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## An extracellular transglutaminase is required for apple pollen tube growth

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**Running title:** *extracellular pollen transglutaminase*

**Abbreviations used:** BSA, bovine serum albumin; DMC, N',N'-dimethyl casein; DTT,  
dithiothreitol; ECM, extra-cellular matrix; FA, focal adhesion; FITC, fluorescein  
isothiocyanate; GFP, green fluorescent protein; gpl TGase, guinea pig liver  
transglutaminase; HIC, hydrophobic interaction chromatography; PA, polyamine; PBS,  
phosphate-buffered saline; RFU, relative fluorescence units; SD, spermidine; SDS-  
PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TGase,  
transglutaminase; tTGase, tissue transglutaminase; TRITC, tetramethyl rhodamine iso-  
thiocyanate.

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651 **SYNOPSIS**

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53 An extracellular form of the  $\text{Ca}^{2+}$ -dependent protein-cross-linking enzyme  
54 transglutaminase (TGase) was demonstrated to be involved in the apical growth of  
55 *Malus domestica* pollen tube. Apple pollen transglutaminase and its substrates were co-  
56 localized within aggregates on the pollen tube surface, as determined by indirect  
57 immuno-fluorescence staining and the *in situ* cross-linking of fluorescently labeled  
58 substrates. Transglutaminase-specific inhibitors and an anti-TGase monoclonal  
59 antibody blocked pollen tube growth, whereas incorporation of a recombinant  
60 fluorescent mammalian TGase substrate (histidine-tagged green fluorescent protein:  
61 His<sub>6</sub>-Xpr-GFP) into the growing tube wall enhanced tube length and germination,  
62 consistent with a role of TGase as modulator of cell wall building and strengthening.  
63 The secreted pollen TGase catalyzed the cross-linking of both polyamines (PAs) into  
64 proteins (released by the pollen tube) and His<sub>6</sub>-Xpr-GFP into endogenous or  
65 exogenously added substrates. A similar distribution of TGase activity was observed *in*  
66 *planta* on pollen tubes germinating inside the style, consistent with a possible additional  
67 role for TGase in the interaction between the pollen tube and the style during  
68 fertilization.

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

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THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20100291

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## 74 INTRODUCTION

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

96 The interactions between the pollen tube and the psECM are reminiscent of the  
97 cell-ECM interactions in animal cells [5]. One key modulator of the ECM in mammals  
98 is tissue transglutaminase (tTGase also known as TGase 2), a member of a large family  
99 of enzymes [16, 17] that catalyzes the calcium dependent posttranslational modification  
100 of proteins, by forming covalent cross-links between glutamyl- and either lysyl-residues  
101 ( $N^{\epsilon}$ ( $\gamma$ -glutamyl) lysine isodipeptide) or PAs (*mono* and *bis*-( $\gamma$ -glutamyl) polyamines)  
102 resulting in a bridge between two or more proteins [18]. In animal cells, extracellular  
103 tTGase is concentrated at focal adhesions (FA), which are sites of communication  
104 between the ECM and intracellular signaling molecules [19-21]. After secretion,  
105 tTGase becomes a non-enzymically active structural component of the ECM, forming a  
106 close association with the glycoprotein fibronectin and heparan sulphate proteoglycans  
107 important in cellular adhesion and migration [20, 22, 23]. Transglutaminases have been  
108 shown to be present in plant tissue [24], including apple pollen, where it is an  
109 intracellular  $Ca^{2+}$ -dependent enzyme catalysing the incorporation of PAs into  $\alpha$ -tubulin,  
110 actin monomers and uncharacterised large protein complexes [25]. Recently  
111 monoclonal antibodies raised against mammalian tTGase (e.g. ID10) have been shown  
112 to cross-react with apple pollen TGase and that the enzyme's *in vitro* activity can be  
113 blocked by an animal TGase inhibitor. In addition, this partially purified apple pollen  
114 TGase has been shown to be capable of catalysing the *in vitro* cross-linking of a  
115 number of purified cytoskeletal components. This suggests that pollen TGase may be  
116 capable of modulating the properties of the pollen cytoskeleton and possibly the  
117 subsequent development of pollen tubes [26]. The presence of TGase substrates in  
118 germinating apple pollen has also been demonstrated by their *in situ* visualization in the  
119 pollen grain, and the elongated pollen tubes as well as the pollen surface [27]. Other  
120 studies have reported the presence of a TGase like activity in the cell wall of higher  
121 plant parenchyma or of lower plant cells whose cell wall composition is different from  
122 that of pollen tubes [24, 28, 29]. The above data suggests that an extracellular TGase  
123 maybe present in the cell wall of apple pollen tubes, and may contribute to the

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124 interaction of pollen proteins with psECM proteins, by catalyzing protein-protein  
125 linkages.

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

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136

## 137 MATERIAL AND METHODS

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### 139 Chemicals and Antibodies

140

141 All chemicals (unless otherwise indicated) were obtained from Sigma-Aldrich (Milan,  
142 Italy). The tTGase inhibitors 283 (1,3-dimethyl-2[(oxopropyl)thio]imidazolium) [30]  
143 (now available also at Zedira, Darmstadt, Germany) and 281 (N-Benzyloxycarbonyl-  
144 L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine), two specific site-directed  
145 inhibitors of TGase, developed to covalently bind the Cys-based active site of the  
146 calcium activated form of mammalian TGase [31- 34] were synthesised at Nottingham  
147 Trent University, UK. The mouse monoclonal antibody ID10 raised against purified  
148 guinea pig liver (gpl) TGase (tTGase) was produced at Nottingham Trent University  
149 and selected by its reactivity to gpl TGase and ion exchange-purified extracts of 14 day  
150 old *Pisum sativum* leaf tissue and germinated *Vicia faba* cotyledons [35]. The TGase-  
151 reactive clone antibody was partially purified from hybridoma culture supernatant by  
152 cation exchange chromatography on Fast Flow S-sepharose (Amersham Biosciences,  
153 Little Chalfont, UK). ELISA-positive fractions were pooled, concentrated in a dialysis  
154 tube against PEG 8,000 and stored in aliquots at  $-20^{\circ}\text{C}$ . The immuno-reactivity of ID10  
155 for pollen TGase, purified by hydrophobic interaction chromatography, was compared  
156 to TGase antibody, AbIII (Neomarker, Fremont, CA). Reactivity of ID10 was also  
157 tested against different cell fractions of *Agaricus* and compared to the cross-reactivity  
158 obtained by CUB7402 on the same cells (personal communication of M. Della Mea,  
159 University of Bologna).

160

161 Guinea pig liver TGase was obtained from Sigma-Aldrich (Milan, Italy) and  
162 further purified in our laboratory. Recombinant His<sub>6</sub>-Xpr-GFP (a TGase substrate) was  
163 produced at the Tokyo Institute of Technology (Yokohama, Japan) [36]. Primary  
164 antibodies include: monoclonal anti-TGase 2 Ab-I [clone Cub7402] (Neomarker,  
165 Fremont, CA), polyclonal antibody AtPng1P (produced in chicken at the CSIC of  
166 qBarcelona, Spain, as already reported [37]; 81D4 monoclonal anti-N<sup>ε</sup>( $\gamma$ -  
167 glutamyl)lysine (Covalab, Lyon, France); 2c10 monoclonal anti-horse albumin (a gift  
168 from Prof. E.E. Billett, Nottingham Trent University). The following antibodies were  
169 obtained from Sigma-Aldrich (Milan, Italy) unless otherwise indicated. T1A2 (anti-  
170 tyrosinated  $\alpha$ -tubulin subunit); B512 (anti-total  $\alpha$ -tubulin); non-immune mouse IgG  
171 (used as negative control); polyclonal anti  $\alpha$ -tubulin antibody. The list of secondary  
172 antibodies includes: HRP-conjugated rabbit anti-mouse IgG; FITC-conjugated rabbit  
anti-mouse IgG (DakoCytomation, Glostrup, Denmark); FITC-conjugated goat anti-

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173 mouse IgG secondary antibody (Cappell, Malvern, PA); FITC-conjugated goat anti-  
174 rabbit IgGs; goat anti-mouse IgG 15nm gold conjugated (Biocell).

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### 176 **Plant material and growth**

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

181

### 182 ***In vitro* germination**

183

184 Pollen was re-hydrated and allowed to germinate as previously described [25] into glass  
185 Petri dishes or in N',N'-dimethyl casein (DMC) coated 96-well microtitre plates (Nunc  
186 Life Technologies, Paisley, UK) or 96-well microtitre fluo black plates (Nunc Life  
187 Technologies, Paisley, UK) for up to 120 min, in the presence and absence of His<sub>6</sub>-Xpr-  
188 GFP, TGase inhibitors (281, 283, iodoacetamide, cystamine) and anti-TGase antibodies  
189 added as specified in the figure legends at zero time of germination or for the last 30  
190 min to allow pollen tube to partially grow. As the monoclonal antibody ID10 gave the  
191 strongest labeling it was used for further experiments. Light microscopy digital images  
192 of at least three non-overlapping fields covering the central part of each chamber were  
193 captured using a video digital camera (Olympus DP10) and quantified in terms of  
194 germinated versus ungerminated pollen, when the pollen tube was either absent or  
195 shorter than the pollen grain diameter. Pollen viability was assessed by staining with  
196 fluorescein diacetate (10 µg ml<sup>-1</sup>).

