An extracellular transglutaminase is required for apple pollen tube growth

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An extracellular form of the calcium-dependent protein-cross-linking enzyme TGase (transglutaminase) was demonstrated to be involved in the apical growth of *Malus domestica* pollen tube. Apple pollen TGase and its substrates were co-localized within aggregates on the pollen tube surface, as determined by indirect immunofluorescence staining and the *in situ* cross-linking of fluorescently labelled substrates. TGase-specific inhibitors and an anti-TGase monoclonal antibody blocked pollen tube growth, whereas incorporation of a recombinant fluorescent mammalian TGase substrate (histidine-tagged green fluorescent protein: His_6–Xpr–GFP) into the growing tube wall enhanced tube length and germination, consistent with a role of TGase as a modulator of cell wall building and strengthening. The secreted pollen TGase catalysed the cross-linking of both PAs (polyamines) into proteins (released by the pollen tube) and His_6-Xpr-GFP into endogenous or exogenously added substrates. A similar distribution of TGase activity was observed *in planta* on pollen tubes germinating inside the style, consistent with a possible additional role for TGase in the interaction between the pollen tube and the style during fertilization.

Key words: extracellular localization, *Malus domestica*, pollen tube growth, polyamine, protein cross-link, transglutaminase.

INTRODUCTION

In angiosperms, sexual reproduction requires the apical growth of pollen tubes through the style, towards the female gametes located into the gynoecium. In the apical zone of the pollen tube, the growth of plasma membrane and cell wall is maintained by the continuous supply of precursors that are provided by the cytoplasm. The dynamics of vesicle and organelle movement along the pollen tube is sustained by the cytoskeletal network controlled by the influx of calcium at the apical tip [1]. The cell-wall strength and focal anchorage are essential requisites to push the extending pollen tip through the style, especially for solid stylar transmitting tracts, as in *Rosaceae* [2]. A number of yet to be completely characterized pollen proteins, either newly synthesized or located in the pollen wall intine, are released into the style [3]. During pollen growth, some of these pollen proteins interact with stylar glycoproteins to anchor the pollen tube to the psECM [pollen/stylar ECM (extracellular matrix)], via molecular mechanisms that are not fully understood [4,5]. PAs (polyamines) are among other secreted molecules necessary for the apical growth of germinating pollen [6,7], helping to sustain pollen germination as demonstrated with inhibitors of PA-biosynthetic enzymes [7–11]. The role of PAs may be related to the structure and assembly of vegetative cell walls [12,13] or the organization of the pollen cell wall [14], and these functions may be relevant to pollen from a variety of plant species [15]. The precise role of PAs secreted from the germinating pollen tube and their interaction with the psECM are not completely understood.

The interactions between the pollen tube and the psECM are reminiscent of the cell–ECM interactions in animal cells [5]. One key modulator of the ECM in mammals is tTGase (tissue transglutaminase; also known as TGase 2), a member of a large family of enzymes [16,17] which catalyses the calcium-dependent post-translational modification of proteins, by forming covalent cross-links between glutamyl- and either lysyl-residues [N^{ε}(γ-glutamyl) lysine isodipeptide] or PAs [*mono* and *bis*-(γ-glutamyl) polyamines] resulting in a bridge between two or more proteins [18]. In animal cells, extracellular tTGase is concentrated at FAs (focal adhesions), which are sites of communication between the ECM and intracellular signalling molecules [19–21]. After secretion, tTGase becomes a non-enzymically active structural component of the ECM, forming a close association with the glycoprotein fibronectin and heparan sulfate proteoglycans important in cellular adhesion and migration [20,22,23]. TGases have been shown to be present in plant tissue [24], including apple pollen, where it is an intracellular calcium-dependent enzyme catalysing the incorporation of PAs into α-tubulin, actin monomers and uncharacterized large protein complexes [25]. Recently monoclonal antibodies raised against mammalian tTGase (e.g. ID10) have been shown to cross-react with apple pollen TGase and the *in vitro* activity of the enzyme can be blocked by an animal TGase inhibitor [ref?]. In addition, this Q1 partially purified apple pollen TGase has been shown to be capable of catalysing the *in vitro* cross-linking of a number of purified cytoskeletal components. This suggests that pollen TGase may be capable of modulating the properties of the pollen cytoskeleton and possibly the subsequent development of pollen tubes [26]. The presence of TGase substrates in germinating apple pollen has also been demonstrated by their *in situ* visualization in the pollen grain, and the elongated pollen tubes, as well as the pollen surface [27]. Other studies have reported the presence of a TGase-like activity in the cell wall of higher plant parenchyma or of lower plant

Abbreviations used: DMC, N′,N′-dimethyl casein; DTT, dithiothreitol; ECM, extracellular matrix; GFP, green fluorescent protein; gpl, guinea pig liver; HIC, hydrophobic interaction chromatography; HRP, horseradish peroxidase; PA, polyamine; RFU, relative fluorescence units; TGase, transglutaminase; tTGase, tissue transglutaminase.

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cells whose cell-wall composition is different from that of pollen tubes [24,28,29]. The above data suggest that an extracellular TGase may be present in the cell wall of apple pollen tubes, and may contribute to the interaction of pollen proteins with psECM proteins, by catalysing protein–protein linkages.

The present study aims to demonstrate the presence of an extracellular TGase linked to the cell wall of the pollen tube or released into the ECM. Apple pollen germination was studied *in vitro* (by incubating the pollen in the germination medium) confirming the presence of TGase, and this was also verified *in planta* using pollen germinated inside the style. The role of TGase in the modulation of pollen tube apical growth was investigated by the application of specific TGase inhibitors, antibodies towards both mammalian and plant TGases, and by assaying the activity of the enzyme in the presence of known TGase protein substrates and PAs.

MATERIALS AND METHODS

Chemicals and antibodies

All chemicals (unless otherwise indicated) were obtained from Sigma–Aldrich. The tTGase inhibitors 283 {1,3-dimethyl-2[(oxopropyl)thio]imidazolium} [30] (now available also at Zedira) and 281 (*N*-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine), two specific site-directed inhibitors of TGase developed to covalently bind the cysteine-based active site of the calcium-activated form of mammalian TGase [31–34] were synthesized at Nottingham Trent University, Nottingham, U.K. The mouse monoclonal antibody ID10 raised against purified gpl (guinea pig liver) TGase was produced at Nottingham Trent University and selected by its reactivity to gpl TGase and ion-exchange-purified extracts of 14-day-old *Pisum sativum* leaf tissue and germinated *Vicia faba* cotyledons [35]. The TGase-reactive clone antibody was partially purified from hybridoma culture supernatant by cation-exchange chromatography on Fast Flow S-Sepharose (Amersham Biosciences). ELISA-positive fractions were pooled, concentrated in a dialysis tube against PEG [poly(ethylene glycol)] 8000 and stored in aliquots at -20°C. The immunoreactivity of ID10 for pollen TGase, purified by HIC (hydrophobic interaction chromatography), was compared with the TGase antibody AbIII (Neomarker). Reactivity of ID10 was also tested against different cell fractions of *Agaricus* and compared with the cross-reactivity obtained by CUB7402 on the
Q2 same cells (M. Della Mea, personal communication).

gpl TGase was obtained from Sigma–Aldrich and further purified in our laboratory. Recombinant His6–Xpr–GFP (GFP is green fluorescent protein) (a TGase substrate) was produced at the Tokyo Institute of Technology (Yokohama, Japan) [36]. Primary antibodies include: monoclonal anti-TGase 2 Ab-I (clone Cub7402; Neomarker); polyclonal antibody AtPng1P (produced in chicken at the CSIC of qBarcelona, Spain, as reported previously [37]); 81D4 monoclonal anti-N^{ε}(γ-glutamyl)lysine (Covalab); 2c10 monoclonal anti-horse albumin (a gift from Professor E.E. Billett, Nottingham Trent University, Nottingham, U.K.). The following antibodies were obtained from Sigma–Aldrich unless otherwise indicated: T1A2 [anti-(tyrosinated α-tubulin subunit)]; B512 [anti-(total α-tubulin)]; non-immune mouse IgG (used as a negative control); and polyclonal anti-(α-tubulin) antibody. The list of secondary antibodies includes: HRP (horseradish peroxidase)-conjugated rabbit anti-mouse IgG; FITC-conjugated rabbit anti-mouse IgG (DakoCytomation); FITC-conjugated goat anti-mouse IgG secondary antibody (Cappell); FITC-conjugated goat anti-rabbit IgGs; and goat anti-mouse IgG 15nm gold-conjugated (Biocell).

Plant material and growth

Mature pollen of *Malus domestica* Borkh. cv Golden Delicious was collected from plants grown in experimental plots (Dipartimento di Colture Arboree, University of Bologna, Bologna, Italy). Handling and storage were performed as reported previously [25].

