mM NaCl and 1% Triton X-100 (v/v), pH 7.5. In both cases 3 mg protein per ml buffer were added. Proteins were separated by SDS-PAGE according to Laemmlı (1970) in a Mini-Protein III system (Bio-Rad, Hercules, CA, USA) and further transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK) on wet systems (Bio-Rad) according to manufacturer’s instructions. Membrane blocking was performed with non-fat dried milk (5% or 10%, w/v) in PBS and washed in PBS with 0.1% to 0.3% Tween 20 (v/v). Immunodetection was carried out with the specific anti-maize TGase antibody PAB 18QA and anti-LHCII commercial antibodies (Ibchb, 2 and 3) from Agrisera AB (Vännäs, Sweden). Antibodies were diluted in PBS to 1:1000 in the case of the PAB 18QA antibody, to 1:5000 in the case of the anti-lhcb antibodies, and to 1:15,000 in the case of the secondary antibody (anti-rabbit IgG). Immunodetection was obtained using the secondary antibody (Ab rabbit IgGs) conjugated to horseradish peroxidase (HRP) and ECL substrates (GE Healthcare). Western blot images were acquired in a Phospho-Imager (Bio-Rad), band intensities quantified using the ImageJ 1.41 program (Bio-Rad) and expressed in optical density units. Broad range protein markers (Bio-Rad) were used for estimation of protein molecular weight.

Protein fractionation

Thylakoid membrane proteins were solubilised sequentially with different buffers, based on the procedure described by Kleffmann et al. (2004). Thylakoid extracts from mature chloroplasts (0.9 mg) were resuspended in 1 ml of the following buffers: (i) 40 mM Tris–HCl, 5 mM MgCl₂ and 1 mM DTT, pH 8.5; (ii) 8 mM urea, 20 mM Tris, 5 mM MgCl₂ and 20 mM DTT; (iii) 7 mM urea, 2 mM thiourea, 20 mM Tris, 40 mM DTT, 2% CHAPS (w/v) and 1% Triton X-100 (v/v); or (iv) 40 mM Tris, 4% SDS (w/v) and 40 mM DTT. Solubilised proteins were collected by ultracentrifugation at 100,000 g and 4 °C. Each extraction was repeated twice for each specific buffer. Protein samples were stored at -20 °C.

Two-dimensional gel electrophoresis of proteins

Thylakoid extracts from mature maize chloroplasts (180 μg protein) were precipitated according to Costa et al. (1998) in 1 ml of precipitation solution. Proteins were solubilised with 25 μl of the following solubilisation solution: 7 mM urea, 2 mM thiourea, 4% CHAPS (w/v), 65 mM DTT and 0.8% resolutes, pH 3–10 (v/v). Protein samples (100 μg protein) were diluted to 125 μl with solubilisation solution, and isolectric focusing was performed in 7 cm immobile dry strips, pH 3–10 (GE Healthcare) with the following programme: 30 V (12 h); 100 V (150 V/h); 250 V (250 V/h); 1000 V (1500 V/h); 2500 V (2500 V/h), 30 min gradient to 8000 V, followed by constant voltage (8000 V) until 12,000 V/h was attained. An IPGphor instrument (Amersham Pharmacia Biotech, Uppsala, Sweden) was used. Strip equilibration was performed as described by Görg et al. (2000), with second-dimension electrophoresis in acrylamide gels (12%, w/v) at 30 V for 15 min and 100 V until the migration front reached the bottom of the gel.