197

### 198 **Pollen protein extraction**

199

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

205

### 206 **Partial purification of pollen TGase**

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208 The partial purification was performed by a hydrophobic interaction chromatography  
209 (HIC) with Phenyl-Sepharose 6 Fast Flow (Sigma-Aldrich, Milan, Italy) at pH 8 [26].  
210 Partially purified TGase was immuno-detected after SDS-PAGE and western blotting  
211 with two tTGase antibodies (ID10 and AbIII).

212

### 213 **Immuno-precipitation of pollen TGase**

214

215 Aliquots (200 µl) of HIC purified pollen extract (0.2 mg ml<sup>-1</sup>) were incubated with  
216 increasing amount of ID 10 monoclonal antibody for 2 h on ice, and the samples  
217 immuno-precipitated as described [23]. The supernatant was assayed for TGase activity  
218 [19] and compared to that of the pre-cleaned sample obtained after incubation of HIC  
219 purified pollen extract (see above) directly with protein G-agarose beads (control)  
220 (Sigma-Aldrich, Poole, UK). The difference between the activities in the supernatants  
221 obtained after ID10 treatment and the control gave a quantitative indication of the

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222 TGase activity lost due to the binding of the pollen TGase to the protein G-coupled  
223 ID10.

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### 225 **SDS-PAGE and Western blot analysis**

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227 A total of 50 µg of germinated pollen proteins obtained after (i) extraction after low  
228 speed centrifugation or from pollen cell walls, or (ii) following purification via HIC or  
229 (iii) after the concentration of the germination medium, were electrophoresed on 10%  
230 (w/v) polyacrylamide SDS-PAGE slab gels [40], using 0.2 µg gpl TGase as a standard.  
231 Western blotting was performed as described previously and protein bands that cross-  
232 reacted with ID10, AbIII, CUB 7402 or AtPng1P revealed accordingly to the literature  
233 [37].

234

### 235 **Transglutaminase activity assays**

236

237 Extracellular pollen TGase activity was measured by the conjugation of biotinylated  
238 cadaverine to DMC as described [19], with minor modifications. Native hydrated  
239 pollen grains (1 mg ml<sup>-1</sup> in germination medium) were allowed to germinate in the  
240 wells for 120 min at 30°C in germination medium (see above) buffered with 5 mM  
241 MES pH 6.5, containing 0.1 mM biotinylated cadaverine and 50 µM DTT. Germination  
242 was monitored by light microscopy throughout. After removal of germinated pollen by  
243 extensive washings with phosphate-buffered saline (PBS), the level of biotinylated  
244 cadaverine incorporated into immobilised DMC was revealed according to an  
245 established protocol [41] with EGTA replacing EDTA in negative controls. Data are  
246 expressed as Ca<sup>2+</sup>-dependent increase in A<sub>450</sub>, after subtraction of the value of the 5 mM  
247 EGTA-treated control. Specific activity was determined as a change in A<sub>450</sub> of 0.1 per  
248 hour per mg of non-hydrated pollen.

249

250 To further verify extracellular pollen TGase protein cross-linking activity, His<sub>6</sub>-  
251 Xpr-GFP was substituted for the biotin cadaverine. Pollen was germinated for 90 min  
252 into 96-microwell fluo black plate (Nunc Life Technologies, Paisley, UK) pre-coated  
253 with DMC in buffered germination medium. His<sub>6</sub>-Xpr-GFP (25 µg ml<sup>-1</sup>) was added for  
254 the last 30 min of germination in the absence or in the presence of increasing  
255 concentrations of anti-TGase antibody ID10 (3.5 - 21 µg ml<sup>-1</sup>), or 283 and 281 (0.05 - 5  
256 mM) inhibitors. After removal of the pollen (utilised for the confocal analysis of the  
257 His<sub>6</sub>-Xpr-GFP cross-link to pollen tube wall proteins) and of the germination medium,  
258 the microwells were extensively washed with PBS. The fluorescence of His<sub>6</sub>-Xpr-GFP  
259 cross-linked to immobilised DMC was measured in an ELISA plate reader (Titertek  
260 Multiscan ELISA Spectrophotometer, Flow Laboratories, Irvine, UK) at 485 nm  
261 excitation and 535 nm emission. The cross-linking activity was quantified as relative  
262 fluorescence units (RFU). Data were expressed as RFU with 1 mM Ca<sup>2+</sup> in the reaction  
263 buffer minus background values with 0.125 mM EDTA and autofluorescence. Positive  
264 control values were adjusted to 100%, with the samples incubated with antibody or  
265 inhibitors reported as percentage (mean ± SD) of the control.

266

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

268

269 Proteins (0.3 mg) secreted into the culture medium by pollen germinated for 2 h were  
270 assayed for extracellular TGase activity and for pollen natural substrates [42] in the  
presence of 10 µl of [1,4(n)-<sup>3</sup>H] spermidine (specific activity 1.5 TBq mmol<sup>-1</sup>; NEN,

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271 Milan, Italy), 40 mM MES pH 6.5, 10 mM DTT, and 5 mM CaCl<sub>2</sub> in a final volume of  
272 300 µl. In the negative control, CaCl<sub>2</sub> was substituted with 10 mM EDTA.

273

### 274 **Histochemical staining of germinating pollen *in vitro***

275

276 The distribution of immuno-reactive TGase and its cross-linked reaction products was  
277 determined by immuno-histochemical and immuno-fluorescence staining. Intracellular  
278 immuno-staining of TGase was carried out on pollen germinated for up to 120 min,  
279 after fixation, by digesting pollen for 7 min with 0.75% (w/v) pectinase (from  
280 *Rhizopus*) and 0.75% (w/v) cellulase (from *Penicillium*) [43]. Cell wall digestion and  
281 membrane permeabilization was omitted for extracellular staining of TGase. Non-  
282 specific protein binding was blocked by incubating fixed pollen with 3% (w/v) BSA in  
283 PBS pH 7.4, for 2 hours at 37°C. For immuno-fluorescence staining, further blocking  
284 with 20% (v/v) non-immune serum was carried out. Once transferred on poly-lysine  
285 coated-slides, pollen was incubated with the following mouse monoclonal antibodies:  
286 T1A2 (anti-tyrosinated α-tubulin subunit), B512 (antitotal α-tubulin), ID10 (anti-  
287 TGase) or 81D4 (anti-cross-link) using non-immune mouse IgG as a negative control.  
288 Antibody-antigen binding was revealed using either HRP-conjugated rabbit anti-mouse  
289 IgG followed by incubation with 3-amino-9-ethylcarbazole (AEC) as a peroxidase  
290 substrate, or FITC-conjugated rabbit anti-mouse IgG. Staining was observed by a video  
291 digital camera (Olympus DP10) producing digital images of at least 6 non-overlapping  
292 fields covering the central portion of each sample. In some experiments, germinated  
293 pollen was also treated with 2 M NaCl, 1% (w/v) SDS or 1% (w/v) DTT either prior to  
294 or after fixation. Polyclonal anti α-tubulin antibody was used as the positive control and  
295 non-immune mouse IgGs raised in rabbit as the negative control, which were revealed  
296 by FITC-conjugated anti-rabbit IgGs. Immuno-gold detection of TGase was performed  
297 exactly as previously described [44]; the primary anti-TGase antibody was used at the  
298 dilution of 1:5 while the secondary goat anti-mouse IgG 15-nm gold conjugated was  
299 used at 1:20. *In situ* TGase activity was visualized by incubation of germinated pollen  
300 with His<sub>6</sub>-Xpr-GFP, a specific glutamine and lysine rich fluorescent substrate for ECM  
301 mammalian TGase modified as reported [36] at different concentrations (from 0.25 to  
302 50 µg ml<sup>-1</sup>). Fluorescence staining, was observed using a Leica TCSNT confocal laser  
303 scanning microscope system (Leica Lasertechnik, Heidelberg, Germany), with the  
304 photo-multiplier tube (PMT) adjusted to minimize autofluorescence emission in the  
305 negative control. Images from at least 10 random fields, were scored for the distribution  
306 of immuno-reactive TGase and N<sup>ε</sup>(γ-glutamyl)-PA or lysine cross-link, present in  
307 pollen proteins with the aid of the Leica TCSNT (version 1.5-451) image processing  
308 menu. For double staining, germinated pollen was incubated with recombinant His<sub>6</sub>-  
309 Xpr-GFP as described before, and after extensive washes, further incubated with either  
310 81D4 or ID10, which were revealed by TRITC-conjugated anti-mouse antibody.  
311 Double staining was performed by adjusting the argon/krypton laser at 488 and 560 nm  
312 for fluorescein and rhodamine excitation, respectively. As a further control, individual  
313 antibody staining was also performed in parallel to double staining.

