

Determination of Transglutaminase Activity in Plants

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Abstract

Transglutaminase (TGase; E.C. 2.3.2.13) catalyzes the acyl-transfer reaction between one or two primary amino groups of polyamines and protein-bound Gln residues giving rise to post-translational modifications. One increasing the positive charge on a proteins surface and the other results in the covalent crosslinking of proteins. Pioneering studies on TGase in plants started in the middle of the 1980's but the methodology designed for use with animal extracts was not directly applicable to plant extracts. Here we describe radioactive and colorimetric methods adapted to study plant TGase, as well as protocols to analyze the involvement of TGase and polyamines in the functionality of cytoskeletal proteins.

Key words Transglutaminase assay, Polyamine–protein interaction, Glutamyl-polyamines, Actin filament binding assay, Microtubule binding/motility assay

1 Introduction

Aliphatic polyamines (PAs) have been the subject of numerous investigations over many years. The historical focus of the work on PAs has been predominantly on soluble PA's in both plant and animal cells, whereas the conjoined forms of PAs has received less attention. However, due to their chemical characteristics, PAs can be covalently or non-covalently linked to several molecules (Fig. 1). Towards the end of the 1950's PAs linked by hydrogen bonds to nucleic acids attracted the attention of pioneering researchers. In the same period, other researchers found the activity of an enzyme (Transglutaminase; TGase) that was able to covalently conjugate PAs to animal proteins. In the 1980's Jack Folk and collaborators [1] published a milestone paper containing a method to quantify this enzyme-catalysed polyamine conjugation to proteins, which was later on modified by Beninati and collaborators [2]. Using this methodology, the enzyme-catalysed polyamine incorporation into proteins was also studied in plant extracts, to detect a Ca^{2+} -dependent TGase activity by its unequivocal products (the conjugated PA residues, i.e., (γ -glutamyl) –PAs, Fig. 2) in

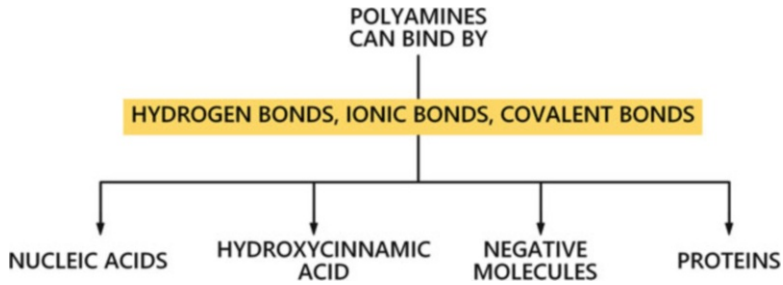


Fig. 1 PAs can be linked to several molecules with different linkages

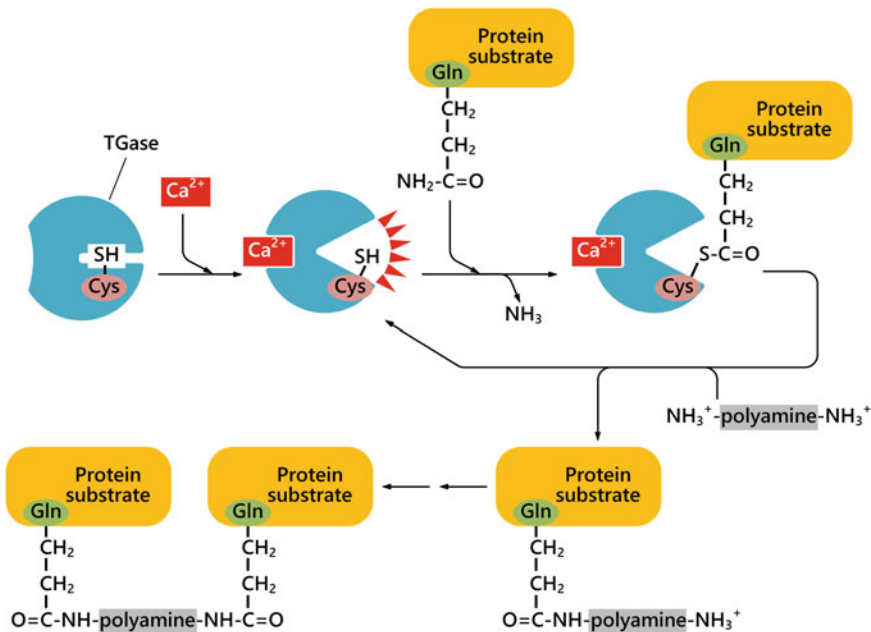


Fig. 2 The two steps of transamidase reaction of TGase. In the presence of Ca^{2+} the enzyme could change its conformation and cysteine in the active site became ready to catalyze the reaction that involves the formation of a thioester acylenzyme intermediate with the release of ammonia. Then, a molecule with a primary amine group, like a polyamine or lysine, performs a nucleophilic attack to the endo-Gln residue of the protein substrate, causing the covalent binding of polyamine to protein. Moreover, diamines and polyamines might act as a bridge in a bis-glutaminy insert between two acceptor molecules, with the length of the bridge depending on the length of polyamine involved in the reaction

several plants in different physiological conditions. This method also reveals the metabolism of polyamines in the cell. Some problems emerged which indicated that the radioactive polyamine incorporation assay designed for animal cell extracts was not directly applicable to all plant cells extracts. The major problem was the presence of diamine oxidase activity in some plant extracts capable of incorporating radioactive putrescine into substrate protein in a calcium-independent fashion [3]. Consequently, this led to

difficulties in the purification and sequencing of the plant polyamine incorporating enzyme due to the length and complexity of the method reported above to identify the enzyme activity.

Alternative assays were designed to overcome these difficulties. The use of ethyldimethylaminopropyl carbodiimide (EDC)-modified casein [4] as a substrate to measure plant TGase calcium-dependent polyamine incorporation initially showed promise but was superseded by the use of biotinylated cadaverine and *N,N*-dimethyl casein as substrates in the microtiter plate TGase assay developed by Slaughter and collaborators [5]; selective sample preparation resulted in the demonstration of calcium-dependent transglutaminase enzyme in plant tissue. Confirmation of plant TGase activity was obtained by the identification of the products of TGase protein transamidase and crosslinking activities (γ -glutamyl PA residues and ϵ -(γ glutamyl) lysine isodipeptide respectively) after digestion of plant protein extracts with a variety of proteolytic enzymes prior to cation exchange chromatography [4, 6].

Despite difficulties it became clear that a plant TGase enzyme was able to conjugate PAs into proteins (and form protein cross-links), but the enzyme had a low cellular activity in most plant cells. However, exceptions to this general rule emerged with higher activities detected in apical meristematic cells, in vitro stimulated dormant or immature tissues, germinating pollen, salt-stressed unicellular algae and in the dark/light transition of photosynthetic cells/chloroplasts, i.e., during a change of developmental fate or activity. A relatively high activity was detected in chloroplasts in the presence of light [7]. Additional work based on the cross reactivity of antibodies directed to animal TGases (e.g., tissue TGase or TG2) indicated that there are more than one plant TGase enzymes which are present in different plant cell compartments [8, 9].

In animals and plants, TGase activity catalyses a two-step conjugation of PAs (or ϵ -lysine) to glutamyl derivatives of specific proteins (Fig. 2). This is referred to as transamidation (polyamine incorporation or protein crosslinking) where in the second step of the catalytic pathway an amine acts as the nucleophile. In the absence of an amine, a water molecule can act as the nucleophile resulting in the deamidation of protein-bound glutamine. Transglutaminase PA incorporating activity and protein cross linking activity has been demonstrated in animal and plant extracts but a user friendly assay to measure TGase deamidating activity has yet to be published. The seed storage proteins of dicotyledonous plants are known to undergo deamidation prior to proteolysis. A TGase deamidation assay based on this observation is currently in preparation. There are other enzymic activities associated with TGase which have been studied in animal cells [10]; at present, they have not been studied in plants cells but the opportunity does exist for a determined researcher.

2 Materials

Prepare all solutions using ultrapure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$ at 25°C) and analytical-grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

Plant materials concern: *Helianthus tuberosus* sprout apices, dormant tubers, isolated chloroplasts, rosaceae (*Malus*, *Pyrus*), *Citrus* and *Corylus* pollens, *Arabidopsis thaliana* tissues, *Nicotiana tabacum* flowers and leaves, *Dunaliella salina* unicellular green algae, *Zea mays* calluses, leaves and isolated chloroplasts, *Oryza sativa* transgenic plants, *Cucumis sativa* isolated plastids, *Hordeum vulgare* plastids, *Beta vulgaris*, *Lactuca sativa* leaves [11–13].

2.1 TGase Assay for the Incorporation of Radioactive Polyamines

1. 1.0 M Tris/HCl pH 8.2: 121.1 g of Tris in 900 ml of H_2O , adjust the pH to 8.2 with HCl and made to a final volume of 1.0 l.
2. 100 mM CaCl_2 : 0.11 g CaCl_2 in 10.0 ml H_2O
3. 100 mM ethylene glycol-bis(2-aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA): 3.80 g EGTA in 100 ml H_2O .
4. 100 mM Dithiothreitol (DTT): 0.154 g in 10 ml H_2O .
5. 10 mM putrescine or spermidine: 0.016 g Put or 0.025 g Spd in 10 ml H_2O .
6. $[1,4 \text{ (n)}\text{-}^3\text{H}]\text{Put}$ (35.7 Ci/mmol, 1 mCi/ml) or $[1,4 \text{ (n)}\text{-}^3\text{H}]\text{Spd}$ (34.8 Ci/mmol, 1 mCi/ml); 5 μl (5 μCi) of radioactive PA are used in the assay (see **Note 1**).
7. Protein substrate (N,N' -dimethylcasein (DMC), (1 mg/ml) (see **Note 2**)
8. Trichloroacetic acid (TCA) 20% (w/v) containing 2 mM cold PA (Put or Spd).

