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MINIREVIEW ARTICLE

The plant extracellular transglutaminase: what mammal analogues tell

Stefano Del Duca · Elisabetta Verderio · Donatella Serafini-Fracassini · Rosa Iorio · Giampiero Cai

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Abstract The extracellular transglutaminases (TGs) in eukaryotes are responsible for the post-translational modification of proteins through different reactions, cross-linking being the best known. In higher plants, extracellular TG appears to be involved in roles similar to those performed by the mammalian counterparties. Since TGs are pleiotropic enzymes, to fully understand the role of plant enzymes it is possible to compare them with animal TGs, the most studied being TG of type 2 (TG2). The extracellular form of TG2 stabilizes the matrix and modulates the interaction of the integrin-fibronectin receptor, causing the adhesion of cells to the extracellular matrix; TG2 plays a role also in the pathogenicity. Extracellular TGs have also been identified in the cell wall of fungi, such as *Candida* and *Saccharomyces*, where they cross-link structural glycoproteins, and in *Phytophthora*, where they are involved in pathogenicity; in the alga *Chlamydomonas*, TGs link polyamines to glycoproteins thereby favouring the strengthening of cell wall. In higher plants, TG localized in the cell wall of flower petals appears to be involved in the structural reinforcement as well as senescence and cell death of the flower corolla. In the pollen cell wall an extracellular TG co-localizes with substrates and cross-linked products; it is required for the apical growth of pollen tubes. The pollen TG is also secreted into the extracellular matrix possibly allowing the migration of pollen tubes during fertilisation. Although pollen TGs seem to be secreted via vesicles transported along actin filaments, a different mechanism from the classical ER-Golgi pathway is possible, similar to TG2.

Keywords Transglutaminase · Polyamine · Secretion · Cell wall · Extracellular medium

Abbreviations

BC	Biotin-cadaverine
CD	Cadaverine
ECM	Extracellular matrix
HSPG	Heparan sulphate proteoglycan syndecan
PAs	Polyamines
PCD	Programmed cell death
PU	Putrescine
SD	Spermidine
SM	Spermine
TGs	Transglutaminases
TG2	Transglutaminase type 2
TGN	Trans golgi network

S. Del Duca (✉) · D. Serafini-Fracassini · R. Iorio
Dipartimento di Scienze Biologiche, Geologiche ed Ambientali, Università di Bologna, Bologna, Italy
e-mail: stefano.delduca@unibo.it

E. Verderio
Biomedical, Life and Health Sciences Research Centre School of Science and Technology, Nottingham Trent University, Nottingham NG11 8NS, UK

G. Cai
Dipartimento Scienze della Vita, Università di Siena, Siena, Italy

The enzyme

This review focuses on plant extracellular TG. Although experimental evidence on the presence of TGs in the extracellular compartment of plants are still limited and no data are available on the phylogenetic origin of TGs in plants, the topic is new and opens insights into the relationship between homologous and heterologous plant cells. There are many experimental evidence from many

different groups on the expression and roles of extracellular TGs in mammalians, which offer a model for the search of extracellular TGs in plants, even though plant and mammalian cells are different. For this reason mammalians extracellular TGs will also be discussed to present the main features. Since intracellular TGs in plants have been previously reviewed by us (Serafini-Fracassini and Del Duca 2008) they will not be considered here, unless related to cell wall/extracellular matrix (ECM). Prokaryotic TGs, that have relevance especially for biotechnological purposes are also not reviewed.

Even if plant and mammalian cells are different, in fact animal cells lack the cell wall, we will discuss the mammalian TGs, in particular their role in the ECM, as they offer useful suggestions especially for the pollen model and its interactions with the female counterpart. Similarities and differences between plant and animal TGs were previously reported in this Journal (Serafini-Fracassini et al. 2009; Beninati et al. 2013). Phylogenetic tree analysis of the known animal TGs confirms that two main branches probably arose from a common ancestral gene: one lineage includes TG1 and FXIIIA, and the second comprises the genes for erythrocyte band 4.2, TG2, TG3, TG5, TG6 and TG7. The band 4.2 is on a separate branch near to TG2 (Lorand and Graham 2003). There are too few known sequences to perform a similar analysis for plant TG. In lower Eukaryotes *Phytophthora sojae* TG did not share significant primary sequence similarity with known TGs from mammals or bacteria. In "higher" plants TGs shows little sequence homology with the best-known animal enzymes: the only two annotated sequences of plant origin are the recombinant TG of *Arabidopsis thaliana*, AtPng1p (Q9FGY9) (PNG1_ARATH) (Della Mea et al. 2004) and the two related sequences detected in *Zea mays* (Q6KF61 and Q6KF70, not included in SwissProt) (Villalobos et al. 2004). Whereas the TG of *Arabidopsis* showed in its sequence the TG catalytic triad, sharing a possible structural homology (Della Mea et al. 2004; Serafini-Fracassini et al. 2009), the maize sequences, derived by isolation of an *Helianthus* chloroplast TG, did not allow to identify in their sequence (which includes only two cysteins), neither the typical TG domain nor the catalytic triad. Furthermore searches by the PFAM multisequence alignment (http://pfam.sanger.ac.uk), and amino acid composition (aac) showed that the maize sequences are not comparable to the animal TG family.