314

### 315 **Histochemical staining of germinating pollen *in planta***

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317 Fresh pollen was applied to the styles in apple flowers and after 48 h the styles were  
318 collected and solubilized in buffer (100 mM Pipes-KOH pH 6.8, containing 10 mM  
319 MgCl<sub>2</sub>, 10 mM EGTA, 0.1%w/v NaN<sub>3</sub>, and 0.05% v/v Triton X-100) supplemented  
320 with fixatives (1.5% w/v paraformaldehyde, 0.05% v/v glutaraldehyde). After 60 min at

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321 4°C the styles were cut longitudinally into four pieces using razor blades with the aid of  
322 a stereo microscope. The cell walls were digested with 0.75% (w/v) cellulysine and  
323 0.75% (w/v) pectinase in buffer for 7 min in the dark. After washing with buffer, the  
324 primary antibody (ID10, diluted 1:20 in buffer) was added to samples and incubated for  
325 1 h at 37°C. Samples were then washed with buffer and incubated for 1 h at 37°C with  
326 the FITC-conjugated goat anti-mouse IgG secondary antibody (Cappell, Malvern, PA)  
327 diluted 1:50 in buffer. Styles were finally mounted in anti fade mountant (Citifluor,  
328 London, UK) and observed with a Zeiss Axiophot fluorescence microscopy equipped  
329 with an AxioCam MRc5 and a 63x oil-immersion objective (Zeiss Inc., Oberkochen,  
330 Germany).

331

### 332 **Statistics**

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334 The values reported are expressed as mean  $\pm$  SD and represent one of at least 3 or 4  
335 different experiments undertaken in triplicate. Differences between sample sets were  
336 determined by the Student's t-test with 95% confidence limits. When indicated  
337 statistical analysis was performed using GraphPad Prism (version 5.0a McIntosh  
338 GraphPad software, San Diego, CA, USA). Percent data from pollen germination were  
339 analyzed after arcsin $\rightarrow$ p transformation. Pearson correlation analysis was performed  
340 when necessary.

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## 343 **RESULTS**

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### 345 **Localization of transglutaminase in pollen tubes by immunological techniques**

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347 To localize TGase in pollen tubes, pollen was allowed to germinate for 120 min *in*  
348 *vitro*, after which the pollen tube wall was incubated with or without cellulase and  
349 pectinase, and its membrane permeabilized [43] prior to probing with the ID10 anti-  
350 TGase monoclonal antibody. This monoclonal antibody has been shown to immuno-  
351 react with partially purified TGase extracted from apple pollen [26]. Two well-  
352 characterized anti-tubulin antibodies were utilised as positive controls and anti-mouse  
353 IgGs as negative controls. Cellulase-digested and membrane permeabilized pollen tubes  
354 resulted in intracellular staining for TGase in the neck region and along the shank of the  
355 pollen tube, especially at the apical tip (Figure 1A a-c). Anti  $\alpha$ -tubulin staining was  
356 distributed along the tube, whereas tyrosinated  $\alpha$ -tubulin was enriched at the growing  
357 tip (Figure 1A d-f), in agreement with previous findings [45-46]. The distribution of  
358 TGase along the pollen tube was confirmed by confocal immuno-staining (Figure 1B).  
359 To specifically visualize extracellular TGase, the staining with ID10 was replicated in  
360 non-digested and non-permeabilised germinating pollen (Figure 1D a-c). In these  
361 conditions, extracellular TGase was detected along the pollen tube surface, in  
362 aggregates outside the tube and also on the pollen grain. Treatment with 2 M NaCl, 1%  
363 (w/v) SDS or 1% (w/v) DTT prior to fixation and immuno-labeling did not dissociate  
364 the TGase from the tube wall, suggesting either a covalent or a stable interaction  
365 between the enzyme and the pollen tube cell wall had occurred (Figure 1D a-b).  
366 Fixation did not affect the immuno-labeling, as incubation with the antibody prior to  
367 fixation led to a similar pattern of TGase staining (Figure 1D c). Digestion of the cell  
368 wall with cellulase and pectinase following the immuno-staining with ID10 resulted in  
369 an almost complete loss of the detection of extracellular TGase, except from around the  
370 pollen grain wall (which is protected by the cellulase-resistant sporopollenin) (Figure

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371 1D d). No sign of osmotic stress was observed. The localization of TGase was also  
372 investigated by immuno-gold labeling, which showed the presence of TGase in the  
373 pollen tube wall and surface (Figure 1E, arrows). Western blotting utilizing anti-TGase  
374 antibody revealed two immuno-reactive bands of 70 and 75 kDa, the latter more  
375 marked, in crude extracts of germinating pollen (Figure 1C, lane CE), in concentrated  
376 germination medium in which germinated pollen had been removed by filtration  
377 (Figure 1C, lane GM), and in cell walls (Figure 1C lane CW). A weakly reactive band  
378 of lower molecular weight (around 55 kDa) was observed in the cell wall extracts  
379 (Figure 1C lane CW). As a positive control, western blotting was conducted on partially  
380 purified 70 kDa TGase isolated from pollen supernatant by hydrophobic ion  
381 chromatography [26], (Figure 1C, lane C, WB). This EGTA-eluted fraction was  
382 immuno-detected in a dose dependent manner by ID10 and by AbIII, another  
383 monoclonal anti-TGase antibody that recognizes plant TGases (Supplementary Figure  
384 S1) as reported by Della Mea *et al.* [29]. The partially purified 70 kDa TGase catalyses  
385 transamidating and cross-linking reactions, as described by Del Duca *et al.* [26].  
386

### 387 **Inhibition of extracellular TGase activity leads to inhibition of pollen germination** 388 **and tube apical growth**

389  
390 Evidence for the activity of the extracellular TGase immuno-detected in viable pollen  
391 tubes was obtained by allowing pollen to germinate in the wells of microtitre plates (to  
392 78±5% germination) followed by *in situ* determination of Ca<sup>2+</sup>-dependent TGase-  
393 mediated incorporation of biotin cadaverine into DMC immobilized in the plate wells  
394 (EGTA-treated samples were used as the control). Typical values of extracellular  
395 TGase activity are shown in Figure 2A,B (control bars, bottom histograms). The pH  
396 optimum for the extracellular pollen TGase activity was determined to be 6.5, which is  
397 also the optimum pH of the medium to allow pollen germination. Extracellular pollen  
398 TGase activity (Figure 2, bars), percentage germination (Figure 2, lines) and tube  
399 growth (Figure 2, top images) were then measured in the presence of TGase inhibitors  
400 and a TGase specific antibody (Figure 2A,B, respectively). All three events were  
401 inhibited in a dose dependent manner by the monoclonal antibody ID10 (Figure 2A)  
402 and by the two site-directed irreversible inhibitors of TGase activity, 283 and 281  
403 (Figure 2B). The presence of the inhibitors resulted in shorter and thicker pollen tubes  
404 with a decreased growth rate within the first 30 min of germination, followed by an  
405 inability to further extend the tip, between 30 and 60 min after germination (Figure  
406 2A,B). Eventually, tube-burst occurred in a dose dependent manner for antibody  
407 concentrations higher than 7 µg ml<sup>-1</sup> and at inhibitor concentrations above 100 µM.  
408 Inhibition of TGase activity and pollen tube growth were not observed with the control  
409 antibody (Figure 2A). The significant correlation between pollen germination and  
410 TGase activity when inhibited by ID10, 281 and 283 respectively, is supported by high  
411 value Pearson coefficients. In a similar fashion, other less specific inhibitors of TGase  
412 activity, cystamine and iodoacetamide (50 –1000 µM) inhibited enzyme activity and  
413 pollen tube growth in a dose dependent manner; at 1 mM, cystamine and iodoacetamide  
414 inhibited the TGase activity by 57±3% and 44±5% respectively; the *in vivo* supply of 1  
415 mM cystamine inhibited the tube growth by 40±4%. A competitive substrate of TGase  
416 activity, putrescine (assayed in the range 1 µM-2.5 mM) led to an inhibition of tube  
417 growth between 30±4% and 53±2%. Further confirmation that ID10 inhibited pollen  
418 TGase was obtained by immuno-precipitation of partially purified pollen TGase [26],  
419 which led to a dose-dependent inhibition of TGase activity, whereas immuno-  
420 precipitation using the negative control antibody (monoclonal anti-horse albumin)

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421 produced no effect (Supplementary Figure S2). Western blot analysis to determine the  
422 specificity of ID10 showed that it reacted only to a single 75 kDa band corresponding  
423 to TGase 2 in gpl homogenates and did not recognize TGase 1 or TGase 3 confirming  
424 an anti-TGase 2 antibody (Supplementary Figure S3).