2.2 Solutions for Separation of Glutamyl-Polyamines by Reversed Phase HPLC

1. 0.1 N NaOH.
2. 0.8 M morpholine acetate buffer pH 8.1.
3. Enzyme for the digestion of TGase polyamine adducts: pronase type XXI from *Streptomyces griseus*.
4. The buffers composition which are necessary to separate glutamyl-PAs by HPLC are reported in Table 1.
5. Column: Anion exchanger: DIONEX DC- 6A, 4.5 mm $\times$ 80 mm, Resin Ultropac 8 Na^+ .

2.3 TGase Assay for the Incorporation of Biotin-Cadaverine

1. 100 mM Tris-HCl pH 8.5: 12.11 g of Tris in 900 ml of H_2O . Adjust the pH to 8.5 with HCl and made up to a final volume of 1 l.
2. 150 mM phosphate buffered saline (PBS)-Tween 80: 137 mM sodium chloride, 2.6 mM potassium chloride, 8.1 mM

Table 1
Buffers necessary to separate glutamyl-PAs by HPLC

Buffer	Conc (N)	pH	Na + -citrate	NaCl	Brij	phenol	Final vol
A	0.6	5.80	1.96 g	33.9 g	1 ml	1 ml	1 l
B	1.5	5.51	3.90	85.3 g	“	“	“
C	3.0	5.55	15.70	166 g	“	“	“
D	5.0	5.50	26.0	280 g	“	“	“
E	0.2	3.31	19.60	–	“	“	“
F	0.2	4.31	19.60	–	“	“	“

disodium hydrogen orthophosphate (Na_2HPO_4), 1.47 mM potassium dihydrogen orthophosphate (KH_2PO_4) and Tween 80, 0.005% (w/v). For 1.0 litre: 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.15 g of disodium hydrogen orthophosphate, 0.2 g of potassium dihydrogen orthophosphate and 500 μl of Tween 80 dissolved in 900 ml of deionized H_2O . Adjust the pH to 7.4 and make to a final volume of 1.0 l with deionized H_2O .

3. 100 mM sodium acetate pH 6.0: 8.2 g of sodium acetate in 900 ml of water. Adjust the pH value to 6.0 with acetic acid and make the volume to 1 l.
4. Assay buffer containing calcium (CaCl_2) positive control, 100 mM Tris-HCl pH 8.5, 6.67 mM calcium chloride, 13.3 mM DTT, 225 μM of biotin-cadaverine. Dissolve 0.30 g Tris, 0.025 g calcium chloride, 0.051 g DTT and 2.5 μg of biotin-cadaverine in 20 ml of deionized water. Adjust the pH to 8.5 with HCl and the final volume to 25 ml with water. The DTT should be added fresh prior to the assay.
5. Assay buffer containing EGTA, negative control, 100 mM Tris-HCl pH 8.5: 20 mM EGTA, 13.3 mM DTT and 225 μM of biotin-cadaverine. Dissolve 0.30 g Tris, 0.05 g EGTA, 0.051 g DTT and 2.5 μg biotin-cadaverine in 20 ml of deionized water. Adjust the pH to 8.5 with HCl and make the final volume to 25 ml with deionized H_2O . The DTT should be added fresh prior to the assay.
6. 10 mg/ml Tetramethyl benzidine (TMB): 10.0 mg TMB diluted in 1.0 ml of dimethyl sulfoxide (DMSO).
7. 3% (w/v) hydrogen peroxide: 10 μl 30% (v/v) hydrogen peroxide in 90 μl ultrapure water.
8. Development solution: 150 μl of 10 mg/ml TMB and 25 μl of 3% (v/v) hydrogen peroxide are added to 20 ml of 100 mM sodium acetate pH 6.0.

9. DMC protein substrate: 50.0 g of DMC dissolved in 500 ml of water overnight (take care this reagent is odourous) to produce a 10× stock. The DMC can be stored in aliquots at -20°C .

2.4 TGase Assay for the Incorporation of Biotin TVQQEL Peptide into Casein (Simulating Protein Crosslinking)

The reagents for this assay are similar to the reagents above described for the biotin-cadaverine assay (Subheading 2.3). The assay buffer contains 5–20 μM biotinylated TVQQEL peptide from a 10 mg ml^{-1} stock dissolved in DMSO instead of biotin-cadaverine. DMC is replaced with bovine casein.

2.5 In vitro TGase Assay During Pollen Germination

1. Composition of pollen germination medium: 68.46 mg/ml sucrose, 300 $\mu\text{g/ml}$ calcium nitrate, 20 $\mu\text{g/ml}$ boric acid in ultrapure water.
2. 1 mg/ml Biotin-cadaverine stock solution.

2.6 Polyamine-Microtubule Assay

1. TGase reactivation buffer: 20 mM Tris-HCl pH 8.0 containing 1.5 mM DTT.
2. Tubulin dilution buffer (TDB): 80 mM HEPES pH 7.5 containing 1 mM EGTA, 1 mM MgCl_2 , 2.0 mM GTP (add immediately prior to use), 20% (v/v) glycerol.
3. Cushion buffer (CB): 80 mM HEPES pH 7.5 containing 1 mM EGTA, 1 mM MgCl_2 , 20% (w/v) sucrose.
4. Microtubule dilution buffer (MDB): 80 mM HEPES pH 7.5 containing 1.0 mM EGTA, 1 mM MgCl_2 , 1 mM GTP (add immediately prior to use).
5. PEM buffer: 80 mM HEPES pH 7.5 containing 1.0 mM EGTA, 1.0 mM MgCl_2 .
6. Motility buffer: 80 mM HEPES pH 7.5 containing 7.0 mM EGTA, 1 mM MgCl_2 , 1 mM DTT, 20 μM taxol, 5 mM ATP (add immediately prior to use).
7. Perfusion chamber: made by a glass-slide and a coverslip separated by two strips of double-sided tape. Both glass-slide and coverslip must be repeatedly washed to clean their surface, they then can be stored for weeks in a beaker containing 20% (v/v) ethanol. At time of use, glass-slide and coverslips are grasped with forceps and the ethanol is removed with the flame of a Bunsen burner. After cooling, glass-slides and coverslips are assembled to form the perfusion chamber in the way indicated in Fig. 3. The volume inside the chamber should be approximately 10–15 μl .
8. PEM buffer + taxol: 80 mM HEPES pH 7.5, containing 1 mM EGTA, 1 mM MgCl_2 , 20 μM taxol.
9. Blocking buffer: PEM buffer containing 1 mg ml^{-1} casein.

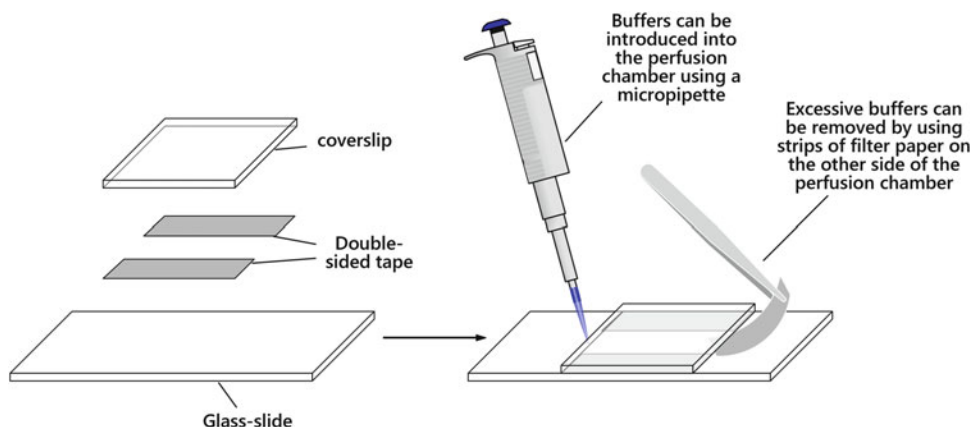


Fig. 3 Schematic representation of the assembly of a perfusion chamber using a glass-slide, a coverslip and two pieces of double-sided tape. The right part of the figure shows how to influx specific volumes of solution using a micropipette while removing previous content with filter paper

2.7 Polyamine-Actin Assay

1. TGase reactivation buffer: 20 mM Tris-HCl pH 8 containing 1.5 mM DTT
2. Buffer A (plus ATP): 5 mM Tris-HCl pH 8 containing 0.2 mM CaCl_2 , 0.2 mM ATP (add before use).
3. 10 $\times$ polymerization buffer (plus ATP): 0.5 M KCl pH 8.0, 20 mM MgCl_2 , 10 mM ATP (add immediately before use).
4. Rhodamine-phalloidin solution: 10 μl 10 $\times$ Polymerization Buffer, 25 μl of rhodamine-phalloidin stock (6.6 μM) and 65 μl of water.
5. ATPase activity assay buffer: 20 mM Pipes pH 7.0 containing 10 mM KCl, 1 mM EGTA, 2 mM MgCl_2 , 5 mM ATP (add immediately before use).