Blue native and SDS-PAGE

The BN/SDS-PAGE technique provided an excellent alternative to IEF/SDS-PAGE for 2D separation of the most hydrophobic thylakoid proteins. IEF (isolectric focusing) in the first dimension excludes some integral membrane proteins as a result of poor solubility in detergents that must be used. Thylakoid extracts were centrifuged at 10,000 g for 20 min at 4 °C. Membrane pellets were resuspended in 750 mM aminocaproic acid, 50 mM Bis-Tris and 0.5 mM Na-EDTA, pH 7.0, to a final protein concentration of 6.0 mg·ml⁻¹. Triton X-100 stock solution (20%, v/v) was added to the membrane sample to a concentration of 1.3% (v/v). Proteins were solubilised in ice for 30 min, with slow mixing, and centrifuged to eliminate insoluble material. BN sample loading buffer with 5% Coomassie blue G-250 (w/v) and 750 mM aminocaproic acid was added to the protein sample in a ratio of 1:10. Protein complexes were separated in blue native (BN)-PAGE according to Reisinger & Eichacker (2006), containing a low quantity of Comassie blue to maintain the integrity of the complexes. Polyacrylamide gradient gels of 5.0–13.5% were used. To separate the subunits of protein complexes obtained according to their molecular weight, BN-PAGE analysis was followed by SDS-PAGE, according to Reisinger & Eichacker (2006). The first dimension BN-PAGE lane was incubated in 2% SDS (w/v), 66 mM Na₂CO₃, 0.67% β-mercaptoethanol (β-ME, v/v) for 20 min, washed briefly with water and transferred immediately to the second dimension gel (SDS PAGE) and overlaid with agarose (0.5%, w/v) in electrophoresis buffer. Electrophoresis started at 30 V and increased to 100 V for protein separation in separating gel (10% w/v, polyacrylamide). Gels were stained with Coomassie blue colloidal according to Neuhoff et al. (1988). Native protein standards (Invitrogen, Carlsbad, CA, USA) were used for estimation of molecular weight of thylakoid protein complexes.

Mass spectrometry and protein identification

Proteins were isolated from gels and subjected to trypsin digestion as described by (Pandey et al. 2000). Sample
Maize plastidial transglutaminase and thylakoid proteins

Jose, CA, USA) and online eluted on an analytical RP micro LC-MS. Protein digests from BN-PAGE gels were also analysed from the gel and that calculated for the identified protein. The similarity between the protein molecular mass calculated from the gel and that calculated for the identified protein. Protein digests from BN-PAGE gels were also analysed by micro LC-MS/MS (LTQ mass spectrometer, Thermo Finnigan) as described by Santos et al. (2009). Briefly, peptides were concentrated and desalted on an RP pre-column (0.18 x 30 mm, BioBasic18; Thermo Electron, San Jose, CA, USA) and online eluted on an analytical RP column (0.18 x 150 mm, BioBasic18; Thermo Electron) operating at 2 μl·min⁻¹. Peptides were eluted using 33-min gradients from 5 to 60% solvent B (solvent A: 2% formic acid, 5% acetonitrile; solvent B: 0.1% formic acid, 80% acetonitrile). The linear ion trap was operated in data-dependent ZoomScan and MS/MS switching mode using the three most intense precursors detected in a survey scan from 450 to 1600 m/z (three microscans). Singly charged ions were excluded for MS/MS analysis. ZoomScan settings were: maximum injection time, 200 ms; zoom target parameter, 3000 ions; and number of microscans, three. Normalized collision energy was set to 35%, and dynamic exclusion was applied during 10-s periods to avoid fragmenting each ion more than twice in each chromatographic peak. Peptide MS/MS data were evaluated using Bioworks® 3.3.1 software (Thermo Scientific, Waltham, MA, USA). Searches were performed against an indexed UniRef 100 database (downloaded April 2008, 5,888,655 entries, http://www.uniprot.org) and a specific database containing the sequences.

RESULTS

TGase activity in thylakoid membrane extracts

Analysis of TGase activity from etioplast membrane extracts and from thylakoid extracts of differentiating and mature chloroplasts (Fig. 1) showed that, when the enzymatic assays were performed in the light, enzyme activity in light-grown plants was practically double that of in etiolated plants. However, although in developing and mature chloroplasts this TGase activity was significantly higher than that measured in the dark (27-fold higher in light with respect to dark, in mature chloroplasts), there were not significant differences in the case of etioplasts.