In vitro germination

Pollen was re-hydrated and allowed to germinate as previously described [25] into glass Petri dishes or in DMC (N′,N′-dimethyl casein)-coated 96-well microtitre plates (Nunc Life Technologies) or 96-well microtitre fluo black plates (Nunc Life Technologies) for up to 120 min, in the presence and absence of His6–Xpr–GFP, TGase inhibitors (281, 283, iodoacetamide and cystamine) and anti-TGase antibodies added as specified in the Figure legends at zero time of germination or for the last 30 min to allow the pollen tube to partially grow. As the monoclonal antibody ID10 gave the strongest labelling it was used for further experiments. Light microscopy digital images of at least three non-overlapping fields covering the central part of each chamber were captured using a video digital camera (Olympus DP10) and quantified in terms of germinated compared with ungerminated pollen, when the pollen tube was either absent or shorter than the pollen grain diameter. Pollen viability was assessed by staining with fluorescein diacetate (10 μg · ml^{-1}).

Pollen protein extraction

Pollen was collected after germination and separated from the culture medium by filtration with a vacuum pump on Millipore disks (5 μm pore size). Pollen proteins and those secreted in the germination medium were extracted following a method described previously [38] and quantified [26]. Proteins were also isolated from pollen cell walls according to a previously published method [39].

Partial purification of pollen TGase

The partial purification was performed by HIC with Phenyl–Sepharose 6 Fast Flow (Sigma–Aldrich) at pH 8 [26]. Partially purified TGase was immunodetected after SDS/PAGE and Western blot analysis with two tTGase antibodies (ID10 and AbIII).

Immunoprecipitation of pollen TGase

Aliquots (200 μl) of HIC-purified pollen extract (0.2 mg · ml^{-1}) were incubated with increasing amounts of the monoclonal antibody ID10 for 2 h on ice, and the samples immunoprecipitated as described previously [23]. The supernatant was assayed for TGase activity [19] and compared with that of the pre-cleaned sample obtained after incubation of HIC-purified pollen extract (see above) directly with Protein G–agarose beads (control) (Sigma–Aldrich). The difference between the activities in the supernatants obtained after ID10 treatment and the control gave a quantitative indication of the TGase activity lost due to the binding of the pollen TGase to the Protein G-coupled ID10.

SDS/PAGE and Western blot analysis

A total of 50 μg of germinated pollen proteins obtained after (i) extraction after low-speed centrifugation or from pollen cell walls, (ii) following purification via HIC or (iii) after the concentration of the germination medium, were electrophoresed on 10 % (w/v) polyacrylamide SDS/PAGE slab gels [40], using 0.2 μg of gpl TGase as a standard. Western blotting was performed as described previously and protein bands that cross-reacted with ID10, AbIII, CUB 7402 or AtPng1p revealed according to previously published studies [37].

TGase activity assays

Extracellular pollen TGase activity was measured by the conjugation of biotinylated cadaverine to DMC as described previously [19], with minor modifications. Native hydrated pollen grains (1 mg · ml^{-1} in germination medium) were allowed to germinate in the wells for 120 min at 30 °C in germination medium (see above) buffered with 5 mM Mes (pH 6.5), containing 0.1 mM biotinylated cadaverine and 50 μM DTT (dithiothreitol). Germination was monitored by light microscopy throughout. After removal of germinated pollen by extensive washing with PBS, the level of biotinylated cadaverine incorporated into immobilized DMC was revealed according to an established protocol [41], with EGTA replacing EDTA in negative controls. Data are expressed as Ca^{2+}-dependent increases in A_{450}, after subtraction of the value of the 5 mM EGTA-treated control. Specific activity was determined as a change in A_{450} of 0.1 per h per mg of non-hydrated pollen.

To further verify extracellular pollen TGase protein cross-linking activity, His6–Xpr–GFP was substituted for the biotin cadaverine. Pollen was germinated for 90 min into 96-microwell fluo black plates (Nunc Life Technologies) pre-coated with DMC in buffered germination medium. His6–Xpr–GFP (25 μg · ml^{-1}) was added for the last 30 min of germination in the absence or in the presence of increasing concentrations of anti-TGase antibody ID10 (3.5–21 μg · ml^{-1}), or 283 and 281 (0.05–5 mM) inhibitors. After removal of the pollen (used for the confocal analysis of the His6–Xpr–GFP cross-link to pollen tube wall proteins) and of the germination medium, the microwells were extensively washed with PBS. The fluorescence of His6–Xpr–GFP cross-linked to immobilized DMC was measured in an ELISA plate reader (Titertek Multiscan ELISA Spectrophotometer, Flow Laboratories) at wavelengths of 485 nm excitation and 535 nm emission. The cross-linking activity was quantified as RFU (relative fluorescence units). Data were expressed as RFU with 1 mM Ca^{2+} in the reaction buffer minus background values with 0.125 mM EDTA and autofluorescence. Positive control values were adjusted to 100 %, with the samples incubated with antibody or inhibitors reported as a percentage (means $\pm$ S.D.) of the control.

Analysis of *mono*- and *bis*-γ-glutamyl-polyamine derivatives

Proteins (0.3 mg) secreted into the culture medium by pollen germinated for 2 h were assayed for extracellular TGase activity and for pollen natural substrates [42] in the presence of 10 μl of [1,4(n)-^{3}H]spermidine (specific activity 1.5 TBq · mmol^{-1}; NEN), 40 mM Mes (pH 6.5), 10 mM DTT and 5 mM $CaCl_2$ in a final volume of 300 μl. In the negative control, $CaCl_2$ was substituted with 10 mM EDTA.

Histochemical staining of germinating pollen *in vitro*

The distribution of immunoreactive TGase and its cross-linked reaction products was determined by immunohistochemical and immunofluorescence staining. Intracellular immunostaining of TGase was carried out on pollen germinated for up to 120 min, after fixation, by digesting pollen for 7 min with 0.75 % pectinase (from *Rhizopus*) and 0.75 % cellulase (from *Penicillium*) [43]. Cell-wall digestion and membrane permeabilization was omitted for extracellular staining of TGase. Non-specific protein binding was blocked by incubating fixed pollen with 3 % (w/v) BSA in PBS (pH 7.4) for 2 h at 37 °C. For immunofluorescence staining, further blocking with 20 % (v/v) non-immune serum was carried out. Once transferred on to poly-lysine coated-slides, pollen was incubated with the following mouse monoclonal antibodies: T1A2 [anti-(tyrosinated α-tubulin subunit)], B512 [anti-(total α-tubulin)], ID10 (anti-TGase) or 81D4 [anti-(cross-link)] using non-immune mouse IgG as a negative control. Antibody–antigen binding was revealed using either HRP-conjugated rabbit anti-mouse IgG followed by incubation with AEC (3-amino-9-ethylcarbazole) as a peroxidase substrate, or FITC-conjugated rabbit anti-mouse IgG. Staining was observed by a video digital camera (Olympus DP10) producing digital images of at least six non-overlapping fields covering the central portion of each sample. In some experiments, germinated pollen was also treated with 2 M NaCl, 1 % (w/v) SDS or 1 % (w/v) DTT either prior to or after fixation. A polyclonal anti-(α-tubulin) antibody was used as the positive control and non-immune mouse IgGs raised in rabbit as the negative control, which were revealed by FITC-conjugated anti-rabbit IgGs. Immunogold detection of TGase was performed exactly as described previously [44]; the primary anti-TGase antibody was used at a dilution of 1:5, whereas the secondary goat anti-mouse IgG 15-nm gold conjugated was used at 1:20. *In situ* TGase activity was visualized by incubation of germinated pollen with His6–Xpr–GFP, a specific glutamine- and lysine-rich fluorescent substrate for ECM mammalian TGase modified as reported previously [36] at different concentrations (from 0.25 to 50 μg · ml^{-1}). Fluorescence staining, was observed using a Leica TCSNT confocal laser scanning microscope system (Leica Lasertechnik), with the PMT (photo-multiplier tube) adjusted to minimize autofluorescence emission in the negative control. Images from at least ten random fields were scored for the distribution of immunoreactive TGase and N^{ε}(γ-glutamyl)-PA or lysine cross-link present in pollen proteins with the aid of the Leica TCSNT (version 1.5-451) image processing menu. For double-staining, germinated pollen was incubated with recombinant His6–Xpr–GFP as described above and, after extensive washes, further incubated with either 81D4 or ID10, which were revealed by a TRITC-conjugated anti-mouse antibody. Double-staining was performed by adjusting the argon/krypton laser at 488 and 560 nm for fluorescein and rhodamine excitation respectively. As a further control, individual antibody staining was also performed in parallel with double-staining.