2.8 Proteolytic Digestion of Plant Proteins for the Analysis of ϵ -(γ -Glutamyl) Lysine Isodipeptide Derived from Protein Crosslinks Following TGase Activity

1. 72% (w/v) TCA: 72 g of solid TCA dissolved in 100 ml distilled water.
2. 10% (w/v) TCA: 10 g of solid TCA dissolved in 100 ml distilled water.
3. 50:50 (ethanol:diethylether): 25.0 ml diethylether added to 25.0 ml ethanol. Store on ice.
4. 100 mM ammonium carbonate pH 10.0: 1.57 g ammonium carbonate dissolved in 90.0 ml of distilled water. Adjust the pH to 10.0 using concentrated sodium hydroxide. Make up to 100.0 ml with distilled water.
5. Leucine amino peptidase activation: 250 μl leucine amino peptidase (250 units), 225 μl 10 mM Tris-HCl pH 8.0, 25 μl 50 mM manganese chloride. The solution is incubated at 37°C for 2 h to activate the leucine amino peptidase.

6. Prolidase activating solution: 50 μ l prolidase (100 units), 200 μ l distilled water, 200 μ l 10 mM Tris-HCl pH 8.0, 50 μ l manganese chloride. The solution is incubated at 37°C for 2 h to activate the prolidase.
7. 0.54 M magnesium chloride: 1.09 g magnesium chloride (hexahydrate) dissolved in 10.0 ml of distilled water.
8. Chloroform:methanol:HCl (2000:1000:2): 100.0 ml chloroform, 50.0 ml methanol, 1.0 ml of concentrated HCl.

3 Methods

Plant tissue contains a variety of phenol and amine oxidase, that could interfere with the TGase assay. For some plant extract the most vulnerable assay is the radioactive polyamine incorporation assay [2, 3]; if problems are suspected and only the activity should be measured switch to either the biotin-cadaverine incorporation assay [4] or the TVQQEL peptide crosslinking assay (simulating protein crosslinking) [14].

In all plant tissue extractions for TGase activity it is wise to plan ahead and ensure that all the reagents have been precooled. Additions, such as proteinase inhibitors, DIECA, polyvinylpolypyrrolidone, 2-mercaptoethanol and ascorbic acid are advisable. After extraction, the pH should be adjusted to between pH 8.0–9.0 before clarification at $12,000 \times g$ for 30 min at 4 °C. The supernatant should then be subjected to protein precipitation using solid ammonium sulphate (0.76 g/ml). This will concentrate the protein and remove the low molecular weight contaminants. The protein ammonium sulphate mixture should be stirred and the protein precipitate collected by centrifugation $12,000 \times g$ for 30 min at 4 °C. The pellet can be dissolved in a minimum volume of 0.1 M Tris buffer pH 8.5 before being dialysed or desalted using size exclusion chromatography to remove excess ammonium ions. It is recommended that the extract is then put through at least one additional chromatography step such as anion exchange chromatography to separate the plant TGase from contaminating oxidase activity before being aliquoted and stored at the lowest temperature available [15].

3.1 Radioactive Assay of Transglutaminase Activity

This method (Fig. 3) can be performed (a) by supplying labelled PAs to either total plant cell extracts or isolated organelles or membranes in the test tube, or to living cells and organs (i.e., germinating pollen, unicellular algae, leaves, etc) or to in vitro cultures (preferably grown in liquid medium). (b) Alternatively, purified protein substrates, like DMC, fibronectin (FN), actin, tubulin, light harvesting complex II (LHCII) etc., or specific substrates of gplTGase (commercial Guinea pig liver TGase) such as N-benzyloxycarbonyl-L- γ -glutamyl-L-leucine, or the histidine-

tagged green fluorescent protein (His₆-X-press-GFP) a fluorescent protein derivatized with GFP, can be assayed for the plant enzymes [16]. On the other hand, if a plant substrate must be evaluated, TGases purified from either plant or animal cells can be assayed. An example of the method applied to pollen extract or entire germinating pollen is described here; some advertise for other plant samples are reported.

Reaction for transglutaminase assay occurs in 1.5 ml tubes. In details, the incubation mixture contains:

1. 15 μ l solution 1.0 M Tris/HCl pH 8.2 + 3 μ l solution 100 mM CaCl₂ (or 100 mM EGTA in case of negative control, *see Note 3*) + 30 μ l solution 100 mM DTT + 6 μ l solution 10 mM Put or Spd + 5 μ l solution [1,4 (n)-³H]Put (35.7 Ci/mmol, 1 mCi/ml) or [1,4 (n)-³H]Spd (34.8 Ci/mmol, 1 mCi/ml) + 50 μ l solution protein substrates (1 mg ml⁻¹) i.e., DMC or others (*see Note 2*) + ultrapure water up to 200 μ l + 100 μ l soln plant extract (0.5 mg ml⁻¹)
2. Incubate samples for 2 h at 30 °C.
3. The reaction is stopped with 100 μ l solution TCA 20% (w/v) containing cold PA (Put or Spd) 2 mM (5% (w/v) is the final concentration of TCA in order to remove trapped free PAs).
4. The mixture is stored at 4 °C for 24 h (or 2 h at -20 °C) and then centrifuged at 13,000 $\times g$ for 10 min at 4 °C.
5. The pellets were solubilized overnight with 0.1 M NaOH at 37 °C and then re-precipitated twice with 5% (w/v) TCA. The supernatants and final pellet, washed with anhydrous diethyl ether, were proteolytically digested as described [1, 2] with some modifications.

3.1.1 Analysis of the Products of the Enzymatic Assay

The TCA pellet obtained after the enzymatic assay can be used for different treatments, as schematically shown in Fig. 4. Direct measure of the radioactivity due to covalently conjugated polyamines: after last precipitation, the TCA pellet, re-suspended in 0.1 N NaOH, is prepared to detect the total radioactivity by a liquid scintillator counter. This simple analysis presents the possibility to erroneously detect PAs bound by a non-enzymatic activity, as it occurs when some “sticky” molecules might be present; PAs can be “glued” to these kind of molecules, severely affecting the value of results. This problem can occur especially with chlorophylls of green tissues or when phenols are present; moreover PAs can be oxidized or otherwise metabolized, thus it is important to detect glutamyl-PAs, as reported in Subheading 3.1.3. (*see Note 4*).

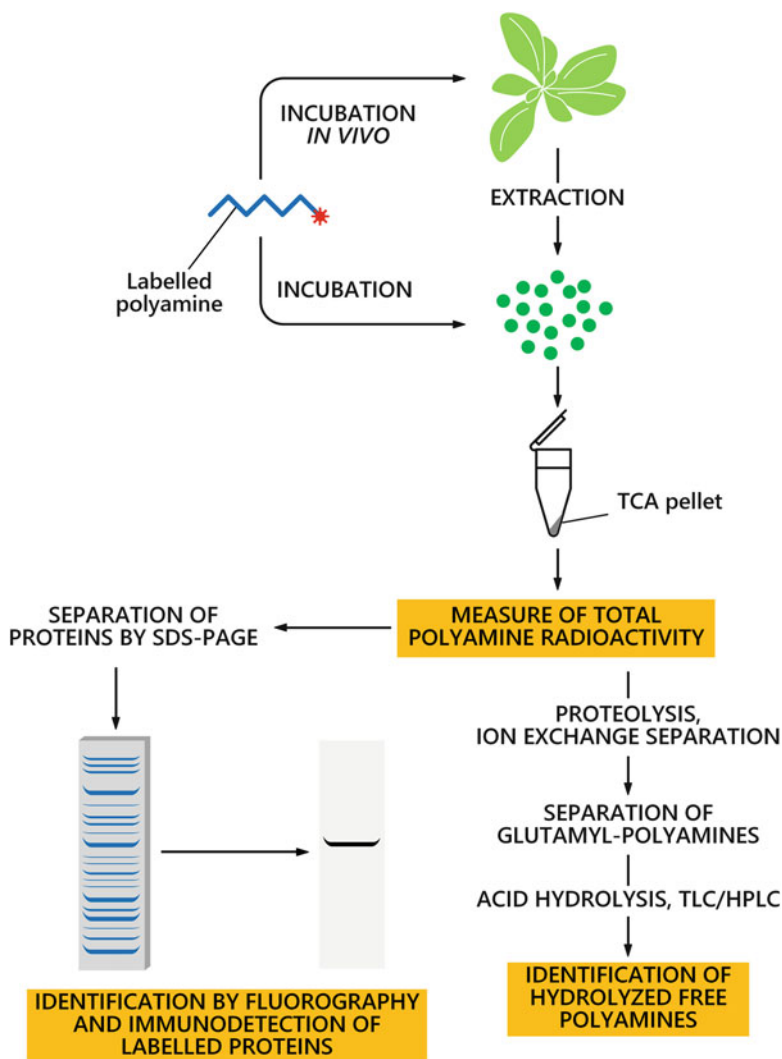


Fig. 4 Procedure that could be completely or partially performed for the study of TGase activity with radioactive PAs