Maize TGase immunodetection in membrane extracts

To evaluate the membrane extract fraction containing maize chlTGase, a first expression analysis with mature chloroplasts using the specific PAB/18QA polyclonal antibody was conducted (Fig. 2A). A 58-kDa protein band was detected in the soluble fraction of thylakoid membrane extracts only after protein concentration (Fig. 2A, lane 4). However, in the insoluble fraction solubilised with Triton X-100 and buffers (Fig. 2A, lane 3), a 58-kDa protein band was intensely detected, indicating that the 58 kDa TGase is preferentially associated with the membrane fraction of thylakoid extracts. This treatment was shown to be sufficient to solubilise the protein, since almost no protein was detected in the remaining membrane pellet after solubilisation with SDS buffer (Fig. 2A, lane 5). In addition, another 34 kDa TGase form was detected in the two fractions and in all solubilisation conditions tested (Fig. 2A, lanes 3–5). Analysis of all samples with pre-immune serum did not reveal any protein band (Fig. 2A lane 6, Fig. 2B lane 4), confirming the specificity of the designed antibody to recognise maize chlTGase.

From these results, TGase expression in the thylakoid extract insoluble fraction of the three differentiating chloroplast fractions solubilised with Triton X-100 was subsequently analysed. The 58 kDa TGase form was scarcely detected by Ab PAB/18QA in membrane extracts from etioplasts (Fig. 2B, lane 1) but was markedly present in extracts in differentiating and mature chloroplasts (Fig. 2B, lanes 2 and 3). Densitometric band quantification demonstrated a 4.5-fold increase in the 58 kDa TGase form in differentiating chloroplasts and a 15-fold increase of this form in mature chloroplasts, in relation to etioplasts. The 34-kDa band was present in all stages of differentiating chloroplast at similar levels of expression. Furthermore, instead of the 58-kDa band, an intense
A band of 48 kDa was detected in etioplast membrane extracts (Fig. 2Ba, lane 1). The 29- and 21-kDa bands detected only in etioplast samples (Fig. 2Ba, lane 1) suggest the presence of a proteolytic process. It is also relevant that the highest levels of expression of the 58-kDa form in thylakoid membrane extracts of light-grown plants coincides with the presence of lhcb1, 2 and 3 proteins (Fig. 2Bb, lanes 2 and 3).

Characterisation of the 58 kDa TGase form by two-dimensional gel electrophoresis

Figure 3A shows the 2D gel electrophoresis (2-DE) separation of solubilised proteins from thylakoid extracts of mature chloroplasts. Western blot with Ab PAB18QA allowed detection of two bands with estimated MW between 64 kDa and 70 kDa, and pI between 7 and 8 (Fig. 3B). This discrimination of the two TGase forms is, most likely, due to the increase in protein separation capacity provided by 2DE in comparison to the SDS-PAGE approach, in which only one form of 58 kDa could be detected (Fig. 2).

Characterisation of the 58 kDa TGase form by sequential fractionation

To identify the membrane fraction in which chITGase is present, the maize thylakoid membrane proteome from mature chloroplasts was fractionated using four different buffers of increasing protein solubilisation capacity. The obtained protein fractions were further analysed by SDS-PAGE and Western blot (Fig. 4). Each fraction obtained presented a distinct protein profile in SDS-PAGE, indicating that this approach is suitable as a first fractionation step for the maize membrane proteome (Fig. 4A). The 58 kDa TGase form was detected with Ab PAB18QA in the protein fraction solubilised with 8M urea and 20 mM DTT (buffer 2) (Fig. 4B, lane 3). According to the procedures used, this fraction contains peripheral proteins, indicating that the TGase has similar physical–chemical properties. Confirming these data by peptide mass fingerprinting using MALDI-TOF mass spectrometry, we were able to identify three subunits of the ATPase complex and other soluble and membrane-associated proteins (Table S1). Integral membrane proteins, such as lhcb2, were only solubilised with buffers containing the detergents CHAPS, Triton X-100 or SDS (buffers 3 and 4) (Fig. 4A–C, lanes 4 and 5).