Histochemical staining of germinating pollen *in planta*

Fresh pollen was applied to the styles in apple flowers and after 48 h the styles were collected and solubilized in buffer [100 mM Pipes/KOH (pH 6.8), containing 10 mM $MgCl_2$, 10 mM EGTA, 0.1 % sodium azide and 0.05 % Triton X-100] supplemented with fixatives [1.5 % (w/v) paraformaldehyde and 0.05 % glutaraldehyde]. After 60 min at 4 °C the styles were cut longitudinally into four pieces using razor blades with the aid of a stereo microscope. The cell walls were digested with 0.75 % cellulysine and 0.75 % pectinase in buffer for 7 min in the dark. After washing with buffer, the primary antibody (ID10, diluted 1:20 in buffer) was added to samples and incubated for 1 h at 37 °C. Samples were then washed with buffer and incubated for 1 h at 37 °C with the FITC-conjugated goat anti-mouse IgG secondary antibody (Cappell) diluted 1:50 in buffer. Styles were finally mounted in anti-fade mountant (Citifluor) and observed with a Zeiss Axiophot fluorescence microscopy equipped with an AxioCam MRc5 and a 63× oil-immersion objective (Zeiss).

Statistics

The values reported are expressed as means ± S.D. and represent one of at least three or four different experiments undertaken in triplicate. Differences between sample sets were determined using a Student's *t* test with 95 % confidence limits. When indicated, statistical analysis was performed using GraphPad Prism (version 5.0a McIntosh GraphPad software). The percentage data from pollen germination were analysed after arcsin → p transformation. Pearson correlation analysis was performed when necessary.

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RESULTS

Localization of TGase in pollen tubes by immunological techniques

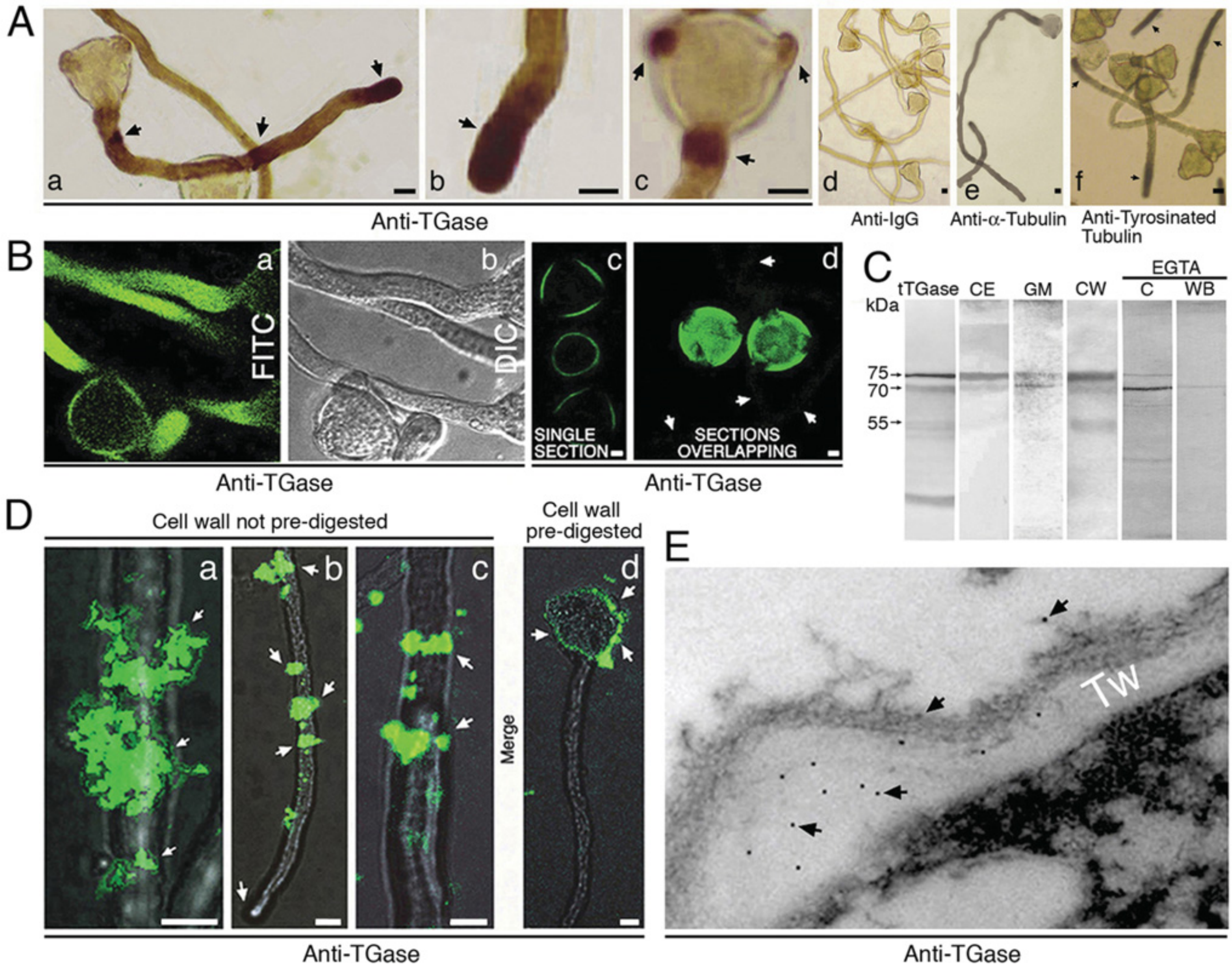
To localize TGase in pollen tubes, pollen was allowed to germinate for 120 min *in vitro*, after which the pollen tube wall was incubated with or without cellulase and pectinase, and its membrane permeabilized [43] prior to probing with the ID10 anti-TGase monoclonal antibody. This monoclonal antibody has been shown to immunoreact with partially purified TGase extracted from apple pollen [26]. Two well-characterized anti-tubulin antibodies were used as positive controls and anti-mouse IgGs as negative controls. Cellulase-digested and membrane-permeabilized pollen tubes resulted in intracellular staining for TGase in the neck region and along the shank of the pollen tube, especially at the apical tip [Figure 1A (a–c)]. Anti-(α-tubulin) staining was distributed along the tube, whereas tyrosinated α-tubulin was enriched at the growing tip [Figure 1A (d–f)], in agreement with previous findings [45,46]. The distribution of TGase along the pollen tube was confirmed by confocal immunostaining (Figure 1B). To specifically visualize extracellular TGase, the staining with ID10 was replicated in non-digested and non-permeabilized germinating pollen [Figure 1D (a–c)]. In these conditions, extracellular TGase was detected along the pollen tube surface, in aggregates outside the tube and also on the pollen grain. Treatment with 2 M NaCl, 1 % (w/v) SDS or 1 % (w/v) DTT prior to fixation and immunolabelling did not dissociate the TGase from the tube wall, suggesting that either a covalent or a stable interaction between the enzyme and the pollen tube cell wall had occurred [Figure 1D (a,b)]. Fixation did not affect the immunolabelling, as incubation with the antibody prior to fixation led to a similar pattern of TGase staining [Figure 1D (c)]. Digestion of the cell wall with cellulase and pectinase following the immunostaining with ID10 resulted in an almost complete loss of the detection of extracellular TGase, except from around the pollen grain wall (which is protected by the cellulase-resistant sporopollenin) [Figure 1D (d)]. No sign of osmotic stress was observed. The localization of TGase was also investigated by immunogold labelling, which showed the presence of TGase in the pollen tube wall and surface (Figure 1E, arrows). Western blotting using the anti-TGase antibody revealed two immunoreactive bands of 70 and 75 kDa, the latter more marked in crude extracts of germinating pollen (Figure 1C, lane CE), in concentrated germination medium in which germinated pollen had been removed by filtration (Figure 1C, lane GM) and in cell walls (Figure 1C, lane CW). A weakly reactive band of a lower molecular mass (approx. 55 kDa) was observed in the cell-wall extracts (Figure 1C, lane CW). As a positive control, Western blotting was conducted on partially purified 70 kDa TGase isolated from pollen supernatant by hydrophobic ion chromatography (Figure 1C, lanes C and WB) [26]. This EGTA-eluted fraction was immunodetected in a dose-dependent manner by ID10 and by AbIII, another monoclonal anti-TGase antibody that recognizes plant TGases (Supplementary Figure S1 at http://www.BiochemJ.org/bj/429/bj429ppppadd.htm) as reported by Della Mea et al. [29]. The partially purified 70 kDa TGase catalyses transamidating and cross-linking reactions, as described by Del Duca et al. [26].