3.1.2 Analysis of Proteins Conjugated to PAs Through SDS-PAGE, Immunoblot Fluorography, or Autoradiography

1. Determine the protein content using a modified bicinchoninic acid (BCA) method [17]. Use BSA as the standard protein.
2. Separate proteins through gel electrophoresis. SDS-PAGE of proteins contained in the pellet is done according to Laemmli [18] or other methods. The percentage of acrylamide (10–17%) is adjusted according to the mass of the labelled proteins.
3. Stain gels with Bio-Safe Coomassie (Bio-Rad) or other dyes. The dried gel is blotted for exposure with X-ray slides Kodak X Omat AR for 15–50 days to detect the labelled protein bands. Alternatively, the gel is exposed for the fluorography

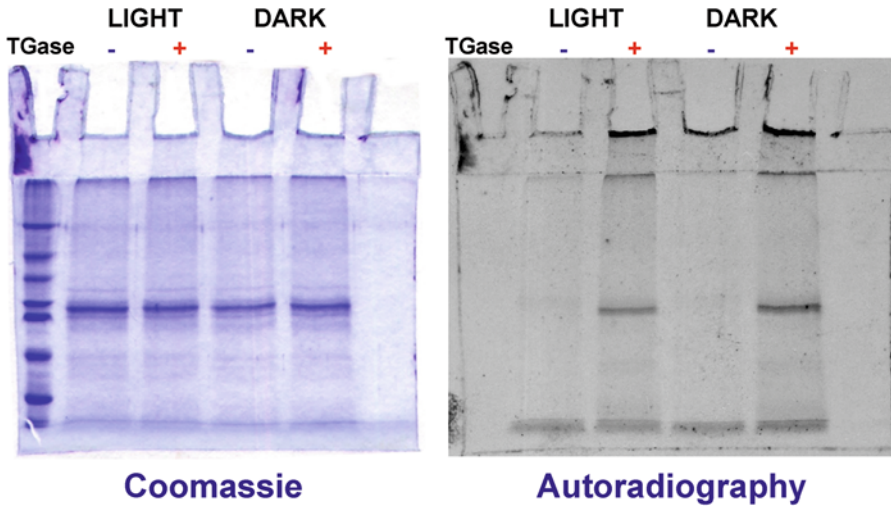


Fig. 5 SDS-PAGE separation of purified LHCII incubated with (+) and without (–) Guinea pig liver TGase (Sigma) and [^{14}C] Spm. Left: Coomassie-stained gel; right: autoradiography of the same gel after drying. Animal TGase conjugates Spm to LHCII, the main band in the gel, and is insensitive to light (third lane incubated to the light and fifth lane incubated in the dark for TGase assay). On the contrary, when chloroplast TGase and LHCII are incubated, the reaction is light-sensitive (data not shown). Bands at the bottom are due to the TGase-independent linkage of Spm with chlorophylls. In the Coomassie-stained gel, the first lane on the left contains MW standards

procedure. In Fig. 5 a Coomassie positive gel is compared with its autoradiography showing which proteins are labelled.

4. To identify the protein, the gel bands can be immunoprobed with specific antibodies. Immunoreactive bands are detected with chemiluminescent reagents available from different suppliers. Immunoblot is performed according to Towbin et al. [19]. Secondary antibodies against mouse and rabbit IgG are conjugated with horseradish peroxidase; secondary antibody to donkey IgG was conjugated with horseradish peroxidase.

3.1.3 Treatment of the Products of the Enzymatic Assay and Detection of Glutamyl-PAs

1. The TGase reaction is stopped with 5% (w/v) (final concentration) TCA also containing 2 mM unlabelled PA (i.e., Put, Spd) in order to remove trapped free polyamines; the mixture is stored at 4 °C for 24 h and then centrifuged at $13,000 \times g$ for 10 min. The pellets are solubilized overnight with 0.1 M NaOH at 37 °C and then precipitated with 5% (w/v) TCA. This procedure is repeated twice. The final pellet is washed with anhydrous diethyl ether to remove TCA residues and digested with proteinases as described [1, 2] with some modifications.
2. After washing TCA pellet with anhydrous diethyl ether, diethyl ether was allowed to evaporate and the precipitate suspended in 100 μl NaOH 0.1 N and left at 37 °C for 1 h to dissolve. The liquid is neutralized with 100 μl HCl 0.1 M.

3. The proteinase solution prepared as follows: 250 mg of pronase type XXI *Streptomyces griseus* are added to 0.5 ml of 0.8 M N-ethyl morpholine acetate buffer pH 8.1. The proteinase solution (50 μ l) is added to 200 μ l of sample (final concentration of pronase is 100 mg ml⁻¹) and the mixture incubated at 37 °C for 48 h.
4. Following digestion, a volume of 10% (w/v) TCA equal to that of the final digestion is added and the precipitate removed by centrifugation at 13,000 $\times g$ for 30 min. The supernatant is extracted three times with diethyl ether and the aqueous layer evaporated under a stream of nitrogen to an appropriate volume for analysis.
5. Then the sample is solubilized in Na-citrate buffer 0.15 M at pH 2.2; 100 μ l the solubilized sample is then injected onto an HPLC for ion exchange chromatographic separations of PAs and PA derivatives (on a Jasco HPLC using a column (0.4 $\times$ 8 cm) of DC-6A resin, Dionex). The anion exchanger DIONEX DC- 6A column, is set at a working temperature of 68 °C.
6. The following program of elution allows the separation of glutamyl-polyamines: Buffer E: 12 min.; Buffer F: 20 min.; Buffer A: 20 min.; Buffer B: 25 min.; Buffer C: 30 min.; Buffer D: 31 min.; Buffer E: 20 min.; Buffer F: 20 min. Flow rate: 0.5 ml/min; Fraction collected every 2 min (1 ml/tube).
7. The identity of the polyamine derivatives is determined by comparison with the corresponding retention times of glutamyl-polyamine standards prepared as reported in Table 2 [2]. Figure 6 shows an example of the separation of the Put and Spd *mono*- and *bis*- derivatives; this separation is performed after the incubation of the chloroplast extract at different pH values of the TGase assay. In these experimental conditions the *bis*-Put eluted at fraction 11, the *mono*-Put co-eluted at fraction 27–28 with *bis*-Spd and *mono*-Spd at fraction 45 (Fig. 6)
8. The identity of conjugated polyamines is determined by TLC or HPLC after release of free polyamines by acid hydrolysis (6 M HCl) of the ion exchange chromatographic fractions. (see **Note 5**).

3.1.4 Synthesis of Glutamyl-PAs as Reference Standards

The method to synthesize glutamyl-Put and glutamyl-Spd as product of TGase reaction, is described in Table 2. These molecules can be used as reference standards in the HPLC separation, to analyze samples tested as above reported in Subheading 3.1 (a) and/or (b) (see **Note 6**).

Table 2
Synthesis of glutamyl-Put and –Spd as reference standards

	Stock solutions	Final concentration	Amount (μl)	Put standard	Spd standard
1	0.5 mM Tris–HCl pH 7.4	50 mM	25	25	25
2	1.5 M NaCl	150 mM	25	25	25
3	25 mM CaCl ₂	2.5 mM	25	25	25
4	100 mM DTT	10 mM	25	25	25
5	2% DMC 20 mg ml ^{–1}	2 mg ml ^{–1}	25	25	25
6	³ H–put (1 μci/μl)	4 μM	10	5	–
Or	³ H–Spd (1 μci/μl)	4 μM	10	–	5
7*	Put 10 mM	1 mM	25 + 10 rad	–	–
Or	Spd 10 mM	1 mM	25 + 10 rad	–	–
8	GplTGase (1 mg ml ^{–1})		5	5	5
9	DDW		Up to 250	115	115

Point 7 could be added to push the reaction towards the formation of *mono*-γ-glutamyl-PAs (*see* Note 5)

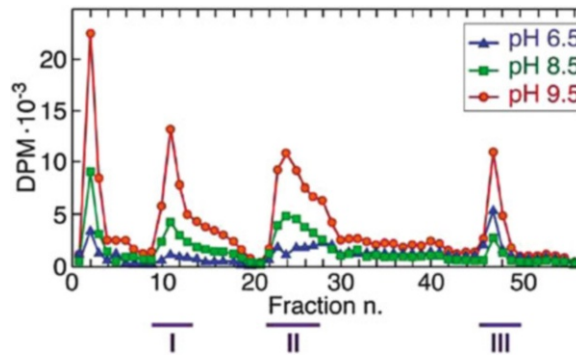


Fig. 6 Separation of glutamyl-PAs by HPLC from chloroplasts incubated with Put at different pH. Roman numbers (I, II and III) indicated the retention times range of elution for fractions of bis-γ-glutamyl Put, mono-γ-glutamyl-Put and free Put respectively. In the region II also bis-γ-glutamyl Spd could be eluted; Spd could be synthesized from Put during incubation assay. To verify if in region II is eluted mono-γ-glutamyl Put or/and bis-γ-glutamyl-Spd, it is necessary to perform an acid hydrolysis (6 M HCl) of the eluted radioactive sample in region II; then an HPLC separation of hydrolyzed sample will allow to identify Put or Spd

3.2 Colorimetric Assay for the Incorporation of the Biotin-Cadaverine into a Protein Substrate (Polyamine Incorporation)

1. For transglutaminase assay, the 96 wells microplate is covered for 16 h (overnight) at 4 °C with 250 μl per well of 100 mM Tris–HCl pH 8.5 containing 10 mg ml^{–1} of DMC or 5 μg ml^{–1} FN (*see* Note 2) well-known substrates for TGase of animal origin. After removing the protein not linked in the well, the plate is washed twice with 150 mM PBS containing 0.05% (v/v) Tween 80 and twice with ultrapure water.