425

#### 426 **Effect of Gln and Lys-rich TGase substrate on pollen germination and tube** 427 **elongation**

428

429 Experiments were performed to evaluate the effect on pollen of His<sub>6</sub>-Xpr-GFP, a  
430 specific glutamine- and lysine-rich substrate of mammalian ECM TGases [36].  
431 Incubation of His<sub>6</sub>-Xpr-GFP in the germination medium during pollen germination and  
432 subsequent *in situ* localization by confocal microscopy, in non-digested and non-  
433 permeabilised pollen, revealed that His<sub>6</sub>-Xpr-GFP was associated extracellularly with  
434 the pollen tube wall (Figure 3A). The addition of His<sub>6</sub>-Xpr-GFP at 0.25 µg ml<sup>-1</sup> gave  
435 rise to a staining located within 20 µm from the tip; increasing the concentrations (1-50  
436 µg ml<sup>-1</sup>) resulted in a proportional increase in the incorporation along the tube and into  
437 aggregates (Figure 3A). At the highest concentration (50 µg ml<sup>-1</sup>), equivalent to that  
438 used for mammalian tissue [36], longer segments of tube were fluorescently labelled,  
439 with the additional formation of larger extracellular structures similar to those  
440 recognized by the anti-TGase monoclonal antibody ID10 (Figure 1D). Extracellular  
441 pollen TGase-mediated crosslinking of His<sub>6</sub>-Xpr-GFP to pollen tube proteins was  
442 confirmed by inhibition with 281, 283 and by the blocking effect of ID10 (Figure 3B).  
443 Unexpectedly, at concentrations between 1 and 25 µg ml<sup>-1</sup>, His<sub>6</sub>-Xpr-GFP resulted in a  
444 significant increase in the percentage of germinated pollen, when compared to the  
445 pollen incubated with control mouse IgG ( $p < 0.05$ ; Table 1). Incubation with 5 µg ml<sup>-1</sup>  
446 of this fluorescent TGase substrate resulted in longer pollen tubes when compared to  
447 incubation with the control mouse IgG (Figure 3C). These effects were not produced by  
448 unmodified GFP, rGFPuv (data not shown). A modified microtiter “fluo black” plate  
449 assay was set up for the detection of cross-linking activity of extracellular TGase of  
450 germinating pollen, which involved incubating pollen in germination medium  
451 containing His<sub>6</sub>-Xpr-GFP in wells coated with immobilised DMC. Fluorescence  
452 measurement showed cross-linking of His<sub>6</sub>-Xpr-GFP to DMC (Figure 3D, control).  
453 This activity was inhibited using both the anti-tTGase ID10 and the inhibitors 281 and  
454 283 (Figure 3D). Both sets of data suggest that during pollen germination the detected  
455 cross-linking is mediated by extracellular TGase activity. Evidence that endogenous  
456 pollen proteins are substrates for pollen TGase was obtained by immuno-histochemical  
457 staining with 81D4 (Figure 3E). This antibody specifically binds TGase-mediated  
458 protein-protein and amine-protein cross-links, reacting with bis-PA derivatives > gln-  
459 lys isodipeptide linkage > mono-PA derivatives but not with their free counterparts [47].  
460 The distribution of anti cross-link staining was observed along the pollen tube surface  
461 (Figure 3E). The ability of pollen extracellular TGase to incorporate the exogenously  
462 supplied His<sub>6</sub>-Xpr-GFP in the same sites of pollen protein-protein and protein-amine  
463 cross-links revealed by 81D4 was demonstrated by their co-localisation in confocal  
464 micrographs (Figure 4A). In addition, the extracellular pollen TGase co-localized  
465 within the same accumulation sites of His<sub>6</sub>-Xpr-GFP cross-link (Figure 4B).

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#### 467 **Presence of pollen extracellular TGase in the germination medium**

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469 The ability of extracellular pollen TGase to conjugate PAs to pollen proteins secreted  
470 during germination *in vitro* was analyzed by incubation with the TGase amine substrate

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471 [<sup>3</sup>H]spermidine as a tracer. The proteins were extracted from the concentrated  
472 germination medium after 120 min of germination and the removal of pollen. The  
473 germination medium contained 1560 DPM and 1816 DPM of *mono*-( $\gamma$ -glutamyl)  
474 spermidine and *bis*-( $\gamma$ -glutamyl) spermidine derivatives per g of germinated pollen,  
475 respectively. These data confirm the presence of TGase catalyzed reaction products in  
476 the pool of *in vitro* secreted pollen proteins.

477

#### 478 ***In planta* localization of extracellular pollen transglutaminase**

479

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

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#### 488 **DISCUSSION**

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

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#### 494 **Localization**

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496 The model presented in Figure 6 summarizes the main results discussed below. In the  
497 non-permeabilised *Malus* pollen tube TGase was found in discrete aggregates outside  
498 the tube both *in vitro* (Figure 1D,E) and *in planta* (Figure 5). In permeabilised  
499 germinated pollen, TGase antibody labelling was observed at the apical tip of the  
500 growing pollen tube (Figure 1A), suggesting that accumulated TGase is present in  
501 and/or released from the pollen tube apex. As the pollen tube elongates during its  
502 growth, the released TGase could be deposited along the tube wall, as shown by  
503 extracellular TGase labeling on the pollen tube surface both *in vitro* and *in planta*  
504 (Figures 1D,E and 5). However, the mechanism by which pollen TGase and tTGase of  
505 mammalian cells [22] is actually secreted in the psECM or ECM respectively remains  
506 unknown. The intracellular distribution of TGase (Figure 1B) in the pollen tubes  
507 appears to be within vesicle-like structures. These may be the same or similar to those  
508 containing cell wall precursors and enzymes that are released from the apical tube tip  
509 [5]. Sub-cellular fractionation of *Nicotiana* petal cells led to the detection of TGase  
510 antigen and activity in the cell wall and microsomal fraction [29], suggesting the  
511 compartmentalisation of plant TGase in membrane structures.

512

513 The cross-reactivity of antibodies raised against animal TGases to react with plant  
514 TGases has previously been reported in studies on different plant tissues [24, 29]. In  
515 germinated apple pollen, two immuno-reactive polypeptides of 75 and 70 kDa (Figure  
516 1C) cross-reacted with the same anti-TGase antibody (ID10) used to visualize the 70  
517 kDa TGase partially purified from ungerminated apple pollen [26]. It is not known if  
518 the two TGase forms have a specific role or different characteristics; they may differ in  
519 their calcium binding properties which allowed only the 70 kDa band to bind to the  
520 hydrophobic interaction column used for purification. Supplementary Figure S1 shows  
521 that 70 kDa band matches with both ID10 and AbIII. The 70 kDa TGase showed *in*

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521 *vitro* Ca<sup>2+</sup>-dependent transamidating activity on cytoskeletal substrates and was  
522 inhibited by TGase inhibitors [26] well characterised by Balklava *et al.* [22] and by  
523 Baumgartner *et al.* [33]. The detection of the 70 and the 75 kDa forms in both the cell  
524 wall and in the germination medium (Figure 1C), suggests that they are both secreted.  
525 We cannot exclude the possibility that the 70kDa form has been derived from the  
526 75kDa form from a post-translational modification and that this may have occurred  
527 prior to or post secretion. The reduction in size has also altered the ability of the 70kDa  
528 forms to interact with an HIC column in the presence of Ca<sup>2+</sup>. Further work will be  
529 needed to characterise the roles of these TGase isoforms in pollen germination.

530 The additional 55 kDa immuno-reactive polypeptide observed on Western blots  
531 (Figure 1C) of the cell wall fraction has the same molecular mass as an active TGase  
532 extracted from *Nicotiana* petal cell wall and may be involved in cell wall strengthening  
533 [29]. A similar role in cell wall organization has also been suggested for a TGase from  
534 the unicellular green alga, *Chlamydomonas reinhardtii* [28]. Only one TGase gene in  
535 apple genome seems to occur similarly to AtPNglp in Arabidopsis genome (personal  
536 communication of Dr. R.Velasco and Dr. A. Cestaro, IASMA).