Inhibition of extracellular TGase activity leads to inhibition of pollen germination and tube apical growth

Evidence for the activity of the extracellular TGase immunodetected in viable pollen tubes was obtained by allowing pollen to germinate in the wells of microtitre plates (to 78 ± 5 % germination) followed by *in situ* determination of calcium-dependent TGase-mediated incorporation of biotin cadaverine into DMC immobilized in the plate wells (EGTA-treated samples were used as the control). Typical values of extracellular TGase activity are shown in Figures 2(A) and 2(B) (control bars, bottom histograms). The pH optimum for the extracellular pollen TGase activity was determined to be 6.5, which is also the optimum pH of the medium to allow pollen germination. Extracellular pollen TGase activity (Figure 2, bars), percentage germination (Figure 2, lines) and tube growth (Figure 2, top images) were then measured in the presence of TGase inhibitors and a TGase-specific antibody (Figures 2A and 2B respectively). All three events were inhibited in a dose-dependent manner by the monoclonal antibody ID10 (Figure 2A) and by the two site-directed irreversible inhibitors of TGase activity, 283 and 281 (Figure 2B). The presence of the inhibitors resulted in shorter and thicker pollen tubes with a decreased growth rate within the first 30 min of germination, followed by an inability to further extend the tip, between 30 and 60 min after germination (Figures 2A and 2B). Eventually, tube-burst occured in a dose-dependent manner for antibody concentrations higher than 7 $\mu g \cdot ml^{-1}$ and at inhibitor concentrations above 100 μM. Inhibition of TGase activity and pollen tube growth were not observed with the control antibody (Figure 2A). The significant correlation between pollen germination and TGase activity when inhibited by ID10, 281 and 283 respectively, is supported by high Pearson coefficient values. In a similar fashion, other less-specific inhibitors of TGase activity, cystamine and iodoacetamide (50–1000 μM), inhibited enzyme activity and pollen tube growth in a dose-dependent manner; at 1 mM, cystamine and iodoacetamide inhibited the TGase activity by 57 ± 3 % and 44 ± 5 % respectively; the *in vivo* supply of 1 mM cystamine inhibited the tube growth by 40 ± 4 %. A competitive substrate of TGase activity, putrescine (assayed in the range 1 μM–2.5 mM) led to an inhibition of tube growth between 30 ± 4 % and 53 ± 2 %. Further confirmation that ID10 inhibited pollen TGase was obtained by immunoprecipitation of partially purified pollen TGase [26], which led to a dose-dependent inhibition of TGase activity, whereas immunoprecipitation using the negative control antibody (monoclonal anti-horse albumin) produced no effect (Supplementary Figure S2 at http://www.BiochemJ.org/bj/429/bj429ppppadd.htm). Western blot analysis to determine the specificity of ID10 showed that it reacted only to a single 75 kDa band corresponding to TGase 2 in gpl homogenates and did not recognize TGase 1 or TGase 3 confirming an anti-TGase 2 antibody (Supplementary Figure S3 at http://www.BiochemJ.org/bj/429/bj429ppppadd.htm).

Effect of glutamine- and lysine-rich TGase substrate on pollen germination and tube elongation

Experiments were performed to evaluate the effect on pollen of His6–Xpr–GFP, a specific glutamine- and lysine-rich substrate



COLOUR

Figure 1 Immunolocalization of pollen TGase during germination

(**A**) Pollen was germinated for 2 h and fixed, permeabilized and probed with monoclonal antibodies directed against TGase 2 (ID10) (a–c), α-tubulin (e) and tyrosinated α-tubulin (f). Negative control: non-specific mouse IgG (d). Magnifications of the distal part (b), the neck region and the proximal parts (c) of germinated pollen stained for TGase. Scale bar: 8 μm. (**B**) Laser-scanning confocal microscopy of germinated pollen tubes treated as in (**A**), and probed with a secondary FITC-conjugated antibody. TGase staining along the tube and at the apex (a) and visualization of the same sample in phase contrast (b). Staining with non-specific mouse IgG followed by secondary antibody as 2 μm-single section (c) or overlaid sections (d); arrows indicate the pollen tubes, whose autofluorescence was minimized. Scale bar: 8 μm. (**C**) TGase-active fractions (50 μg of protein) of germinated pollen extract (lane CE), germination medium (lane GM) and cell-wall extract (lane CW) were analysed by Western blotting with the monoclonal anti-TGase antibody ID10. The partially purified TGase from pollen supernatant (isolated by HIC and eluted with EGTA) was evaluated both by Coomassie Blue gel staining (lane C) and by Western blotting with the anti-TGase antibody ID10 (lane WB). 'tTGase' in the first lane indicates the commercial gpl TGase used as a positive control. (**D**) The cell wall of non-digested and non-membrane-permeabilized 120 min-germinated pollen was treated with 2 M NaCl, 1 % (w/v) SDS or 1 % (w/v) DTT, fixed and then stained with the anti-TGase antibody ID10 (a and b). In order to prove that the staining was not affected by fixation, ID10 was incubated for the last 30 min of germination in the medium prior to fixation (c). The arrows indicate the ID10 immunolabelling. In some cases, the cell wall was further digested and the residual labelling was localized around the grain (arrows) (d). Scale bar: 8 μm. (**E**) Immunogold labelling of apple pollen tubes conducted with the anti-TGase antibody ID10. Labelling was mainly found in association with the amorphous layer of cell wall (arrows) in the flanks of pollen tubes. Scale bar: 200 nm. Tw, tube wall.

of mammalian ECM TGases [36]. Incubation of His6–Xpr–GFP in the germination medium during pollen germination and subsequent *in situ* localization by confocal microscopy in non-digested and non-permeabilized pollen, revealed that His6–Xpr–GFP was associated extracellularly with the pollen tube wall (Figure 3A). The addition of His6–Xpr–GFP at 0.25 $\mu g \cdot ml^{-1}$ gave rise to a staining located within 20 μm from the tip; increasing the concentrations (1–50 $\mu g \cdot ml^{-1}$) resulted in a proportional increase in the incorporation along the tube and into aggregates (Figure 3A). At the highest concentration (50 $\mu g \cdot ml^{-1}$), equivalent to that used for mammalian tissue [36], longer segments of tube were fluorescently labelled, with the additional formation of larger extracellular structures similar to those recognized by the anti-TGase monoclonal antibody ID10 (Figure 1D). Extracellular pollen TGase-mediated cross-linking of His6–Xpr–GFP to pollen tube proteins was confirmed by inhibition with 281, 283 and by the blocking effect of ID10 (Figure 3B). Unexpectedly, at concentrations between 1 and 25 $\mu g \cdot ml^{-1}$, His6–Xpr–GFP resulted in a significant increase in the percentage of germinated pollen, when compared with the pollen incubated with control mouse IgG ($P < 0.05$; Table 1). Incubation with 5 $\mu g \cdot ml^{-1}$ of this fluorescent TGase substrate resulted in longer pollen tubes when compared with incubation with the control mouse IgG (Figure 3C). These effects were not produced by unmodified GFP, rGFPuv (results not shown). A modified microtitre 'fluo black' plate assay was set up for