2. Add 250 μl of 100 mM Tris-HCl pH 8.5 containing 3% (w/v) BSA to each well. Maintain the plate in agitation for 30 min at room temperature. This step allows an optimal saturation and, as a consequence, the reduction of the background signal.
3. The plate is washed again as above with a final washing step with 100 mM Tris-HCl pH 8.5. The last washing of each cycle is necessary to pre-equilibrate the proteins to the new assay conditions used in the following step.
4. After washing, each well is supplemented with 150 μl of 100 mM Tris-HCl pH 8.5 containing 13.3 mM DTT and 6.67 mM CaCl_2 or 150 μl of 100 mM Tris-HCl pH 8.5 containing 13.3 mM DTT and 20 mM EGTA for negative control. Both solutions contain 1 μl of biotin-cadaverine (1 mg ml^{-1}) per ml of buffer.
5. Then, 50 μl of the sample whose enzymatic activity needs to be tested are added in each well. This assay is necessary to check the plant TGase activity on well-known mammal TGase substrates (DMC or FN) in presence of Ca^{2+} (5 mM) or in its absence (condition obtained by 20 mM EGTA). To this aim, the same sample is assayed in triplicate (to obtain a statistical significance) in the presence or absence of calcium. A positive control is obtained incubating on DMC or on FN also the guinea pig liver (gpl) TGase at different concentrations (0.01 $\mu\text{g } \mu\text{l}^{-1}$ e 0.02 $\mu\text{g } \mu\text{l}^{-1}$).
6. The activity obtained in the wells of the gpl TGase on DMC or on FN with biotin-cadaverine in presence of calcium is used to set a titration curve of the purified enzyme. On that basis, it is possible to obtain the TGase calcium-dependent activity of the sample whose putative TGase calcium-dependent activity is to be tested.
7. Plates are incubated at 37 °C for 2 h and the reaction is stopped with a washing step identical to the last described. Then, 200 μl of 100 mM Tris-HCl pH 8.5 containing 1% (w/v) BSA and extravidin peroxidase diluted 1:5000 (4 μl of extravidin peroxidase in 20 ml of buffer) are added to each well. The extravidin links to biotin residues of biotin-cadaverine amplifying the signal obtained by the conjugation of biotin-cadaverine to the glutamyl residues of protein substrate (DMC or FN used to pre-saturate the plate) catalyzed by the enzyme present in the total plant extract.
8. The plate is incubated at 37 °C for 45 min then washed as above replacing the 100 mM Tris-HCl pH 8.5 with 100 mM sodium acetate pH 6.0. The reaction is developed with 200 μl per well of 100 mM sodium acetate pH 6.0 containing 0.310 mM TMB (150 μl TMB diluted in 10 mg ml^{-1} of DMSO, in 20 ml of buffer) and 0.0045 (v/v) H_2O_2 (2.50 μl 30% H_2O_2 in 20 ml of buffer).

9. The staining development should be stopped either at 5 min or 15 min by the addition of 50 μl per well of 5 N H_2SO_4 (*see* Subheading 3.3 below). The yellow colour is relatively stable at this stage.
10. The absorbance is read at 450 nm using a microplate spectrophotometer (e.g., Titertek Multiscan ELISA). The TGase activity is obtained as ΔAbs at 450 nm, between the value obtained in presence of calcium and that obtained in presence of EGTA.
11. This value must be divided for the assay time (this represents the time the extract is in the presence of biotin-cadaverine e.g., 2 h in this procedure, not the development time) and the proteins amount present in the 50 μl of plant extract incubated in each well to obtain the value of Unit (U) of TGase specific activity.

When using this protocol consider what reported in **Note 7**.

3.3 Colorimetric Assay for the Incorporation of the Biotinylated TVQQEL Peptide into a Protein Substrate (Simulating Protein Crosslinking)

1. The method for this assay is similar to the method described above (Subheading 3.2).
2. The 96-well plates are coated with 1.0 mg ml^{-1} bovine casein and incubated overnight at 4 °C before being washed and blocked. The assay constituents include biotin TVQQEL instead of biotin-cadaverine.
3. In both the biotin-cadaverine polyamine and the biotin TVQQEL assay there is a requirement to limit the development stage. Two standard graphs should be prepared, one at a relatively high concentration of pure gplTGase (0–250 ng well^{-1}) developed for exactly 5 min, the other at a relatively low concentration of gplTGase (0–25 ng well^{-1}) developed for 15 min. The TGase used in the standard plots can be pure mammal TGase (hr or gplTGase) and the plant extracts can then be compared and quoted as mammal TGase (i.e. gplTGase) equivalents. To overcome the gradual loss in potency of stored standard mammal TGase each 96-well microplate plate should have a column with a high concentration of pure standard mammal TGase (e.g., 100 ng well^{-1}) and a column with a low concentration of pure standard mammal TGase (e.g., 10 ng well^{-1}). When the development stage of the assay is reached the buffer column, the high standard mammal TGase and the low standard mammal TGase should be developed first (the high standard for 5 min the low standard for 15 min). The plant extracts can then be developed for either 5 or 15 min depending on their potency. Comparison between extracts assayed at different times can then be adjusted depending on the potency of the standard gplTGase. This will help in the comparison of results between different microplates and different laboratories.

3.4 *In vitro* TGase Assay During Pollen Germination

1. Pollen was rehydrated at 30 °C and 100% relative humidity for 30 min. Pollen has been suspended in germination medium at 1 mg ml⁻¹
2. Then it was allowed to germinate into in DMC coated 96-well microtitre plates for up to 120 min, in the presence of 0.1 mM biotin-cadaverine (to check extracellular transamidase activity) supplied for the last 30 min of germination period; negative controls have been performed with TGase inhibitors (i.e., ZDON R283, iodoacetamide, cystamine) added at zero time of germination or for the last 30 min to allow pollen tube to partially grow. Pollen viability was assessed by staining with fluorescein diacetate (10 µg ml⁻¹).
3. The level of enzyme activity was expressed as Ca²⁺-dependent increase in A_{450nm}, after subtraction of the value of the TGase inhibitor treated control. Specific activity was determined as a change in A_{450nm} of 0.1 per hour per mg of pollen protein.

3.5 TGase-Based Covalent Binding of Polyamines to Microtubules and Actin Filaments

The covalent binding assay between polyamines and tubulin/actin in the presence/absence of TGase seeks to establish whether this aspect of plant TGase activity might affect specific processes that require the full functionality of cytoskeletal proteins. In particular, here we propose to analyse their polymerization, ability to bind motor proteins, and to support the movement and activity of motor proteins. This analysis can provide information on the role of polyamines during the cellular processes that involve microtubules and actin filaments. The protocol requires an initial phase in which microtubules and actin filaments are assembled starting from the monomeric tubulin/actin, followed by the enzymatic reaction in which TGase covalently links polyamines with preformed microtubules/actin filaments. Subsequently, microtubules and actin filaments can be analyzed by means of several tests as schematically depicted in Figs. 7 and 8.

3.5.1 Method for the Polyamine-Microtubule Assay

1. Prepare TGase according to the type of enzyme you are interested in. This depends on the type of organism one is working with. If TGase comes from specific organs or tissues or cells, it must be purified according to specific protocols adapted to the particular conditions [20]. If the enzyme is purchased from companies, usually it comes in lyophilized form that must be resuspended in a minimum volume of enzyme reactivation buffer. It is advisable to leave the enzyme suspension for at least 5 min at room temperature in order to fully reactivate the enzyme. The final concentration of TGase may be critical for subsequent experiments. It is suggested to test different concentrations of enzyme in order to determine the optimum enzyme concentration for each experiment. In our hands, a working concentration of 0.01–0.1 mg ml⁻¹ proved to be effective.