537

### 538 Substrates

539

540 The presence of PAs in apple pollen (ungerminated or germinated), as well as their  
541 release from the pollen tube has previously been established [6, 7]. In kiwi pollen, free,  
542 TCA-soluble and -insoluble conjugated PAs were also detected, with spermidine (SD)  
543 conjugates being the most abundant conjugated PA [9]. Inhibition of PAs biosynthesis  
544 caused a severe inhibition of pollen tube growth, possibly due to reduced binding to cell  
545 wall polysaccharides [11]. The PA presence in the cell wall of higher plants and their  
546 deficiency, which induced loosening of the fibrillar component, are pertinent to the  
547 structure of the cell wall. In vegetative tobacco cells microfibrillar cellulose structures  
548 and pectic substances of the cell wall were altered under treatments with PA  
549 biosynthesis inhibitors [12]. PAs are also suggested to contribute to vegetative cell wall  
550 assembly by forming amide bonds with pectins [13]. Hydroxycinnamic acid (HCA)-  
551 PAs have been found in the pollen of various plant species, thus suggesting a general  
552 role in modulating the rigidity of the cell wall [15]. The triferuloyl-SD conjugate has a  
553 possible role in the organization of the *Arabidopsis* pollen cell wall, and as a  
554 component of the tryphine (constituents of the pollen coat), which is involved in  
555 pollination and in pollen-stigma interaction [14].

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

565 The secreted apple pollen TGase catalyzed the incorporation of two exogenous  
566 substrates (i.e. H<sub>6</sub>-Xpr-GFP and DMC) into the pollen cell wall proteins (Figure 3A,D),  
567 and this incorporation was inhibited by specific TGase inhibitors (Figure 3B).The  
568 presence of TGase mediated protein cross-links was demonstrated by *in situ* immuno-  
569 detection using the anti cross-link monoclonal antibody 81D4 (Figures 3E and 4A). The  
570 identity of these protein substrates is not known.

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571 In pollen, we have shown that TGase activity could be stimulated by high  
572 concentration of substrates (Figure 3A for higher His<sub>6</sub>-Xpr-GFP concentration), which  
573 possibly act as a nucleation centre for further cross-linking. The concomitant  
574 enhancement of pollen tube growth associated with His<sub>6</sub>-Xpr-GFP protein cross-linking  
575 at the pollen tube surface suggests that His<sub>6</sub>-Xpr-GFP facilitates pollen tube apical  
576 growth. The finding that both anti-TGase antibodies and inhibitors (mainly acting  
577 extracellularly) block His<sub>6</sub>-Xpr-GFP binding to cell wall and inhibit pollen tube growth  
578 is a clear indication that the catalytic activity of extracellular pollen TGase is critical for  
579 correct pollen tube growth in *Malus*.

580 The co-localisation between extracellular TGase antigen and cross-linked  
581 products suggests that apple pollen TGase could become embedded, under the effect of  
582 local micro-environmental conditions, into the same accumulation sites of the protein  
583 cross-links catalyzed by its enzyme activity. The localization of TGase-catalyzed cross-  
584 linked aggregates along the shank of pollen tubes is similar to that observed in the cell  
585 wall during the pulsatory growth of pollen tubes in flowering plants with a solid style.  
586 However, the distribution of pectins and AGPs reported in the literature does not fit  
587 exactly with the irregular distribution of the TGase-catalyzed aggregates [48]. These  
588 discontinuous cross-linked structures may reflect oscillations of other factors  
589 influencing TGase activity and pollen growth, e.g. changes in Ca<sup>2+</sup> and pH [1]. These  
590 collective results indicate that when the apple pollen TGase is externalised it remains  
591 biologically active.

#### 592 593 **Common aspects with other TGases**

594  
595 The evidence that extracellular TGase activity from germinating pollen tubes is  
596 inhibited by compounds that inhibit animal tTGase (281 and 283, iodoacetamide and  
597 cystamine), [16, 30-33] and by anti-TGase antibodies (Figure 2A,B) strongly suggests  
598 that the plant enzymes are catalytically similar to animal TGases. In mammalian cells,  
599 imidazolium derivatives such as 283 and 281 have been described as potent inhibitors  
600 of mammalian TGases [22, 30, 32]. The inhibitor 281 is reported to act primarily  
601 outside the cell [33] and to inhibit pollen TGase activity by reducing the fluorescein-  
602 cadaverine cross-linking to pollen proteins during germination [27].

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

612 The above reported similarity between animal tTGase and pollen TGase is  
613 confirmed by the evidence that partially purified 70 kDa TGase extracted from pollen  
614 shares similar properties to gpl TGase in affecting the functional properties of the  
615 cytoskeleton [26]. The inhibition of pollen TGase by 281, which covalently binds the  
616 cysteine at the active site of mammalian TGase, also suggests the presence of a similar  
617 cysteine at the active site in *Malus* pollen TGase. This is in agreement with the  
618 observation that the typical catalytic triad of the TGase family (His-Cys-Asp) [16, 17]  
619 is conserved in *Arabidopsis* TGase (AM745095) [37] and in an EST sequence in the  
620 *Malus* database (Tree Fruit Technology, Michigan State University), which shows 62%

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621 homology with *Arabidopsis* TGase (increasing to 77% homology with TGase active-  
622 site containing regions).

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

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634

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**842 Table 1. Evaluation of the effect of His<sub>6</sub>-Xpr-GFP on pollen germination.**

843 His<sub>6</sub>-Xpr-GFP and control mouse IgG were added at different concentrations into the medium  
 844 containing hydrated pollen at time zero and left up to 120 min germination. The data are  
 845 expressed as percentage of germination (mean ± SD). P values were obtained by paired *t*-  
 846 Student test between control and His<sub>6</sub>-Xpr-GFP-treated pollen of 10 samples of three  
 847 independent experiments. Significantly different at  $p < 0.05$  <sup>(a)</sup>.

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**Table 1.** The effect of His<sub>6</sub>-Xpr-GFP and IgG on pollen germination

μg ml <sup>-1</sup>	% Germination	
	His <sub>6</sub> -Xpr-GFP	IgG
0.10	68 ± 6	67 ± 1
0.25	69 ± 4	72 ± 3
0.50	82 ± 5	77 ± 1
1.00	88 ± 3 <sup>a</sup>	78 ± 5
5.00	88 ± 2 <sup>a</sup>	76 ± 2
25.00	80 ± 2 <sup>a</sup>	70 ± 2
50.00	82 ± 2	76 ± 2

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## 851 LEGENDS

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853 **Figure 1. Immuno-localisation of pollen TGase during germination.**

854 (A) Pollen was germinated for 2 h and fixed, permeabilised and probed with  
 855 monoclonal antibodies directed to TGase 2 (ID10) (a-c),  $\alpha$ -tubulin (e) and  $\alpha$ -tyrosinated  
 856 tubulin (f). Negative control: non-specific mouse IgG (d). Magnifications of the distal  
 857 part (b), the neck region and the proximal parts (c) of germinated pollen stained for  
 858 TGase. Scale bar: 8  $\mu$ m.

859 (B) Laser scanning confocal microscopy of germinated pollen tubes treated as in (A),  
 860 and probed with secondary FITC-conjugated antibody. TGase staining along the tube  
 861 and at the apex (a) and visualization of the same sample in phase contrast (b). Staining  
 862 with non-specific mouse IgG followed by secondary antibody as 2  $\mu$ m-single section  
 863 (ct) or overlaid sections (d); arrows indicate the pollen tubes, whose autofluorescence  
 864 was minimized. Scale bar: 8  $\mu$ m.

865 (C) TGase-active fractions (50  $\mu$ g protein) of germinated pollen extract (lane CE)  
 866 germination medium (lane GM) and cell wall extract (lane CW) were analysed by  
 867 western blotting with the monoclonal anti-TGase antibody ID10. The partially-purified  
 868 TGase from pollen supernatant (isolated by hydrophobic ion chromatography and  
 869 eluted with EGTA) was evaluated both by coomassie gel staining (lane C) and by  
 870 western blotting with anti-TGase antibody ID10 (lane WB). "tTGase" in the first lane  
 871 indicates the commercial gpl TGase used as a positive control.

872 (D) The cell-wall of non digested and non membrane-permeabilised 120 min-  
 873 germinated pollen was treated with 2 M NaCl, 1% (w/v) SDS or 1% (w/v) DTT, fixed  
 874 and then stained with anti-TGase antibody ID10 (a-b). In order to prove that the  
 875 staining was not affected by fixation, ID10 was incubated for the last 30 min of  
 876 germination in the medium prior to fixation (c). The arrows point the ID10 immuno-  
 877 labelling. In some cases, the cell wall was further digested and the residual labeling was  
 878 localized around the grain (arrows) (d). Scale bar: 8  $\mu$ m.

879 (E) Immuno-gold labeling of apple pollen tubes conducted with anti-TGase ID10.  
 880 Labeling was mainly found in association with the amorphous layer of cell wall  
 881 (arrows) in the flanks of pollen tubes. Scale bar: 200 nm.