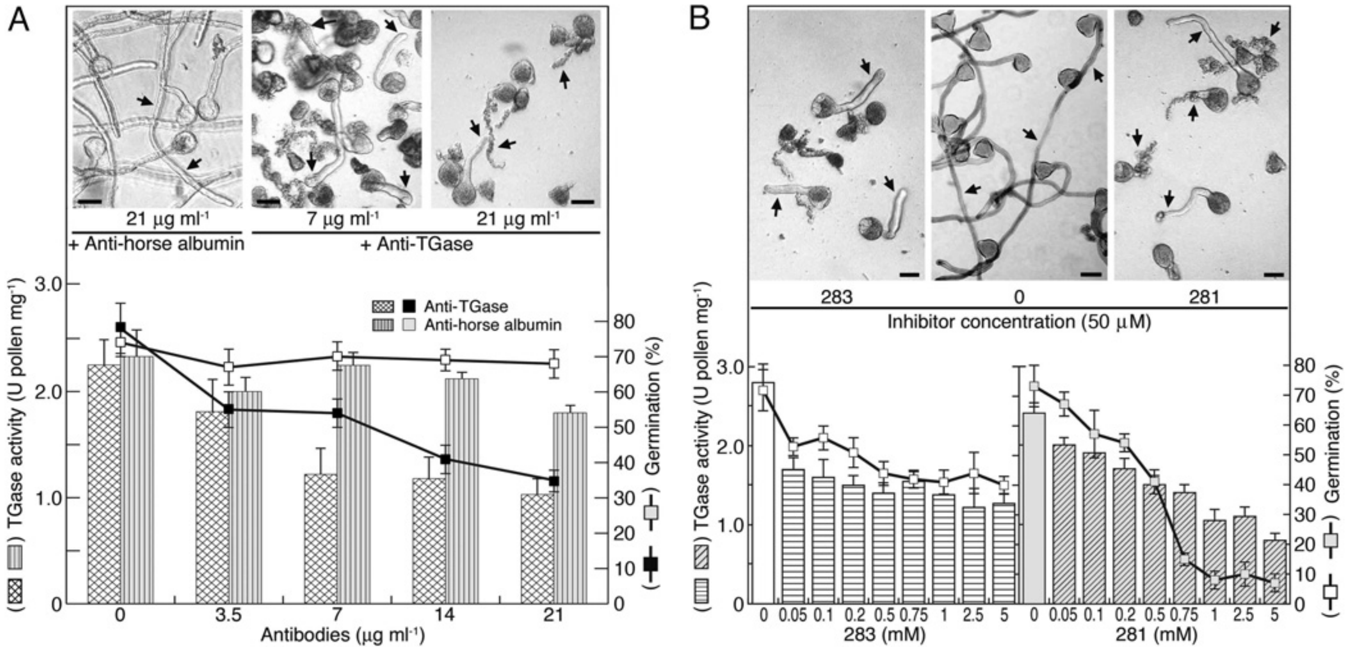


Figure 2 Inhibition of TGase activity affects pollen tube growth

(**A**) Effect of anti-TGase ID10 on pollen tube morphology, germination and extracellular TGase activity. Pollen was incubated at zero time with ID10 or control antibodies against horse albumin, in microtitre plates, and allowed to germinate up to 120 min. Pollen tube morphology was observed by light microscopy (upper panels) in the presence of control anti-horse albumin (left-hand panel) and increasing concentrations of ID10 (middle and right-hand images). Pollen tube morphological malformations and diffuse bursting are indicated by arrows. Scale bar: 24 μm. Bottom panels: lines show the percentage (means ± S.D.; $n = 10$) of germination with ID10 (■) or control antibody (□); microplate assay of TGase activity of germinating pollen (bars) treated with ID10 (crossed lines) or anti-horse albumin (vertical lines) and expressed as units (U) per mg of pollen. The correlation analysis between pollen germination and TGase activity, when inhibited by ID10, gave a Pearson coefficient of $R = 0.92$ ($P = 0.03$). (**B**) Effect of two irreversible inhibitors of TGase on pollen tube morphology, germination and extracellular TGase activity. Pollen was germinated, as described in (**A**), and, at zero time, incubated with 50 μM 283 and 281 or control Mes buffer (0). Pollen tube morphological malformations are indicated by arrows (upper panels). Scale bar: 24 μm. Bottom plot: lines show the percentage of germination (means ± S.D.; $n = 10$) in the presence of inhibitors 283 (white squares) and 281 (grey squares). TGase activity was reported, as in (**A**), in the presence of 283 (bars with horizontal lines) or 281 (bars with oblique lines). The correlation analysis between pollen germination and TGase activity when inhibited by 281 and 283 respectively, gave a Pearson coefficient of $R = 0.94$ ($P < 0.0001$) and 0.92 ($P < 0.0005$).

the detection of the cross-linking activity of extracellular TGase of germinating pollen, which involved incubating pollen in germination medium containing His6–Xpr–GFP in wells coated with immobilized DMC. Fluorescence measurement showed cross-linking of His6–Xpr–GFP to DMC (Figure 3D, control). This activity was inhibited using both the anti-tTGase antibody ID10, and the inhibitors 281 and 283 (Figure 3D). Both sets of data suggest that during pollen germination the detected cross-linking is mediated by extracellular TGase activity. Evidence that endogenous pollen proteins are substrates for pollen TGase was obtained by immunohistochemical staining with 81D4 (Figure 3E). This antibody specifically binds TGase-mediated protein–protein and amine–protein cross-links, reacting with bis-PA derivatives > glutamine–lysine isodipeptide linkage > mono-PA derivatives, but not with their free counterparts [47]. The distribution of anti-cross-link staining was observed along the pollen tube surface (Figure 3E). The ability of pollen extracellular TGase to incorporate the exogenously supplied His6–Xpr–GFP in the same sites of pollen protein–protein and protein–amine cross-links revealed by 81D4 was demonstrated by their co-localization in confocal micrographs (Figure 4A). In addition, the extracellular pollen TGase co-localized within the same accumulation sites of His6–Xpr–GFP cross-link (Figure 4B).

Table 1 Evaluation of the effect of His_6-Xpr-GFP on pollen germination

His_6-Xpr-GFP and control mouse IgG were added at different concentrations into the medium containing hydrated pollen at zero time and left up to 120 min germination. The data are expressed as the percentage of germination (means ± S.D.). *P* values were obtained by using a paired Student's *t* test between control and His_6-Xpr-GFP-treated pollen of ten samples of three independent experiments. Values were significantly different at *$P < 0.05$.

	Percentage of germination	
Concentration (μg · ml^{-1})	His_6-Xpr-GFP	IgG
0.10	68 ± 6	67 ± 1
0.25	69 ± 4	72 ± 3
0.50	82 ± 5	77 ± 1
1	88 ± 3*	78 ± 5
5	88 ± 2*	76 ± 2
25	80 ± 2*	70 ± 2
50	82 ± 2	76 ± 2

Presence of pollen extracellular TGase in the germination medium

The ability of extracellular pollen TGase to conjugate PAs to pollen proteins secreted during germination *in vitro* was analysed by incubation with the TGase amine substrate [^{3}H]spermidine as a tracer. The proteins were extracted from the concentrated germination medium after 120 min of germination and the removal of pollen. The germination medium contained 1560 d.p.m. and 1816 d.p.m. of *mono*-(γ-glutamyl) spermidine and *bis*-(γ-glutamyl) spermidine derivatives per g of germinated pollen respectively. These data confirm the presence of TGase catalysed reaction products in the pool of *in-vitro*-secreted pollen proteins.

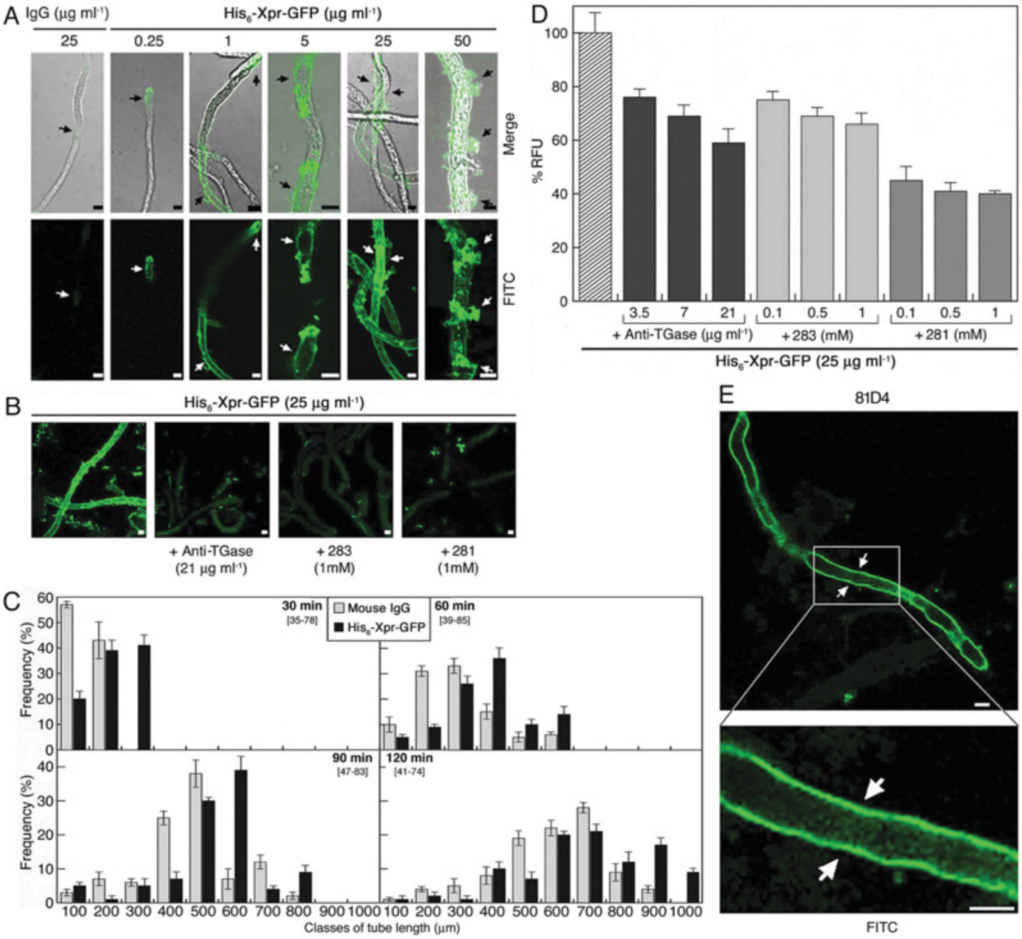


Figure 3 Extracellular TGase cross-linking during pollen germination

(**A**) Laser-scanning confocal microscopy at 120 min-germination of undigested and non-permeabilized pollen, His6–Xpr–GFP was added for the last 30 min of germination in the medium prior to fixation. Superimposition of the GFP labelling on the phase-contrast image (upper panel); GFP labelling (bottom panel). Control: non-specific mouse IgG followed by FITC-antibody. Arrows indicate His6–Xpr–GFP fluorescence. Scale bar: 8 μm. (**B**) Laser confocal microscopy of pollen, incubated and treated as in (**A**), but in the presence of either the anti-TGase ID10 or 281/283 inhibitors for the final 30 min of germination. Scale bar: 8 μm. (**C**) Pollen was germinated from zero time in the presence of $5\,\mu g \cdot ml^{-1}$ His6–Xpr–GFP (black bars) or mouse IgG (grey bars) and the tube length was evaluated via a digital camera at 30, 60, 90 and 120 min and reported as the relative frequency (means $\pm$ S.D. of 100–1000 μm tube length). Values in square brackets report the lowest and the highest number of measured pollen tubes for each optical field. (**D**) Microplate assay of extracellular TGase cross-linking activity in the presence of increasing concentrations of the anti-TGase antibody ID10 and the inhibitors 283 and 281 supplied to pollen for the last 30 min of germination, as indicated above in (**A**). The fluorescence of His6–Xpr–GFP cross-linked by the pollen extracellular TGase to immobilized DMC was measured and compared with the values obtained by standard free gpl TGase (control, oblique line bar). Relative fluorescence units (RFU) of the treated samples were reported as the percentage (means $\pm$ S.D.) of the control normalized to 100 % (12580 $\pm$ 1133 RFU). (**E**) TGase-mediated cross-linking products immunolocalized by 81D4 [monoclonal antibody against N^{ε}(γ-glutamyl)-lysine] on 120-min-germinated unfixed pollen (neither digested nor permeabilized). Arrows indicate the cross-link immunodetection. Scale bar: 8 μm.