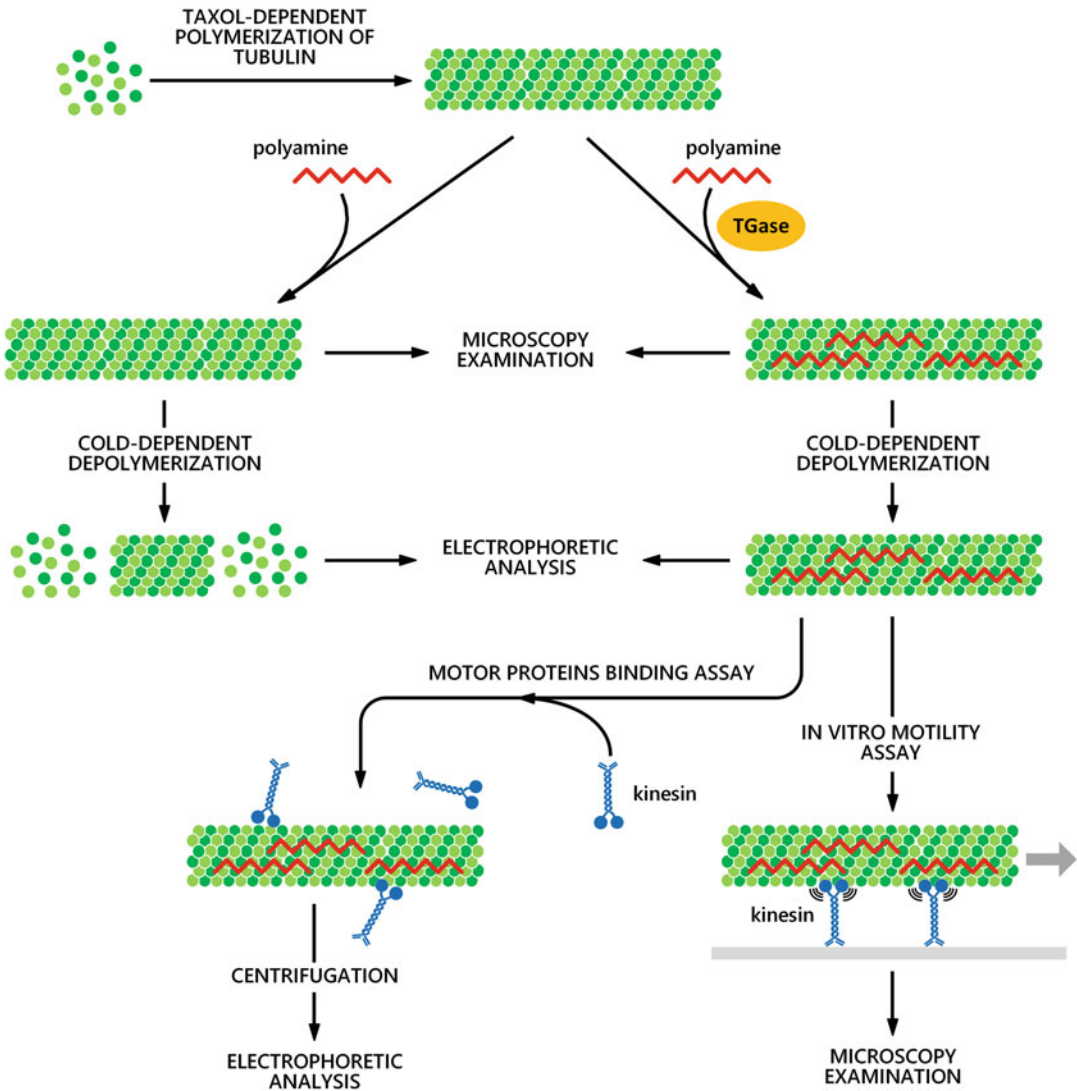


Fig. 7 Diagram of the analytical methods to test the effects of transglutaminase/polyamines on the functionality of microtubules

2. Although tubulin can be purified from different animal and plant sources [21–24], currently it can easily and cheaply be purchased from different companies. Purchased tubulin is effective and pure. Tubulin is delivered either as lyophilized or frozen at specific concentrations. Both lyophilized and frozen samples usually contain the solutes required for tubulin stability at optimal concentration. Anyway, it is suggested to check in the company website or the product brochure. For optimal stability, tubulin must be stored at -80°C .
3. Just before starting the experiment, defrost tubulin aliquots. If tubulin is lyophilized, add the required volume of water to

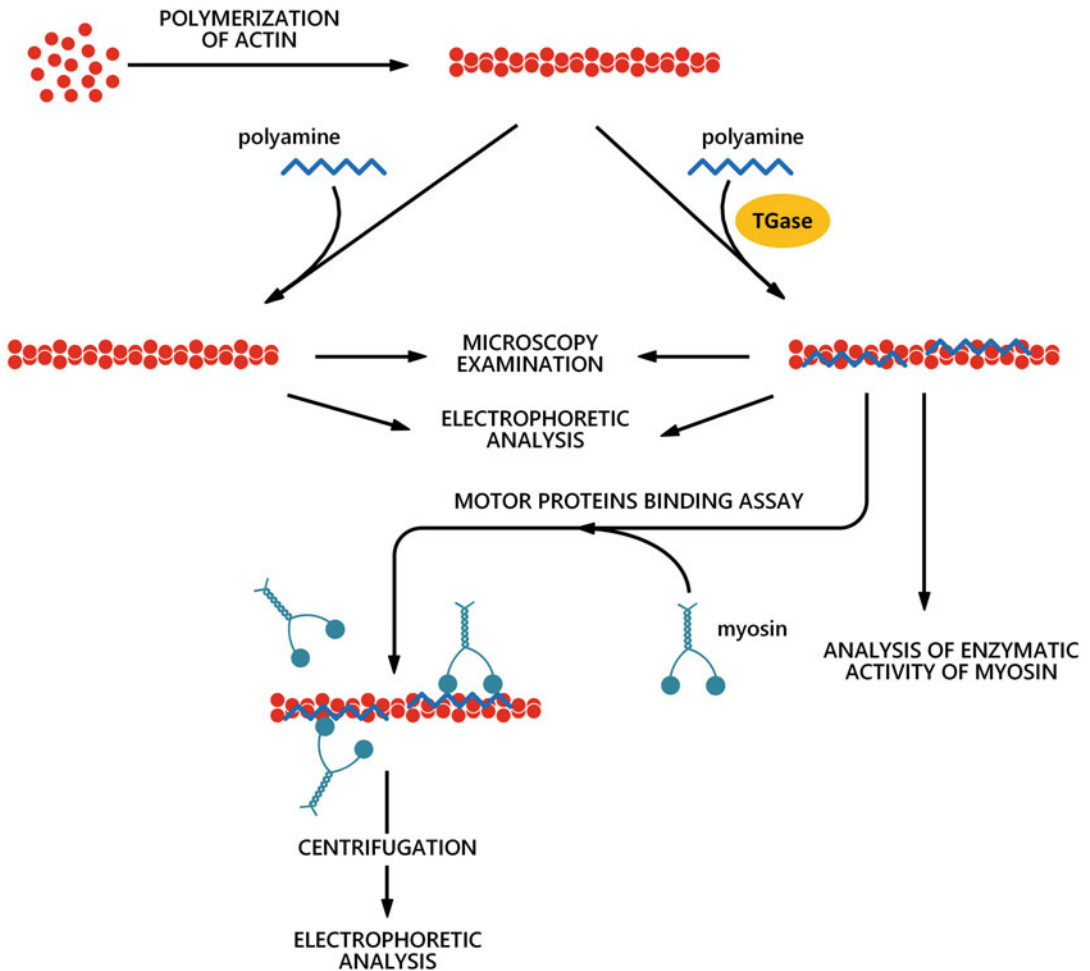


Fig. 8 Diagram of the analytical methods to test the effects of transglutaminase/polyamines on the functionality of actin filaments

adjust the protein concentration at 10 mg ml^{-1} . We usually started with $100 \text{ }\mu\text{l}$ of tubulin at the concentration of 10 mg ml^{-1} . This volume and concentration is generally appropriate for a series of experiments, but the final volume must be calibrated on the specific experimental requirements. For preparation of microtubules, take $25 \text{ }\mu\text{l}$ of tubulin solution ($250 \text{ }\mu\text{g}$) and transfer it into a microfuge tube.

4. Add $25 \text{ }\mu\text{l}$ of TDB to tubulin aliquots. The tubulin solution is now at a concentration of 5 mg ml^{-1} . Add $6.25 \text{ }\mu\text{l}$ of CB into the microfuge tubes. This step initiates tubulin polymerization. In order to enhance polymerization of microtubules, incubate sample at 37°C for 30 min. You can do this by using a water bath set at desired temperature or more precise incubators for microfuge tubes.

5. Mix 400 μl of MDB with 32 μl of 500 μM taxol thereby making the so-called MDB-T buffer. Incubate it at 37 °C.
6. When the incubation has ended, add 400 μl of MDB-T to the microfuge tube containing newly polymerized microtubules. Mix gently and leave the samples at room temperature. Tubulin is now polymerized and at a concentration of approximately 0.5 mg ml^{-1} .
7. For the TGase-mediated incorporation of polyamines into microtubules, prepare the following reaction mixture by mixing 50 μl of microtubules (250 μg) with TGase in a ratio of 10:1 as a starting value. Please note that this ratio has to be determined experimentally on the basis of the specific TGase to be assayed. In addition, add the polyamine of your choice at a concentration of 5 mM and then add 6 mM CaCl_2 .
8. As control, prepare a second mixture containing 50 μl of microtubule solution (250 μg), 5 mM polyamine and 6 mM CaCl_2 .
9. Incubate both samples at 37 °C for 2–3 h. The appropriate incubation time must be determined experimentally and this depends on the specific activity of TGase. Reactions can be stopped by adding EGTA to a final concentration of 6–7 mM. Samples can be stored at room temperature until needed.
10. For observation with a microscope, take 10 μl of TGase/polyamine-modified microtubules and perfuse it within a perfusion chamber. Wash the chamber several times with PEM buffer + taxol in order to remove unpolymerized tubulin and observe samples using a optical microscope with Köhler illumination and a DIC filter (for proper settings, you can refer to the instruction manual of your own microscope) (*see Note 8*). You will need specific software or hardware to remove background noise and to visualize microtubules in the perfusion chamber. For a basic idea of the required system, *see Fig. 9*. You can refer to the literature for further information on the assembly of an optical/computer system suitable for visualization of microtubules [25]. In control condition, microtubules are usually detected as single structures while in TGase/polyamine-treated samples microtubules appear as large bundles or aggregates.
11. To visualize the occurrence of TGase/polyamine-dependent aggregates, centrifuge samples for 60 min at $13,000 \times g$ in a microfuge at 25 °C. Both pellets and supernatants have to be processed for SDS-PAGE analysis. The pellet can be resuspended in a small volume of PEM buffer and then added to appropriate volume of PAGE denaturing buffer [18]. Alternatively, the pellet can be directly dissolved in the denaturing buffer. The supernatant can be directly added to appropriate volumes of denaturing buffer (*see Note 9*). At this step, it is