Maize TGase presence in thylakoid membrane protein complexes

Thylakoid membrane protein complexes from mature maize chloroplasts were solubilised with Triton X-100 (1%, v/v) and separated using BN-PAGE. Protein complexes from 100 kDa up to 1000 kDa were efficiently separated with this technique (Fig. 5, lane 1). Immunode-
tection of lhcb and TGase proteins revealed a complex between 380 and 480 kDa, containing LHCII proteins (Fig. 5, lane 2) and two TGase bands at approximately 410 and 460 kDa (Fig. 5, lane 3). Furthermore, characterisation by LC-MS/MS of the different protein complexes separated in BN-PAGE located different PSII proteins in the same molecular weight range in which TGase was detected (Table 1, Table S2).

To obtain complementary information on the composition of the BN-PAGE protein complexes obtained, these complexes were separated in a second dimension using SDS-PAGE followed by immunoblot detection (Fig. 6A–D). The 58 kDa and 34 kDa TGase forms detected with this approach (Fig. 6C) resolved with several subunits of PSII and ATPase complexes (Fig. 6B) identified by MALDI-TOF and LC-MS/MS analysis, which confirmed the results obtained from BN-PAGE analysis. As in the case of the Triton X-100 solubilised membrane extracts (Fig. 2), the 34 kDa TGase form detected by immunoblotting (Fig. 6C) belongs to the same complex in which LHCII proteins were identified (Fig. 6D; spots 1, 2 and 3). Spot 1 was assigned to LHCII monomer, spot 2 is part of the LHCII trimer complex and spot 3 corresponds to the PSII-LHCII complex (bands 5–8, Table 1, Table S2).

### DISCUSSION

TGase activity and molecular forms detected in differentiating maize chloroplasts

Although TGase activity of mature chloroplasts have been detected in other species, and its dependence on light exposure has been reported (Del Duca et al. 1994; Bernet et al. 1999; Dondini et al. 2003; Villalobos et al. 2004), detection of higher TGase activity in membrane extracts of developing chloroplasts with respect to that of etioplasts demonstrated the relevance of the light-dependent TGase active forms in these plastid developmental phases (Fig. 1). These results also confirmed the characteristics demonstrated for the previously cloned maize chlTGase (Torné et al. 2002; Villalobos et al. 2004). The different TGase forms detected in this work, depending on plastid developmental stage, are directly related to the differences in enzyme activity observed. The main 58-kDa form present in light-grown plastids (Fig. 2Ba), which is almost absent in etioplasts, may be associated with the high
TGase activity observed in those samples. Furthermore, the low enzymatic activity present in etioplasts with respect to the light-differentiating chloroplast stages probably indicates that etioplast TGase forms have only basal activity, as expected by its growth condition. It is also known that etioplasts of higher plants (especially *Zea mays*) contain almost 10-times higher endogenous Put levels than mature chloroplasts (Andreakis & Kotzabasis).

**Fig. 5.** Lane 1, Thylakoid membrane protein complexes from mature maize chloroplasts separated by Coomassie-stained BN-PAGE. Lane 2, Immunodetection of lhcb2 protein complexes. Lane 3, Immunodetection of maize TGase in the corresponding lhcb complex. Thirty micrograms of protein were loaded in each lane. Maize TGase was detected with Ab PAB/18QA. Lhcb2 proteins were detected with commercial Agrisera® antibodies. Arrows indicate TGase protein.

**Table 1.** Characterisation of thylakoid protein complexes separated by BN-PAGE and identified by LC-MS/MS. The position of each protein band in BN-PAGE gels is reported in Fig. 6A. Results of protein identification by LC-MS/MS are given in Table S2.

<table>
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<tr>
<th>protein band</th>
<th>mass range (kDa)</th>
<th>genes for the identified proteins</th>
<th>thylakoid complexes</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>850–1000</td>
<td>psaF; psaA; Lhca3</td>
<td>PSI-LHC1</td>
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</tr>
<tr>
<td>2</td>
<td>720–850</td>
<td>psaF; psaA; Lhca3; psbB; pSbS</td>
<td>PSI-LHC1</td>
<td></td>
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<tr>
<td>3</td>
<td>550–600</td>
<td>psaA; psbB; psaD; psaF; psaH; Lhca1-3; psbB atpA, atpB</td>
<td>PSI-LHC1</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>350–480</td>
<td>psbB; psbD atpA; atpB psaA; psaF petA; petB</td>
<td>PSI-LHC1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>270–350</td>
<td>psbA; psbB; psbC, psbD atpA; atpB atpC petA; petB</td>
<td>PSI-LHC1</td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td>Lhcb1-4</td>
<td>PSI-LHC1</td>
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<td>8</td>
<td>100–150</td>
<td>Lhcb1; Lhcb3-6</td>
<td>LHCII</td>
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</tbody>
</table>

*Thylakoid complexes predicted on the basis of subunits identified and determined molecular mass.