882

883 **Figure 2. Inhibition of TGase activity affects pollen tube growth.**

884 (A) Effect of anti-TGase ID10 on pollen tube morphology, germination and  
 885 extracellular TGase activity. Pollen was incubated at time zero with ID10 or control  
 886 antibodies against horse albumin, in microtiter plates, and allowed to germinate up to  
 887 120 min. Pollen tube morphology was observed by light microscopy (upper panels) in  
 888 the presence of control anti-horse albumin (left) and increasing concentrations of ID10  
 889 (centre and right images). Pollen tube morphological malformations and diffuse  
 890 bursting are indicated by arrows. Scale bar: 24  $\mu$ m. Bottom plot: lines show the  
 891 percentage (mean  $\pm$  SD;  $n = 10$ ) of germination with ID10 (black squares) or control  
 892 antibody (white squares); microplate assay of TGase activity of germinating pollen  
 893 (bars) treated with ID10 (crossed lines) or anti-horse albumin (vertical lines) and  
 894 expressed as units (U)  $\text{mg}^{-1}$  pollen. The correlation analysis between pollen germination  
 895 and TGase activity, when inhibited by ID10, gave a Pearson coefficient of  $R = 0.92$  ( $p$   
 896  $= 0.03$ ).

897 (B) Effect of two irreversible inhibitors of TGase on pollen tube morphology,  
 898 germination and extracellular TGase activity. Pollen was germinated, as in (A), and, at  
 899 time zero, incubated with 50  $\mu$ M 283 and 281 or control MES buffer (0). Pollen tube  
 900 morphological malformations are indicated by arrows (upper panels). Scale bar: 24  $\mu$ m.

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901 Bottom plot: lines show the percentage of germination (mean  $\pm$  SD;  $n = 10$ ) in the  
902 presence of inhibitors 283 (white squares) and 281 (grey squares). TGase activity was  
903 reported, as in (A), in the presence of 283 (bars with horizontal lines) or 281 (bars with  
904 oblique lines). The correlation analysis between pollen germination and TGase activity  
905 when inhibited by 281 and 283 respectively, gave a Pearson coefficient of  $R = 0.94$  ( $p <$   
906  $0.0001$ ) and  $0.92$  ( $p < 0.0005$ ).

907

908 **Figure 3. Extracellular TGase cross-linking during pollen germination.**

909 **(A)** Laser scanning confocal microscopy at 120 min-germination of undigested and  
910 non-permeabilised pollen, His<sub>6</sub>-Xpr-GFP was added for the last 30 min of germination  
911 in the medium prior to fixation. Superimposition of the GFP labeling on the phase  
912 contrast image (upper panel); GFP labeling (bottom panel). Control: non-specific  
913 mouse IgG followed by FITC-antibody. Arrows indicate His<sub>6</sub>-Xpr-GFP fluorescence.  
914 Scale bar: 8  $\mu$ m.

915 **(B)** Laser confocal microscopy of pollen, incubated and treated as in (A), but in the  
916 presence of either the anti-TGase ID10 or 281/283 inhibitors for the final 30 min of  
917 germination. Scale bar: 8  $\mu$ m.

918 **(C)** Pollen was germinated from time zero in the presence of 5  $\mu$ g ml<sup>-1</sup> of His<sub>6</sub>-Xpr-GFP  
919 (black bars) or mouse IgG (grey bars) and the tube length was evaluated via a digital  
920 camera at 30, 60, 90 and 120 min and reported as relative frequency (mean  $\pm$  SD of  
921 100-1000  $\mu$ m tube length). Square brackets report the lowest and the highest number of  
922 measured pollen tubes for each optical field.

923 **(D)** Microplate assay of extracellular TGase cross-linking activity in the presence of  
924 increasing concentrations of the anti-TGase antibody ID10 and the inhibitors 283 and  
925 281 supplied to pollen for the last 30 min of germination, as indicated above in (A).  
926 The fluorescence of His<sub>6</sub>-Xpr-GFP cross-linked by the pollen extracellular TGase to  
927 immobilised DMC was measured and compared to the values obtained by standard free  
928 gpl TGase (control, oblique line bar). Relative fluorescence units (RFU) of treated  
929 samples were reported as percentage (mean  $\pm$  SD) of the control normalized to 100%  
930 ( $12,580 \pm 1,133$  RFU).

931 **(E)** TGase-mediated crosslinking products immuno-localized by the 81D4 (monoclonal  
932 antibody anti-N<sup>ε</sup>( $\gamma$ -glutamyl)-lysine) on 120 min-germinated unfixed pollen (neither  
933 digested nor permeabilized). Arrows indicate the cross-link immuno-detection. Scale  
934 bar: 8  $\mu$ m.

935

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

938 **(A)** Germinating pollen tubes were incubated *in vivo* for the last 30 min of germination  
939 with His<sub>6</sub>-Xpr-GFP prior to fix and, omitting cell wall digestion and permeabilisation,  
940 incubated with 81D4 (monoclonal antibody anti-N<sup>ε</sup>( $\gamma$ -glutamyl)-lysine and -PAs),  
941 followed by TRITC-conjugated secondary antibody. In the different panels, the positive  
942 staining indicates the localisation of TGase-mediated cross-linking products (His<sub>6</sub>-Xpr-  
943 GFP incorporation) (upper), immuno-staining with 81D4 (centre), and superimposition  
944 of the His<sub>6</sub>-Xpr-GFP and 81D4/TRITC immuno-labeled pollen tubes (merge bottom  
945 panel). Scale bar: 8  $\mu$ m.

946 **(B)** Germinating pollen tubes were treated as in A but stained with anti-TGase antibody  
947 ID10. Localization of TGase mediated cross-linking products by His<sub>6</sub>-Xpr-GFP (upper  
948 panel), immuno-staining with ID10 (centre panel), and superimposition of His<sub>6</sub>-Xpr-  
949 GFP and ID10/TRITC immuno-labeling (merge bottom panel). Scale bar: 8  $\mu$ m.

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951 **Figure 5. Immuno-localisation of pollen TGase during germination *in planta*.**

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

957

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

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

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970 **LEGENDS FOR SUPPLEMENTAL FIGURES**

971

972 **Figure S1.** Western blot of an active pollen fraction partially purified from hydrated  
973 pollen by a hydrophobic interaction chromatography (HIC), set up to purify  $\text{Ca}^{2+}$ -  
974 dependent TGases, assayed at 30, 20 and 10  $\mu\text{g}$  of protein and both not purified (NP)  
975 and purified (P) gpl TGase, comparatively probed with anti-TGase antibodies ID10 and  
976 AbIII.

977

978 **Figure S2.** Percentage of residual TGase activity in the pollen SN after immuno-  
979 precipitation obtained with increasing amounts of anti-TGase antibody ID10. Proteins  
980 were extracted from hydrated pollen grains.

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982 **Figure S3. (A)** Western blots of 1  $\mu\text{g}$  and 0.1  $\mu\text{g}$  human recombinant TGase 1 (1),  
983 TGase 2 (2) and TGase 3 (3) probed with the anti-TGase antibody ID10; **(B)** Western  
984 blots of 0.1  $\mu\text{g}$  human recombinant TGase 1 (1), TGase 2 (2) and TGase 3 (3) probed  
985 with anti-TGase antibodies ID10, CUB7402, anti-TGase 1 and anti-TGase 3. These  
986 results show the specificity of ID10 toward TGase 2.