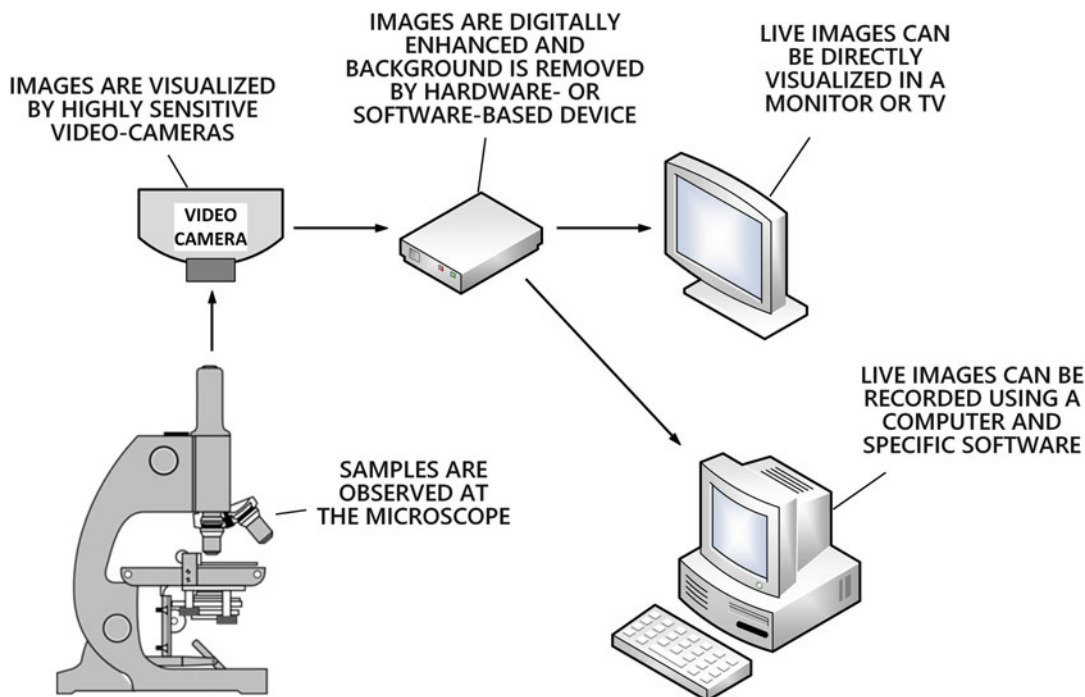


Fig. 9 Basic components of a system for viewing and recording of microtubules in a perfusion chamber. What is represented here is a relatively simple system that can be improved according to the technology offered by various companies

critical to maintain the ratio between pellets and supernatants in order to analyze corresponding volumes. Otherwise, the protein concentration has to be determined using the BCA assay [17] or commercial kits.

12. To test for temperature-dependent depolymerization, aliquots of polyamine-modified microtubules and controls can be incubated for 30 min at 4 °C. This step can be performed using a refrigerators or on ice. While this is happening acclimatize the microfuge to 4 °C.
13. At the end of incubation, proceed to the centrifugation step by centrifuging the sample at $13,000 \times g$ in a microfuge for 60 min at 4 °C. Both the supernatant and pellet can be processed for SDS-PAGE by diluting with PAGE denaturing buffer (*see Note 10*). Again, it is important to maintain the volume ratio between samples.
14. To perform in vitro motility assay, the TGase/polyamine microtubules can be used together with a specific motor protein, such as kinesin. Purified motor proteins can be purchased from different companies. Alternatively, they can be purified from various animal or plant sources [26–28]. It is advisable to buy the purified proteins because they are normally provided at

desired working concentrations and stability. The concentration of the motor protein is not critical at this stage. However, it is important that it is maintained at a constant concentration throughout the experiments. Motor proteins purchased from a supplier are usually delivered as lyophilized proteins and must be reconstituted by adding specific volumes of water.

15. Perfuse 10 μ l of the motor protein solution into a perfusion chamber, then incubate the glass-slide into a moist perfusion chamber for 5 min to allow proteins to adhere.
16. Wash out unbound motor proteins by perfusing 2–3 times with 10–20 μ l of blocking buffer. This step is also used to block sites in the perfusion chamber (perform all these steps while the perfusion chamber is placed within a moist chamber in order to prevent dehydration of samples).
17. Dilute 10 μ l of microtubule solution (treated or untreated with polyamines) with 40 μ l of motility buffer. Add 10 μ l of microtubule solution into the perfusion chamber and incubate for 1 min at room temperature.
18. Rapidly move to the microscope, which should be set up in advance. Observe the samples under Köhler illumination and using DIC filters. A 63 $\times$ or 100 $\times$ objective is required for optimum viewing, as well as a hardware or software device to reduce background noise (Fig. 9). A video camera is also required. Signals acquired by the video camera must be delivered to a video acquisition system, which allows recording of live video clips using dedicated software. The type of software and hardware used for video acquisition might be dependent on the model of video camera (*see Note 11*). It is advisable to acquire video clips at the highest video frame and at the highest frame per second.
19. The velocity of microtubule movement can be analyzed by specific software. We recommend ImageJ as a free alternative (<https://imagej.nih.gov/>), with the plugin “MTrackJ”. The analysis allows comparison of the speed of microtubules under different experimental conditions.
20. For the microtubule binding assay with motor (or non-motor) proteins, the first step is the preparation of the test protein. Again, it is suggested to purchase purified proteins because of their standard concentration and stability. The test can be used to analyze the binding of a specific protein or of a mixture of proteins present in a specific cellular compartment. In the test, we suggest to use fixed volumes of one component (e.g., microtubules) and to vary the volume or concentration of the binding protein(s). The reaction volume can be made equivalent by the addition of the assay buffer.

21. Prepare several tubes containing 30 μl of microtubule solution (treated or untreated with polyamines) then add 2, 5, 10, 20 μl of the binding protein (as an example). Set all reaction volumes to 50 μl . If the assay involves a motor protein in the binding test, the reaction volume should also contain 10 mM ATP or 5–10 mM AMPPNP to release or enhance the binding of motor proteins to the microtubules [29].
22. Samples can be incubated for 30 min at 25 °C, then they can be centrifuged at $13,000 \times g$ for 60 min at 25 °C. Both pellets and supernatants can be analyzed by SDS-PAGE .
23. For a quantitative analysis of the binding reaction, gels can be scanned and their images analyzed with dedicated software. Different equipment and software for this type of analysis are commercially available. In practical terms, this allows converting the protein band(s) of interest into a numerical values and therefore a comparable value.

3.5.2 Method for the Polyamine-Actin Assay

1. Reactivation of TGase: for procedural details, you can refer to what already stated in paragraph 1 of the previous protocol concerning the microtubule assay. Briefly, resuspend the enzyme in a minimum volume of reactivation buffer and let stand at room temperature for 5 min.
2. Thaw 1 aliquot of actin (optimum 1 mg) and dilute the actin to a concentration of 10 mg ml⁻¹ by the addition of 100 μl of A-buffer. Dilute the actin to a concentration of 0.4 mg ml⁻¹ with A-buffer by mixing 100 μl of actin solution with 2.4 ml of A-buffer. Incubate on ice for 1 h. Subsequently, add the polymerization inducing buffer (Polymerization Buffer 1 $\times$ at final dilution) in the following ratio: 2.25 ml of actin plus 0.25 ml of 10 $\times$ Polymerization Buffer. Incubate at room temperature for 1 h. The actin filaments are ready to use and can be stored at room temperature until use. It is suggested that the actin should be purchased from companies; obviously, actin can be purified from plant and animal sources and from tissues/organs selected by the operator [30].
3. The reaction mixture can be prepared as follows. Mix 625 μl of actin ($\approx 250 \mu\text{g}$) with TGase so that the ratio is around 10:1 (again, it is necessary to mention that the exact ratio must be determined experimentally and according to the specific conditions of the operator). Add the polyamine at the concentration of 1 mM and CaCl₂ at 6 mM. In parallel test tubes, mix exactly the same components but without TGase.
4. Incubate all samples at 37 °C for 2–3 h.
5. Stop the reaction by adding EGTA to the final concentration of 7 mM.