**Table 1.** Characterisation of thylakoid protein complexes separated by BN-PAGE and identified by LC-MS/MS. The position of each protein band in BN-PAGE gels is reported in Fig. 6A. Results of protein identification by LC-MS/MS are given in Table S2.

**Fig. 6.** Separation by BN-PAGE/SDS-PAGE of thylakoid membrane protein complexes from mature maize chloroplasts. A: First dimension BN-PAGE separation of thylakoid protein complexes; labelled numbers indicate the protein complexes described in Table 1 and Table S2. B: SDS-PAGE second dimension separation of protein complexes; most relevant identified proteins are labelled. C: immunodetection of maize TGase with Ab PAB/18QA. Arrows indicate TGase forms. D: lhcb2 immunodetection using commercial Agrisera® antibodies. Spot 1, LHCII monomers, spot 2, LHCII trimer complex; spot 3, PSI-LHCII complex.

TGase activity observed in those samples. Furthermore, the low enzymatic activity present in etioplasts with respect to the light-differentiating chloroplast stages probably indicates that etioplast TGase forms have only basal activity, as expected by its growth condition. It is also known that etioplasts of higher plants (especially *Zea mays*) contain almost 10-times higher endogenous Put levels than mature chloroplasts (Andreakis & Kotzabasis).
1996), consequently, it is possible that during the activity assay the free active sites on TGase etioplast membranes could be limited. TGase activity in etioplasts has been reported recently in cucumber cotyledons, but only during the 24 h etioplast–chloroplast differentiation process (Sobieszczuk-Nowicka et al. 2008). Likewise, it is noteworthy that TGase activity in the chloroplast and its fractions is generally considered as the covalent incorporation of polyamines, mainly to LHCII proteins (Della Mea et al. 2004), which are not present in the etioplast membrane extract (Fig. 2Bb).

Co-immunodetection of chlTGase with LHCII proteins during chloroplast development

The use of a specific antibody against maize chlTGase and the proteomic analyses performed, provide a clear indication that the highly abundant 58-kDa form immunoblotted in light-grown chloroplast membrane extracts corresponds to the TGase previously TEM-immunolocalised in the grana of maize chloroplasts by Santos et al. (2007). In the cited work, the pattern of TGase localisation was that of LHCII proteins. In the present work, the same immunoblot expression pattern for TGase and lhcb 1, 2 and 3 proteins was obtained, indicating that the 58 kDa TGase might be the form that utilises these proteins as substrates in light-grown chloroplasts. The present results reinforce once more the thesis that this enzyme, in particular the 58-kDa form, is required during chloroplast development. Accumulation of the 58 kDa TGase form and LHCII proteins in membrane chloroplasts is in agreement with the role hypothesised for chlTGase – as catalyst of polyamine incorporation into LHCII proteins. It is known that polyaminylation of proteins results in a significant change in charge of the target protein, and it is well established that negative charges of LHCII proteins must be neutralised by cations to allow adjacent membranes to stack and grana to form (Standfuss et al. 2005). This kind of charge neutralisation is feasible with monovalent or divalent inorganic cations (Barber 1982) or with organic cations such as polyamines (Ioannidis & Kotzabasis 2007). Chloroplast TGase might play a role in thylakoid stacking and/or assembly of photosynthetic components with respect to light energy assimilation by polyaminylation of LHCII proteins under certain conditions. The increase in grana size and thylakoid-bound polyamines obtained in tobacco transplastomic plants over-expressing the maize chlTGase gene are in agreement with the implication of this enzyme in the described phenomenon (Ioannidis et al. 2009).