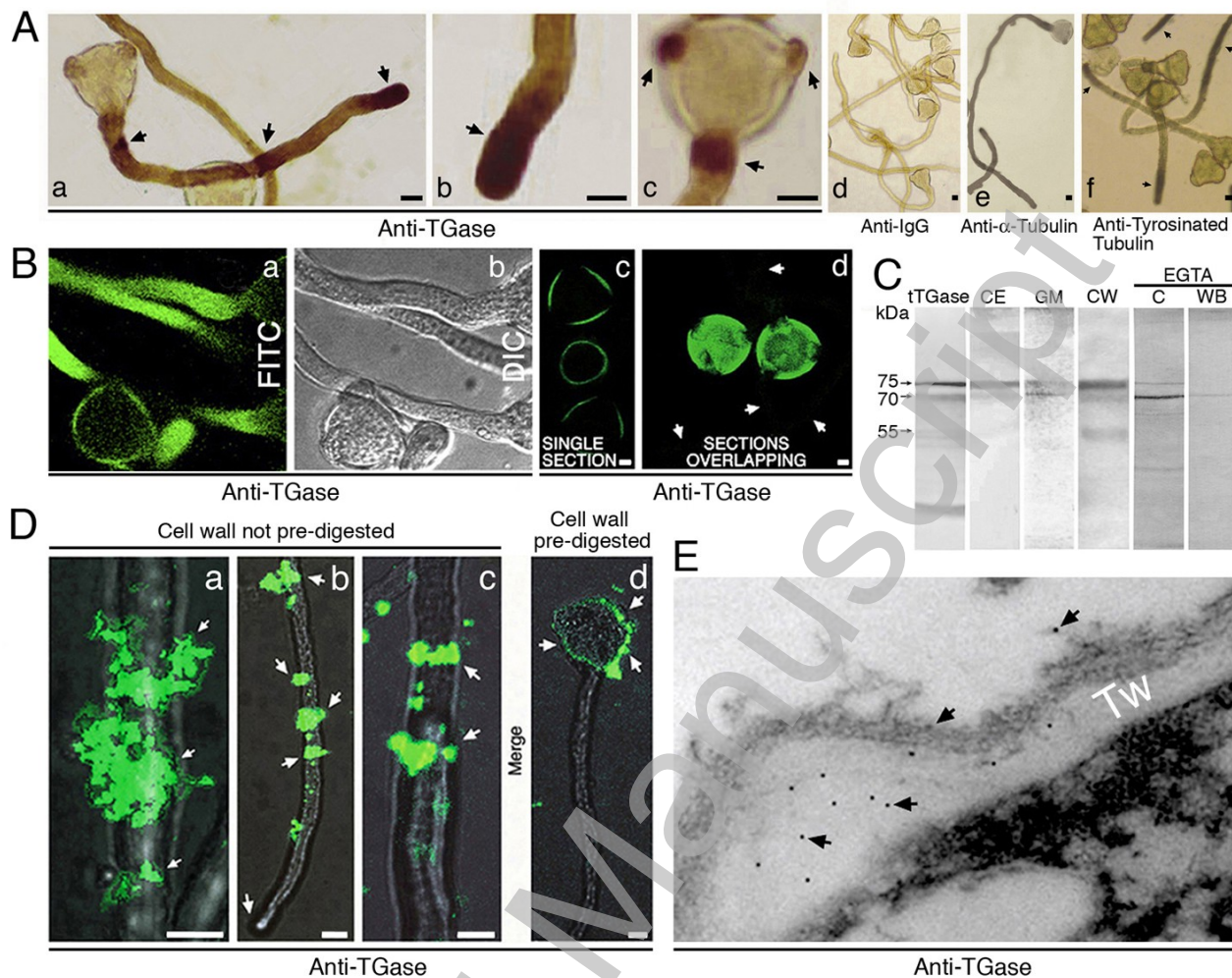
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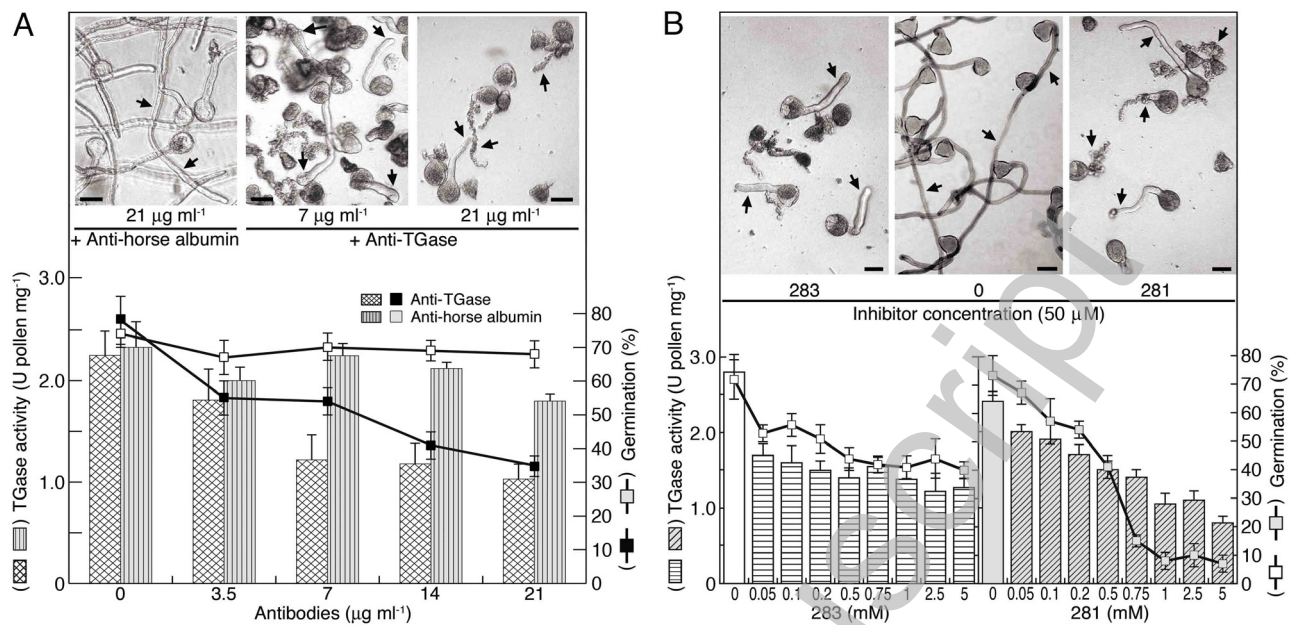
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990 **Figure 1.** Immuno-localisation of pollen TGase during germination.

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993 **Figure 2.** Inhibition of TGase activity affects pollen tube growth.