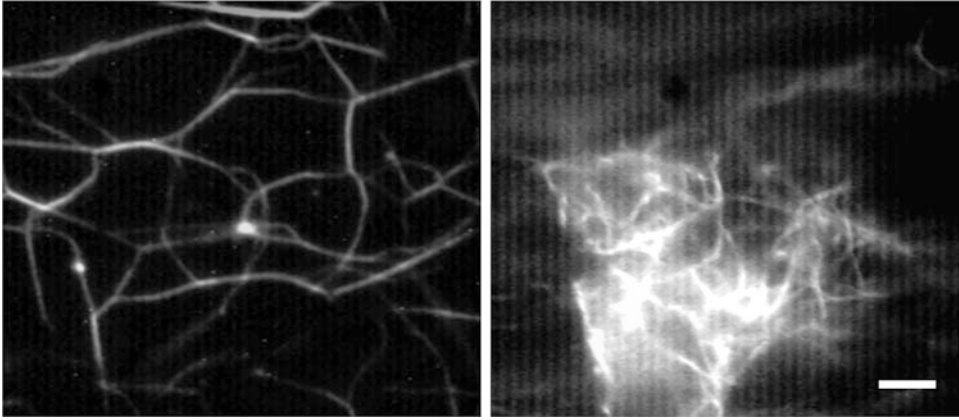


Fig. 10 Rhodamine-labelled F-actin obtained in the presence of plant TGase and Put (viewed in the rhodamine filter). On the left, Rhodamine-labelled F-actin obtained in the absence of plant TGase; on the right, Rhodamine-labelled F-actin obtained after TGase and Put treatment. Scale bar: 5 μ m

6. For the observation at the microscope, perfuse 10 μ l of samples within individual perfusion chambers (*see* above for their preparation) and incubate for 5 min in a moist chamber.
7. To visualize actin filaments, wash the perfusion chambers with 10–15 μ l of the rhodamine-phalloidin solution to label actin filaments. After 5 min incubation, wash unbound labeling molecules and observe the samples using a fluorescent microscope (rhodamine filter). Usually, the sample incubated with TGase and polyamines shows altered actin configuration with the frequent occurrence of amorphous aggregates (Fig. 10).
8. In order to determine if TGase/polyamine alters the polymerization status of actin filaments, samples can be centrifuged in a microfuge at $13,000 \times g$ for 60 min at 25 °C. Both supernatants and pellets can be processed for SDS-PAGE analysis. Again, please take care to maintaining the exact volume ratio between pellets and supernatants in order to evaluate the results correctly.
9. As stated before, gels can be scanned and images can be analyzed for densitometric values in order to evaluate statistically the percentage of actin between pellets and supernatants.
10. The binding assay to motor proteins can be performed using myosin. Actin-based motor proteins can be purchased from different companies at very good quality. Therefore, to increase reproducibility and interpretation of results, we suggest to use commercially available proteins. If myosin is delivered as lyophilized, resuspend the protein in a suitable buffer (4 mM Tris-HCl pH 7.6, 2 mM $MgCl_2$, 100 mM KCl) in order to have a concentration of about 0.5–1 mg ml⁻¹.

11. Add 10 μl of the myosin solution to 100 μl of actin filaments and incubate for 30 min at 25 °C. Centrifuge samples at $13,000 \times g$ for 60 min at 25 °C. Both supernatants and pellets can be denatured for SDS-PAGE analysis. Again, the binding of myosin to actin filaments can be quantified by gel scanning and densitometric evaluation of the band corresponding to the myosin heavy chain (usually around 170–200 kD).
12. The activity of myosin can also be evaluated by using an ATPase assay [31]. For this analysis, one can use different volumes of the actin filament solution as prepared before. For example, 10, 20 or 50 μl of actin filaments (0.4 mg/ml, either treated or untreated with TGase/polyamines) can be mixed with 4 μg of myosin; the ATPase assay buffer can be used to bring the final volume to 100 μl .
13. Samples are incubated at 25 °C for 30 min and the reaction stopped by putting samples on ice.
14. Determine the amount of released inorganic phosphate through one of the several phosphate detection kits commercially available.

3.6 Method for the Proteolytic Digestion of Plant Proteins for the Analysis of ϵ -(γ -Glutamyl) Lysine Isodi-peptide

1. Plant material was homogenized and protein was precipitated from the $80,000 \times g$ supernatant by the addition of an appropriate volume of 72% (w/v) TCA to give a final concentration of 7.2% (w/v).
2. The protein was pelleted by centrifugation at $13,000 \times g$ for 30 min at 4°C.
3. The pellet was washed once in 10% (w/v) TCA, three times in 50:50 ethanol:diethylether and three times in diethylether (each washing was followed by centrifugation at $13,000 \times g$ for 30 min).
4. The pellets were dried by evaporation and re-dissolved in 1.0 ml of 100 mM ammonium carbonate pH 10.0. The pH of each tube was checked with pH indicator paper and adjusted to 10.0 by the addition of sodium hydroxide if necessary.
5. Subtilisin (0.1 mg) was added and incubated 16 h at 37°C. The addition of subtilisin was repeated twice more over 48 h.
6. 150 μg of pronase was added to each tube as 10 μl of a 15 mg ml^{-1} solution. Following a further 16 h incubation, each extract was boiled for 15 min.
7. 10 μl of activated leucine amino peptidase and activated prolidase solution were added along with a 10 μl aliquot of 0.54 M magnesium chloride. The tubes were vortexed and incubated for 24 h.
8. The pH of each digestion was then adjusted to 7.0 with 1.0 M HCl (using pH indicator paper) and 0.1 mg of

carboxypeptidase Y was added as 10 μl of a 10 mg ml^{-1} solution. Following a further 16 h incubation.

9. The digested protein solutions were placed into conical glass centrifuge tubes and added to chloroform:methanol:HCl (200:100:2) at the level of 3.6 ml to 1.0 ml of protein digestion. A further 1.2 ml of chloroform was added and the tube was vortex mixed. The tubes were centrifuged at $2500 \times g$ for 5 min at 4°C . The upper methanol layer containing the liberated TG crosslinked peptide plus amino acids and the central interface containing undigested protein were carefully removed and dried.
10. The methanol phase was re-dissolved in an appropriate volume of amino acid analysis loading buffer and stored at -20°C prior to subsequent amino acid analysis using a cation exchange amino acid analyser (Biochrom UK).
11. The undigested protein was retained and quantified using the a modified BCA assay [17] to determine the efficiency of the hydrolysis.

4 Notes

1. Polyamines can be labelled in the carbon [^{14}C]-PA or in the hydrogen [^3H]-PA of the molecule. According to the target of the experiment, the type and position of the label might be critical. For example, if PAs are oxidized by the cell enzymes, tritium can be removed; but also carbon, if located in trimethylene moiety of Spm or Spd, can be lost if the molecule is catabolized to the respective lower PA (Spd and Put). Thus, the PA used should have the labelled carbon located in the tetramethylene part of the backbone. Alternatively, the molecule can be uniformly labelled.
2. Different protein substrates can be used to check plant TGase activity; among them the most common are: soy globulins, FN, rubisco, LHCII, actin and tubulin.
3. Plant TGase is a Ca^{2+} -dependent enzyme and thus it is inhibited by EGTA.
4. The pigments (i.e., chlorophylls) can be removed from the pellet by washing with ethanol/diethyl-ether 1:1 to remove TCA and 80% (v/v) acetone to remove chlorophylls; however other molecules to which PAs can be “glued”, might be present, severely affecting the value of results.
5. *Advantages of the radioactive method.* This method has the advantage to supply the underivatized PA thus without introducing additional groups (for e.g., fluorescent groups) that can modify the hydrophobicity and the steric hindrance of the

molecule disturbing the reaction. In addition, both the terminal amino-groups are free and consequently the PA is capable to be conjugated to the glutamyl residues of a protein by only one or both amine groups, giving rise respectively to *mono*-glutamyl- or *bis*-glutamyl –PA (Fig. 2). Spd is the only asymmetric PA and thus it can give rise to two different *mono*-derivatives according if –NH_3^+ in position 1 or 8 is engaged. *Disadvantages of the radioactive method.* The entire procedure is very time-consuming. This method does not reveal the catalysis of glutamyl-lysine conjugates occurring via TGase reaction.

6. This method allows to synthesize either *mono*-glutamyl- or *bis*-glutamyl–PAs. To push the synthesis of *mono*-glutamyl–PA it is necessary to increase the concentration of the PA by increasing the cold reagent. Otherwise if it is necessary to synthesize preferably the *bis*-glutamyl –PA, keep the concentration of cold PA very low or do not add the cold PA into the assay.
7. Remember to warm all the reagents to 37 °C before they are used in a TGase microplate assay. Add the reducing agent (e.g., 2-mercaptoethanol or DTT) immediately before use. Close the plate with an adhesive microplate seal after the reagents have been added to the assay. During the development stage of the assay with extravidin peroxidase use exact time intervals e.g., 5 or 15 min.
8. Optical microscopy analysis of microtubules is not an easy technique to perform. It requires a very good system of optical microscopy, specifically equipped with a high-quality 63× or 100× oil-immersion objective. It also requires the expertise of trained staff. For a detailed description of the method for microscopy observation, please refer to the following textbook [25].
9. The electrophoretic analysis can be performed with standard equipments readily available in a biochemical laboratory. Generally, a 6–20% range of polyacrylamide or a linear 8% gel are generally appropriate for the analysis and visualization of TGase-dependent aggregates.
10. After staining gels with either Coomassie Blue or silver staining, the gel lanes containing the control sample and the sample treated with TGase can be compared to detect the protein complexes generated by the enzymatic reaction. The analysis may be simply visual and then descriptive, or the gel may be scanned and analyzed by specific software in order to obtain a more accurate quantitative assessment.
11. The recording of a visual field containing stationary or moving microtubules requires an adequate capturing/recording system. Nowadays, one can record images directly on a computer using video capture systems. Current videocameras directly convert the signal so that it can be immediately read and stored

as a video file using a specific software. It is important to capture the video file to the highest possible resolution and with the highest number of frames per second. The storage format can be proprietary or generic; in this case, a particular video compression codec must be installed on the computer.

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