In planta localization of extracellular pollen TGase

To confirm the presence of extracellular TGase *in vivo*, pollen tubes germinated (*in planta)* in the stylar tissue of carpels were fixed and stained by indirect immunofluorescence using ID10. As shown in Figure 5, anti-TGase reactivity was localized in extracellular aggregates along the pollen tube surface in a similar distribution to that observed *in vitro* (Figures 1D and 3A). By contrast, no ID10 staining was observed on the surface of stylar tissue, as shown by the black background (Figure 5, arrows).

DISCUSSION

The data presented here provides evidence that in germinating apple pollen there is a catalytically active TGase, which is localized extracellularly and is involved in pollen tube growth.

Localization

The model presented in Figure 6 summarizes the main results discussed below. In the non-permeabilized *Malus* pollen tube TGase was found in discrete aggregates outside the tube both *in vitro* (Figures 1D and 1E) and *in planta* (Figure 5). In permeabilized germinated pollen, TGase antibody labelling was observed at the apical tip of the growing pollen tube (Figure 1A), suggesting that accumulated TGase is present in and/or released from the pollen tube apex. As the pollen tube elongates during its growth, the released TGase could be deposited along the tube wall, as shown by extracellular TGase labelling on the pollen tube surface both *in vitro* and *in planta* (Figures 1D, 1E and 5). However, the mechanism by which pollen TGase and tTGase of mammalian cells [22] is actually secreted in the psECM or ECM respectively remains unknown. The intracellular distribution of TGase (Figure 1B) in the pollen tubes appears to be within

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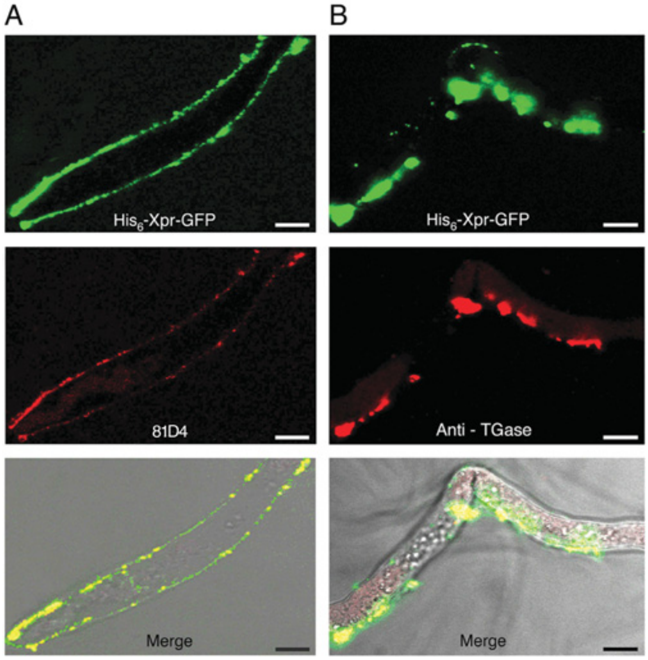

Figure 4 Co-association of protein cross-linking and extracellular TGase on the surface of 120 min-germinated pollen

(**A**) Germinating pollen tubes were incubated *in vivo* for the last 30 min of germination with His6–Xpr–GFP prior to fixing and, omitting cell-wall digestion and permeabilization, incubated with 81D4 [monoclonal antibody against $N^{\varepsilon}(\gamma$-glutamyl)-lysine and -PAs], followed by TRITC-conjugated secondary antibody. In the different panels, the positive staining indicates the localization of TGase-mediated cross-linking products (His6–Xpr–GFP incorporation) (upper), immunostaining with 81D4 (centre) and superimposition of the His6–Xpr–GFP and 81D4/TRITC-immunolabelled pollen tubes (merge bottom panel). Scale bar: 8 μm. (**B**) Germinating pollen tubes were treated as in (**A**) but stained with the anti-TGase antibody ID10. Localization of TGase-mediated cross-linking products by His6–Xpr–GFP (upper panel), immunostaining with ID10 (centre panel), and superimposition of His6–Xpr–GFP and ID10/TRITC immunolabelling (merge bottom panel). Scale bar: 8 μm.

vesicle-like structures. These may be the same or similar to those containing cell-wall precursors and enzymes that are released from the apical tube tip [5]. Subcellular fractionation of *Nicotiana* petal cells led to the detection of TGase antigen and activity in the cell wall and microsomal fraction [29], suggesting the compartmentalization of plant TGase in membrane structures.

The cross-reactivity of antibodies raised against animal TGases to react with plant TGases has previously been reported in studies on different plant tissues [24,29]. In germinated apple pollen, two immunoreactive polypeptides of 75 and 70 kDa (Figure 1C) cross-reacted with the same anti-TGase antibody (ID10) used to visualize the 70 kDa TGase partially purified from ungerminated apple pollen [26]. It is not known whether the two TGase forms have a specific role or different characteristics; they may differ in their calcium-binding properties which allowed only the 70 kDa band to bind to the HIC column used for purification. Supplementary Figure S1 shows that the 70 kDa polypeptide cross-reacted with the two different anti-TGase antibodies (ID10 and AbIII). The 70 kDa TGase showed *in vitro* calcium-dependent transamidating activity on cytoskeletal substrates and was inhibited by TGase inhibitors [26], well-characterized by Balklava et al. [22] and by Baumgartner et al. [33]. The detection of the 70 and the 75 kDa forms in both the cell wall and in the germination medium (Figure 1C), suggests that they are both secreted. We cannot exclude the possibility that the 70 kDa form has been derived from the 75 kDa form from a post-translational modification, and that this may have occured

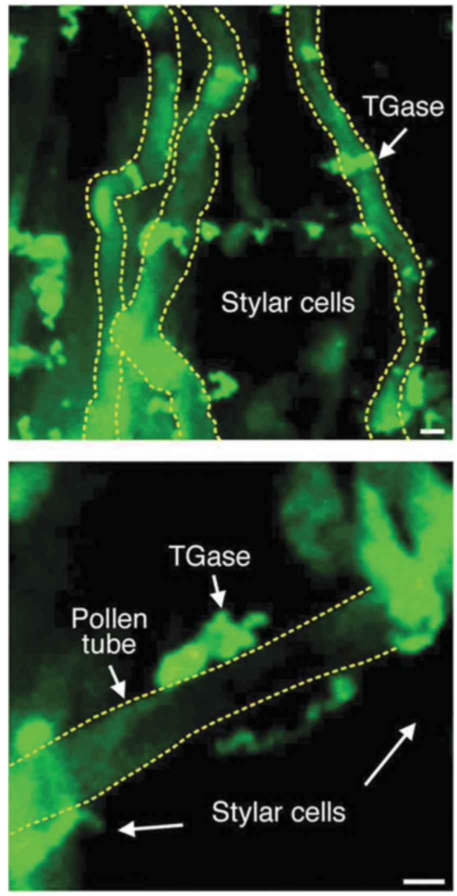

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Figure 5 Immunolocalization of pollen TGase during germination *in planta*

Following flower pollination, the pollen tubes were left to grow for 48 h into the stigma/style. The latter was fixed and anti-TGase ID10-probed (pollen tubes delineated as interrupted lines). Pollen tubes growing inside the style are shown in the upper panel; magnification of the clusters around the pollen tubes is reported in lower panel. Scale bar: 8 μm.

prior to or post secretion. The reduction in size has also altered the ability of the 70 kDa forms to interact with an HIC column in the presence of Ca^{2+}. Further work will be needed to characterize the roles of these TGase isoforms in pollen germination.