The maize chlTGases detected and products of the cloned tgz genes

It is noteworthy that the presented results enable us to confirm that the chlTGases detected here have the same characteristics (pI, molecular weight, expression pattern) as the deduced products of the maize TGase cDNAs cloned by Villalobos et al. (2004) and over-expressed in E. coli by Carvajal-Vallejos et al. (2007). In plants, different TGase forms have been immunodetected in maize leaves (Villalobos et al. 2001, 2004; Della Mea et al. 2004), Helianthus tuberosus leaves (Dondini et al. 2003) and in cucumber cotyledons (Sobieszczuk-Nowicka et al. 2008), but only Villalobos et al. used a plant-specific antibody. The use of an antibody designed against a specific TGase maize sequence in the present work consolidates the obtained results. The difference of 3–4 kDa in the molecular weight estimated for the two proteins in comparison with that predicted for the products of the TGase genes is likely attributed to gel distortion, particularly between pH 6 and 10 (Fig. 3A). It is clear that the 2DE technique increased the separation capacity of proteins, allowing discrimination of two TGase forms, which was not possible with 1D electrophoresis (Fig. 2). With respect to the detected 34-kDa form (Fig. 2), it seems that it presents different solubilisation properties that determined its absence in the 2DE analysis with respect to the 64- and 7-kDa forms. This 34-kDa form may be a post-translational modification of the native TGase protein, related to the activity process, corroborated in part by its presence in the BN/SDS-PAGE gels, and associated with some LHCII protein forms.

The chlTGase is a membrane-associated protein related to a specific thylakoid protein complex of PSII

The maize thylakoid membrane fractionation presented here allowed the first verification that chlTGase behaves as a peripheral membrane protein, interacting on the thylakoid membrane. These results agree with the pI characteristics of the tgz-cloned gene deduced product, as well as with the deduced chloroplast signal peptide, both indicating the stromal orientation of the enzyme on the thylakoid membrane (Villalobos et al. 2004). This membrane interaction was further complemented by results obtained using the BN-PAGE method followed by TGase immunodetection (Figs 5 and 6), leading to detection of two chlTGase bands included in certain thylakoid protein complexes whose compounds were identified by mass spectrometry (Table 1, Table S2). The identified PSII and LHCII-PSII protein complexes were previously reported to be resolved in this range of molecular masses (Ciambella et al. 2005; Granvogl et al. 2006), and chlTGase has now been detected as forming part of these native protein complexes, suggesting an interaction between them. In spite of the association of TGase with the photosynthetic apparatus in higher plants outlined above, experimental data demonstrating the intermolecular interaction of TGase with the photosynthesis machinery of plants is lacking. The approach presented clearly shows that two forms of chlTGase are associated with the LHCII complexes in mature maize chloroplasts. Other putative interactions with PSII and ATPase complexes are also likely to occur. A recent study published by our laboratory (Pinto-Marijuan et al.
adaptation to light stimuli, thylakoid appression and the important role of this enzyme in processes related to the forms and some PSII complex proteins, suggesting an action of both PsbS and LHCII proteins in the PSII light harvesting system in a pH-dependent manner. Further biochemical and proteomic studies of chlTGase-transformed plants will allow us to deepen knowledge on the physiological role of TGase in plants.

CONCLUSION

From the present work, it can be confirmed that the previously cloned maize chlTGase is a constitutive plastid membrane-associated protein; its expression pattern changing according to chloroplast developmental stage and light regime under which the plants are grown. Furthermore, the results provide substantial information at the proteomic level on the relationship between maize chlTGase forms and some PSII complex proteins, suggesting an important role of this enzyme in processes related to the adaptation to light stimuli, thylakoid appression and/or photoprotection. Further biochemical and proteomic studies of chlTGase-transformed plants will allow us to deepen knowledge on the physiological role of TGase in plants.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Protein identification by peptide mass fingerprinting from protein fractionation bands (Fig. 4, lane 3). Accession numbers are from the MSDB database.

Table S2. Protein identification by LC-MS/MS, with respect to results of BN-PAGE separation presented in Table 1 and Figs 5 and 6. Only the results of bands 5 to 8 are presented. Database: uniref100.fasta, 5888655 entries.

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REFERENCES