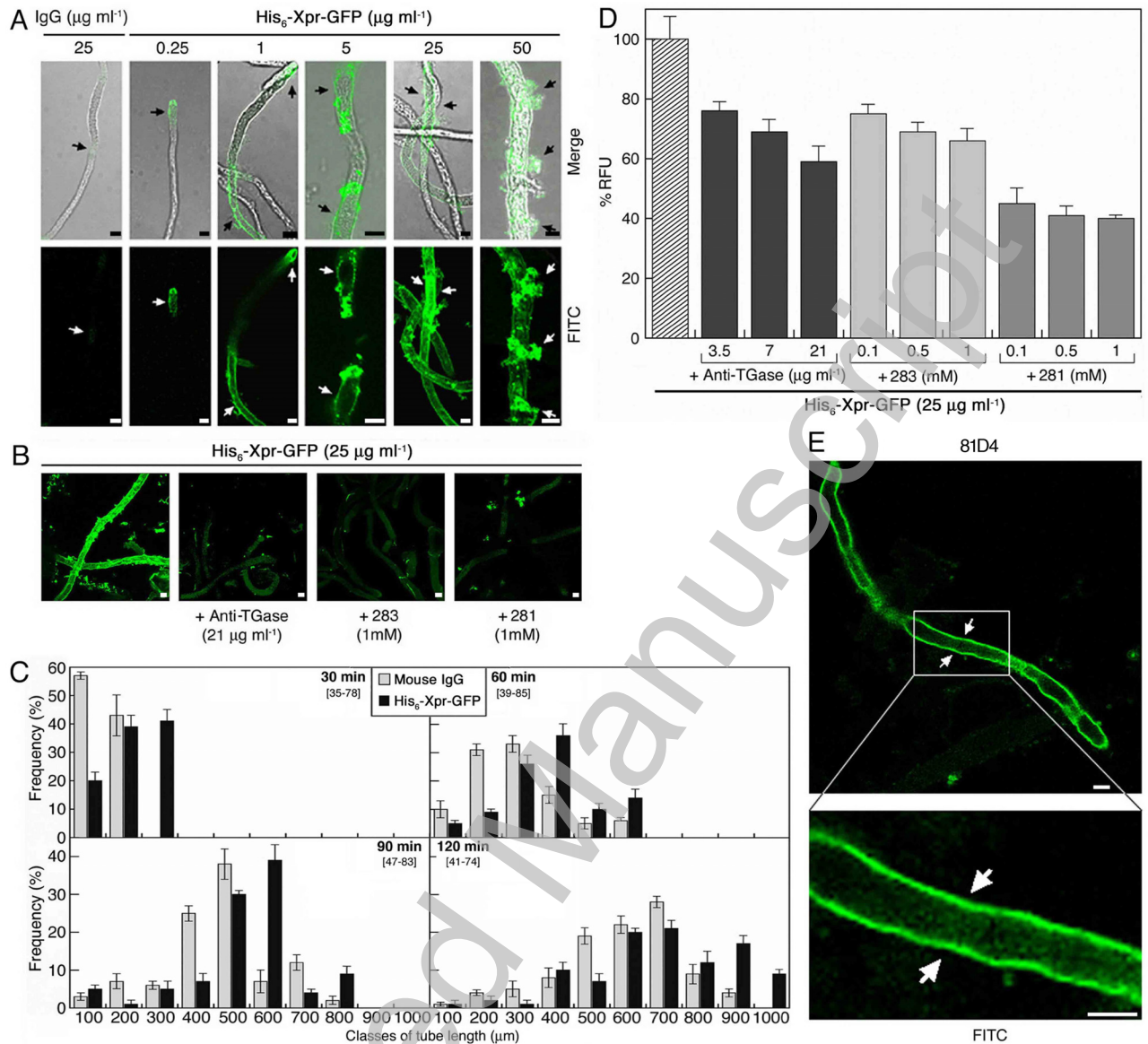
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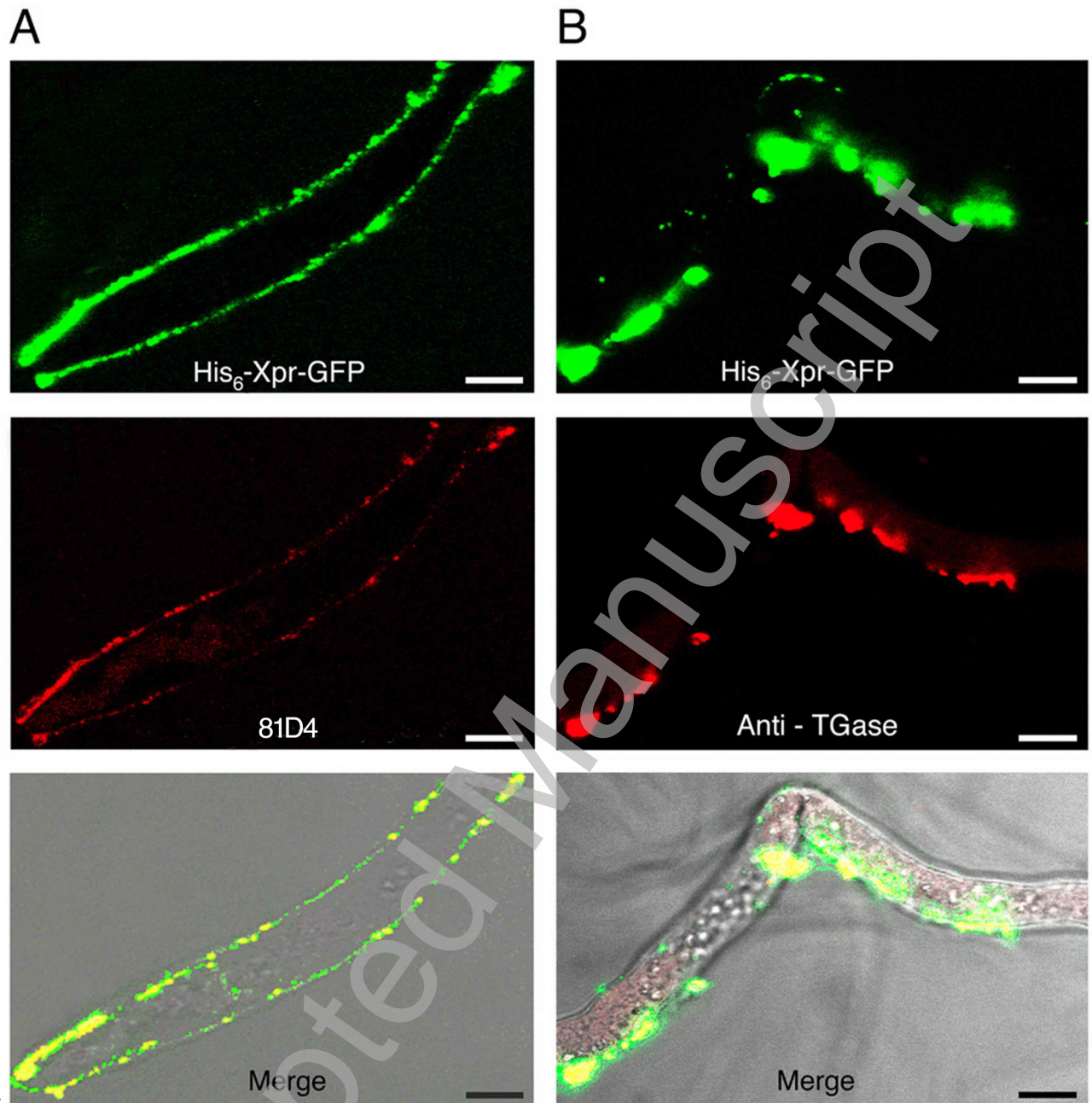
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996 **Figure 3.** Extracellular TGase cross-linking during pollen germination.

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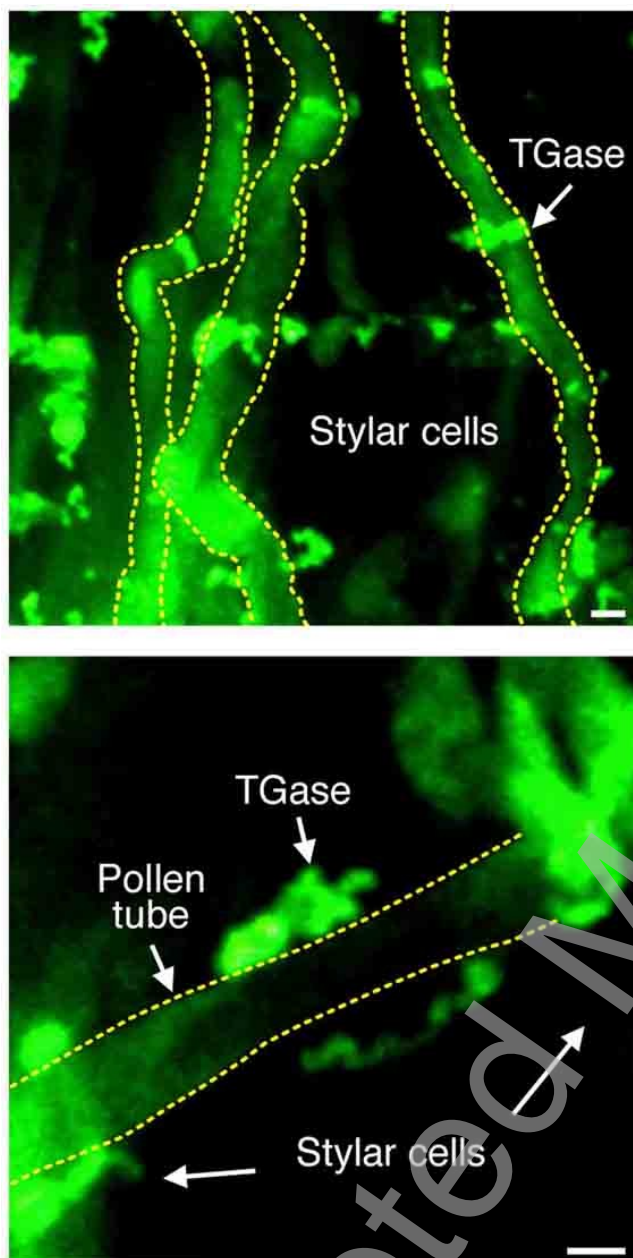
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999 **Figure 4.** Co-association of protein cross-linking and extracellular TGase on the surface of  
1000 120 min-germinated pollen. Scale bar: 8  $\mu$ m.

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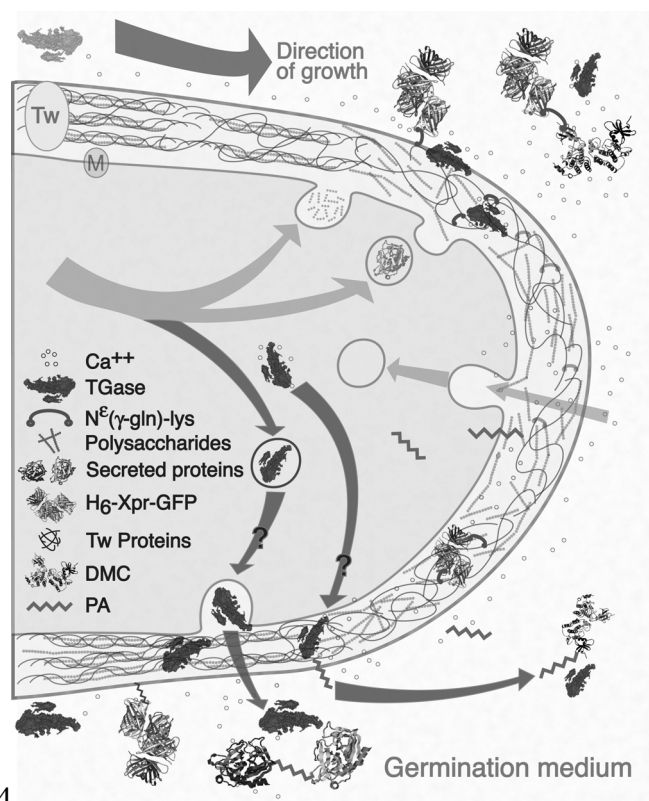
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10021003 **Figure 5.** Immuno-localisation of pollen TGase during germination *in planta*.107  
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**Figure 6.** Model of externalised TGase regulating *in vitro* pollen tube growth.

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