The additional 55 kDa immunoreactive polypeptide observed on Western blots (Figure 1C) of the cell-wall fraction has the same molecular mass as an active TGase extracted from the *Nicotiana* petal cell wall and may be involved in cell-wall strengthening [29]. A similar role in cell-wall organization has also been suggested for a TGase from the unicellular green alga, *Chlamydomonas reinhardtii* [28]. Only one TGase gene in the apple genome seems to occur similarly to AtPNg1p in the *Arabidopsis* genome (R.Velasco and A. Cestaro, personal communication). Q2

Substrates

The presence of PAs in apple pollen (ungerminated or germinated), as well as their release from the pollen tube has previously been established [6,7]. In kiwi pollen, free TCA (trichloroacetic acid)-soluble and -insoluble conjugated PAs were also detected, with spermidine conjugates being the most abundant conjugated PA [9]. Inhibition of PA biosynthesis caused a severe inhibition of pollen tube growth, possibly due to reduced binding to cell wall polysaccharides [11]. The PA presence in the cell wall of higher plants and their deficiency, which induced

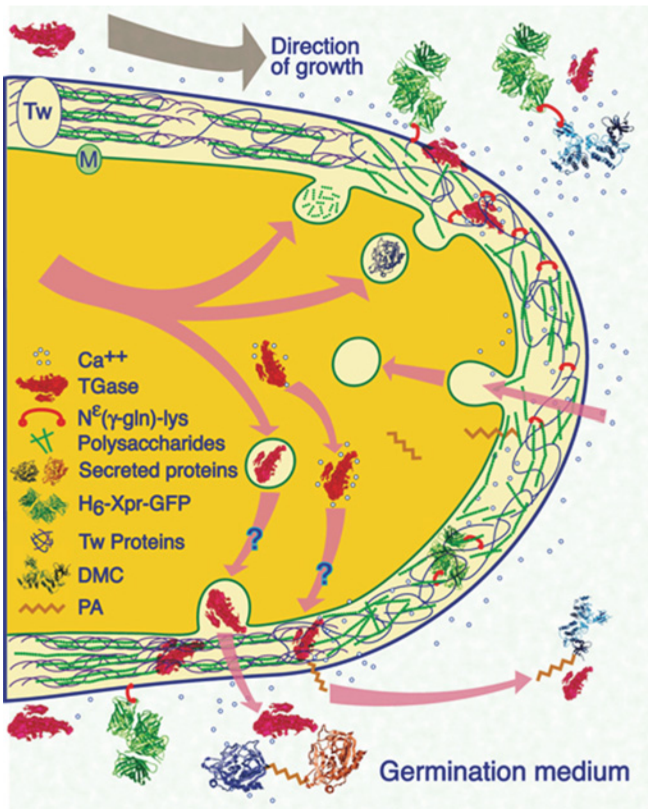

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Figure 6 Model of secreted TGase regulating *in vitro* pollen tube growth

In the pollen tube apical zone distally growing (grey arrows), TGase would accumulate: (i) intracellularly at the tip as such or in vesicles, (ii) outside the membrane (M) in the thin wall (under construction) and (iii) released outside (pink arrows). In the apical tube wall, TGase cross-links tube wall (Tw) proteins without forming aggregates. Once released into the Ca^{2+}-rich germination medium, TGase may cross-link proteins or incorporate PAs into pollen proteins, also secreted. As the pollen tip is continuously elongating during pollen germination, TGase might remain behind along the tube wall and be embedded, under the effect of local micro-environmental conditions, into the same accumulation sites of the protein cross-links catalysed by its enzymic activity.

loosening of the fibrillar component, are pertinent to the structure of the cell wall. In vegetative tobacco cells, microfibrillar cellulose structures and pectic substances of the cell wall were altered under treatment with PA biosynthesis inhibitors [12]. PAs are also suggested to contribute to vegetative cell-wall assembly by forming amide bonds with pectins [13]. HCA (hydroxycinnamic acid)-PAs have been found in the pollen of various plant species, thus suggesting a general role in modulating the rigidity of the cell wall [15]. The triferuloyl–spermidine conjugate has a possible role in the organization of the *Arabidopsis* pollen cell wall, and as a component of the tryphine (constituents of the pollen coat), which is involved in pollination and in pollen–stigma interaction [14].

PAs are known to be substrates for TGase enzymes [18]. The intracellular PAs of apple pollen were reported to be conjugated by TGase to cytoskeletal proteins regulating their functional properties [26]. In the present study the occurrence of pollen protein cross-links in the germination medium was demonstrated by the detection of labelled glutamyl-PA derivatives. The detection of a TGase in the apple pollen cell wall (Figures 3D and 3E) could support a role for protein-conjugated PAs in pollen-tube growth and stability. TGase activity in apple pollen extracts could be detected using a PA-incorporation assay and inhibited by an anti-TGase antibody and specific TGase inhibitors (Figures 2A and 2B).

The secreted apple pollen TGase catalysed the incorporation of two exogenous substrates (i.e. His6–Xpr–GFP and DMC) into the pollen cell-wall proteins (Figures 3A and 3D), and this incorporation was inhibited by specific TGase inhibitors (Figure 3B). The presence of TGase-mediated protein cross-links was demonstrated by *in situ* immunodetection using the anti cross-link monoclonal antibody 81D4 (Figures 3E and 4A). The identity of these protein substrates is not known.

In pollen, we have shown that TGase activity could be stimulated by a high concentration of substrates (Figure 3A for higher His6–Xpr–GFP concentration), which possibly acts as a nucleation centre for further cross-linking. The concomitant enhancement of pollen tube growth associated with His6–Xpr–GFP protein cross-linking at the pollen tube surface suggests that His6–Xpr–GFP facilitates pollen tube apical growth. The finding that both anti-TGase antibodies and inhibitors (mainly acting extracellularly) block His6–Xpr–GFP binding to the cell wall and inhibit pollen tube growth is a clear indication that the catalytic activity of extracellular pollen TGase is critical for correct pollen tube growth in *Malus*.

The co-localization between extracellular TGase antigen and cross-linked products suggests that apple pollen TGase could become embedded, under the effect of local micro-environmental conditions, into the same accumulation sites of the protein cross-links catalysed by its enzyme activity. The localization of TGase-catalysed cross-linked aggregates along the shank of pollen tubes is similar to that observed in the cell wall during the pulsatory growth of pollen tubes in flowering plants with a solid style. However, the distribution of pectins and arabinogalactan proteins reported in the literature does not fit exactly with the irregular distribution of the TGase-catalysed aggregates [48]. These discontinuous cross-linked structures may reflect oscillations of other factors influencing TGase activity and pollen growth, e.g. changes in Ca^{2+} and pH [1]. These collective results indicate that when the apple pollen TGase is externalized it remains biologically active.

Common aspects with other TGases

The evidence that extracellular TGase activity from germinating pollen tubes is inhibited by compounds that inhibit animal tTGase (281 and 283, iodoacetamide and cystamine) [16,30–33] and by anti-TGase antibodies (Figures 2A and 2B) strongly suggests that the plant enzymes are catalytically similar to animal TGases. In mammalian cells, imidazolium derivatives such as 283 and 281 have been described as potent inhibitors of mammalian TGases [22,30,32]. The inhibitor 281 is reported to act primarily outside the cell [33] and to inhibit pollen TGase activity by reducing the fluorescein-cadaverine cross-linking to pollen proteins during germination [27].

When the germination occurs *in planta* inside the style, the secreted TGase could have a potential role in the adhesion of pollen tube to stylar cells, similar to the role of mammalian tTGase in cell–ECM interactions [19–23], thus allowing style anchorage and subsequent tube migration. Interestingly, in mammalian cells extracellular tTGase remains embedded in the ECM thus acting as a structural adhesive protein, as well as a cross-linking enzyme involved in cell adhesion [20]. tTGase has been shown to co-localize with fibronectin and integrin-β1 at focal-adhesion structures [49,50], where it acts as an integrin co-receptor; once secreted, tTGase modifies the ECM by cross-linking fibronectin and collagen thus leading to the modulation of cell adhesions [20].

The above reported similarity between animal tTGase and pollen TGase is confirmed by the evidence that partially purified 70 kDa TGase extracted from pollen shares similar properties to gpl TGase in affecting the functional properties of the cytoskeleton [26]. The inhibition of pollen TGase by 281, which covalently binds the cysteine residue at the active site of mammalian TGase, also suggests the presence of a similar cysteine residue at the active site in *Malus* pollen TGase. This agrees with the observations that (i) the typical catalytic triad of the TGase family (His–Cys–Asp) [16,17] is conserved in *Arabidopsis* TGase (AM745095) [37] and (ii) the deduced polypeptide encoded by an EST (expressed sequence tag) of the *Malus* database (Tree Fruit Technology, Michigan State University), exhibits 62 % sequence identity with *Arabidopsis* TGase (increasing to 77 % identity with TGase active-site containing regions).

In conclusion, the data presented in the present paper suggest that pollen TGase plays an essential role in successful apple pollen tube growth. The enzyme could, therefore, function by *in vitro* protein cross-linking and amine protein conjugation, strengthening the cell-wall scaffold of the extending pollen tube. *In planta*, we hypothesize that the extracellular TGase might be involved in connecting the pollen tube to surrounding stylar cells as it progresses through the psECM. Work in progress will help to further characterize the molecular mechanisms by which pollen TGase facilitates successful pollen tube growth.

Author contribution

The idea to study the TGase in pollen to clarify its role during germination was proposed by Donatella Serafini Fracassini and Stefano Del Duca, being already authors of papers on this subject. The experiments were designed in collaboration with Martin Griffin, at that time head of the laboratory, Philip Bonner, Alan Hargreaves and Elisabetta Verderio, experts of TGase mainly in mammals. The research has been performed in the laboratories of Nottingham Trent University, University of Bologna and University of Siena. Alessia Di Sandro characterized the pollen enzyme and studied the regulation of the growth of pollen tubes. Stefano Del Duca collaborated in the characterization of the pollen enzyme. Elisabetta Verderio designed the confocal microscopy studies. Alan Hargreaves collaborated in the characterization of the pollen enzyme and antibody. Alessandra Scarpellini took care of the co-localization studies at the confocal microscopy. Giampiero Cai, Mauro Cresti and Claudia Faleri collaborated in the immunolocalization of TGase in the *in vivo* germinated pollen using the immunogold technique. Rosa Anna Iorio collaborated in the study of the regulation of pollen germination. Shigehisa Hirose and Yutaka Furutani prepared the recombinant substrate His6–Xpr–GFP. Ian Coutts produced the inhibitors. Martin Griffin contributed by both suggesting experiments and providing the antibody and the inhibitors, prepared in his laboratory. Philip Bonner collaborated by characterizing the enzyme using biochemical methods. Donatella Serafini-Fracassini was the supervisor of the entire study, suggesting new experiments and collaborations with the University of Yokohama and University of Siena. The manuscript preparation and response to reviewer comments were performed mainly by Alessia Di Sandro, Stefano Del Duca, Elisabetta Verderio, Alan Hargreaves, Giampiero Cai, Roda Anna Iorio, Martin Griffin, Philip Bonner and Donatella Serafini-Fracassini.

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SUPPLEMENTARY ONLINE DATA

An extracellular transglutaminase is required for apple pollen tube growth

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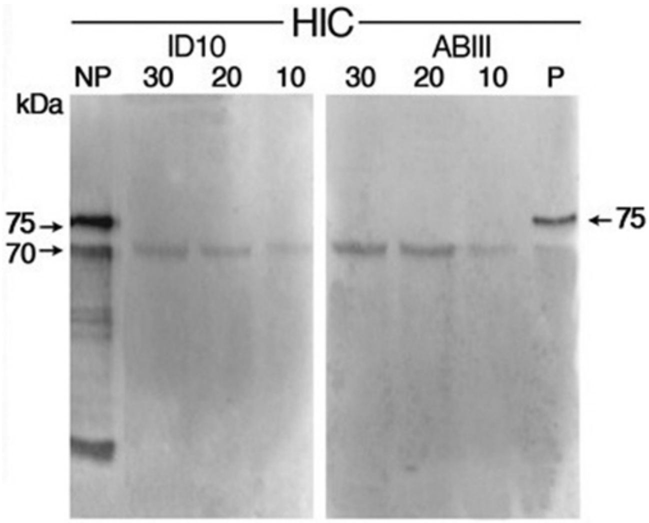

Figure S1 Western blot of an active pollen fraction partially purified from hydrated pollen by hydrophobic interaction chromatography (HIC), set up to purify calcium-dependent TGases, assayed at 30, 20 and 10 μg of protein and both not purified (NP) and purified (P) gpl TGase, comparatively probed with anti-TGase antibodies ID10 and AbIII

The molecular mass in kDa is indicated on the left-hand side.

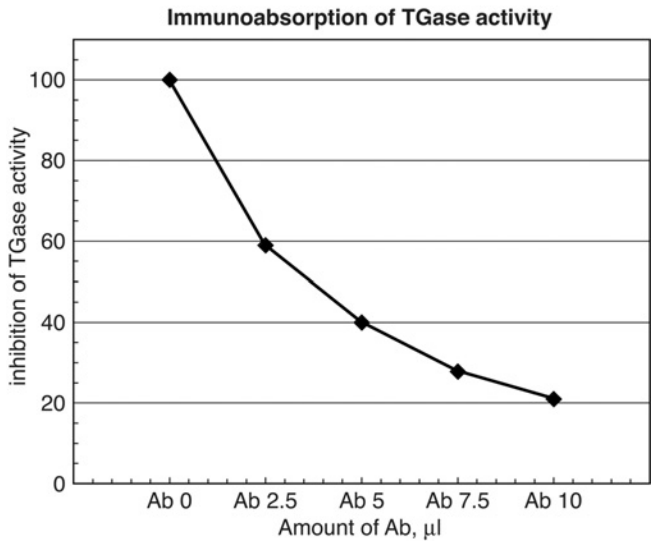

Figure S2 Percentage of residual TGase activity in the pollen SN after immunoprecipitation obtained with increasing amounts of anti-TGase antibody ID10

Proteins were extracted from hydrated pollen grains.

[1] Correspondence may be addressed to either of these authors (email philip.bonner@ntu.ac.uk or donatella.serafini@unibo.it).

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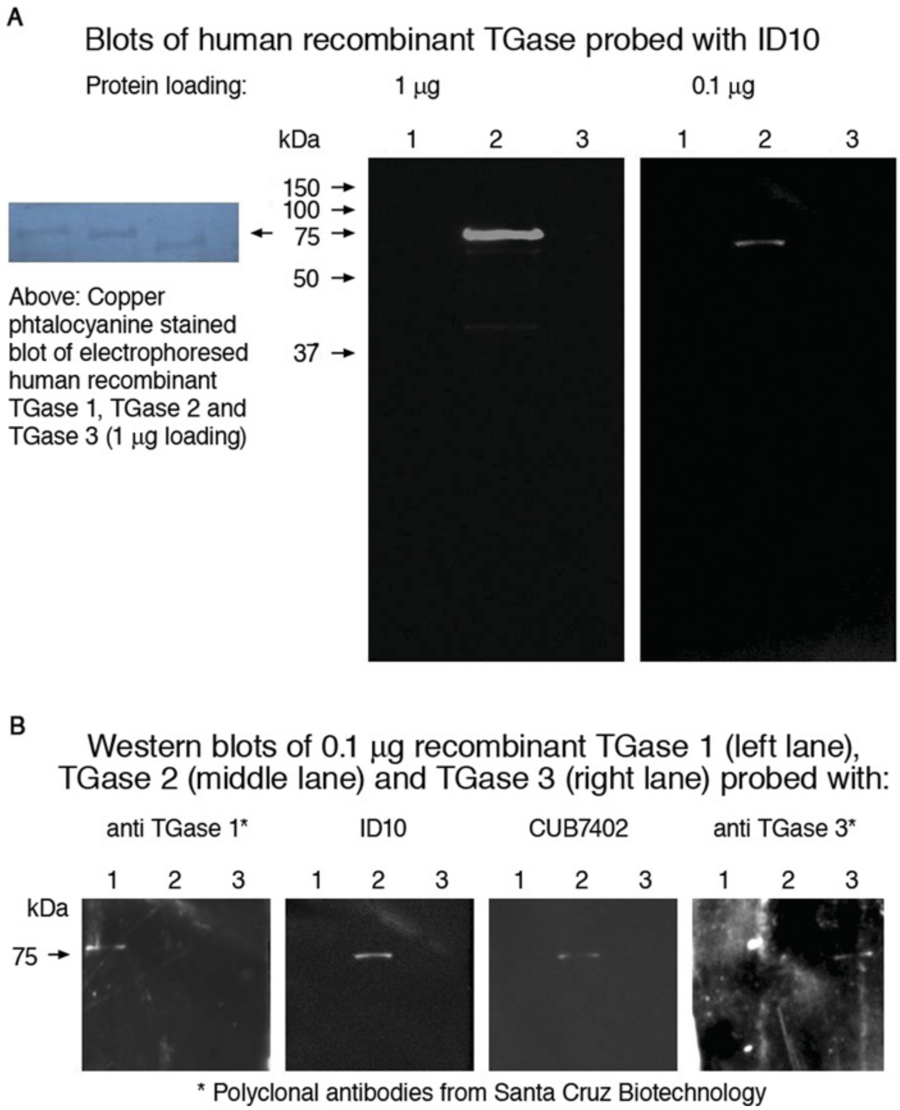

Q4 **Figure S3 ????**

(**A**) Western blots of 1 μg and 0.1 μg of human recombinant TGase 1 (lane 1), TGase 2 (lane 2) and TGase 3 (lane 3) probed with the anti-TGase antibody ID10; (**B**) Western blots of 0.1 μg of human recombinant TGase 1 (lane 1), TGase 2 (lane 2) and TGase 3 (lane 3) probed with anti-TGase antibodies ID10, CUB7402, anti-TGase 1 and anti-TGase 3. These results show the specificity of ID10 towards TGase 2.

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