As in plants there are no many TG gene sequences available to compare for filogenesis purposes (one sequence from *A. thaliana* and two associated sequences from *Z. mays*) we considered also the amino acid composition (aac) of *H. tuberosus* TGs that have been compared to mammalian TGs. In particular, three active TG forms (85, 75 and 58 kDa) from *H. tuberosus* sprout apices were isolated and their diverse aac shared some unexpected similarities with mammalian TGs (Beninati et al. 2013). The *H. tuberosus* 75 kDa enzyme aac was practically identical (with a difference of 0.29 %) to that of the erythrocyte EPB42, and its 3D model perfectly fitted with that of the human TG2; the *H. tuberosus* 85 kDa TG and the TG2 appeared more similar to each other than TG2 to EPB42_HUMAN and PNG1_ARATH (the 82 kDa purified recombinant AtPng1p). These comparisons as well as their catalytic capacities suggest that the 75 and 85 kDa *H. tuberosus* enzymes may be conserved during the evolution. On the contrary, the third enzyme (58 kDa), which is the most frequently detected MW for the TGs in plants (not reported for animal ones), shows quite different characteristics and its phylogenesis is at present undetectable. In the hypothesis of plastid genome codified proteins, being of prokaryotic origin, 58 kDa TG should share some similarity with those of cyanobacteria, but this cannot be verified, as the sequences for TG unfortunately are not available in cyanobacteria genome.

The problem of the unusual way of secretion of the enzyme in plants and mammals will also be treated.

Transglutaminases (TGs) are a widely distributed family of enzymes present in animals, plants and unicellular organisms, which are responsible for the post-translational modification of proteins by different reactions (Fig. 1) (Folk 1980; Griffin et al. 2002; Lorand and Graham 2003; Serafini-Fracassini and Del Duca 2008). These are generally Ca^{2+}-dependent, but in the case of some microorganisms and rodent intestinal mucosa TGs also act as Ca^{2+}-independent enzymes (Griffin et al. 2002; Lorand and Graham 2003); one of the best-known reactions catalysed by TGs is the interaction between acyl acceptor glutamyl residues and amine donors. When the amine donors are the primary amino groups of lysine (Lys) residues the TG reaction gives rise to crosslinks between proteins (Fig. 1); however, also a glutamine (Gln)-containing peptide could be linked to a protein by the acylation of a Lys side chain of the donor protein.

When the donors are polyamines (PAs), the transamidase reaction determines the binding of a PA into an endo-glutamyl residue of proteins (Fig. 1); the aliphatic PAs bear two terminal amino groups and both of them could be involved in the reaction, determining the formation of a bridge between two endo-glutamyl residues of the same protein or of two different proteins. The different lengths of the PAs aliphatic chain have a consequence on the distance between proteins. The two-step reaction is reported in detail in Fig. 2.

In the absence of primary amino group the TG reaction deamidates the neutral Gln residue replacing it with a negatively charged glutamic acid (Glu), which is important

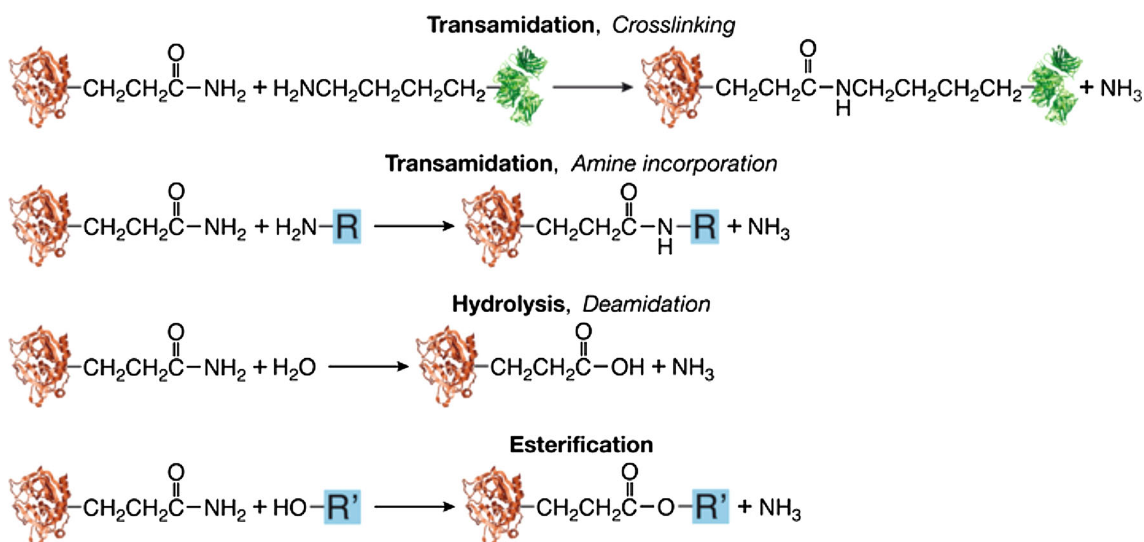


Fig. 1 Biochemical activities of transglutaminase. By transamidation TG can catalyse Ca^{2+}-dependent acyl-transfer reaction between γ-carboxamide group of a specific protein-bound glutamine (Gln) residue (the acceptor residue of the red protein) and the ε-amino group of a distinct protein-bound lysine (Lys) residue (the donor residue of the green protein) giving rise to covalent protein crosslinking, the main in vivo activity. TGs react only with the γ-amides of selected endo-Gln residues in some proteins and peptides showing specificities both for their Gln and Lys substrates. Moreover, TG can cause the incorporation of an amine (H_2NR) into the Gln residue of the acceptor (*red*) protein. In the absence of primary amines and in presence of water TG reaction can catalyse a hydrolysis with the deamidation of an endo-Gln to an endo-Glu and the realease of an aminic group. Esterification can cause the linkage of an endo-Gln residue to the aliphatic chain of some ceramide/fatty acids. R represents the side chain in a primary amine; R′, a ceramide/fatty acid (color figure online)

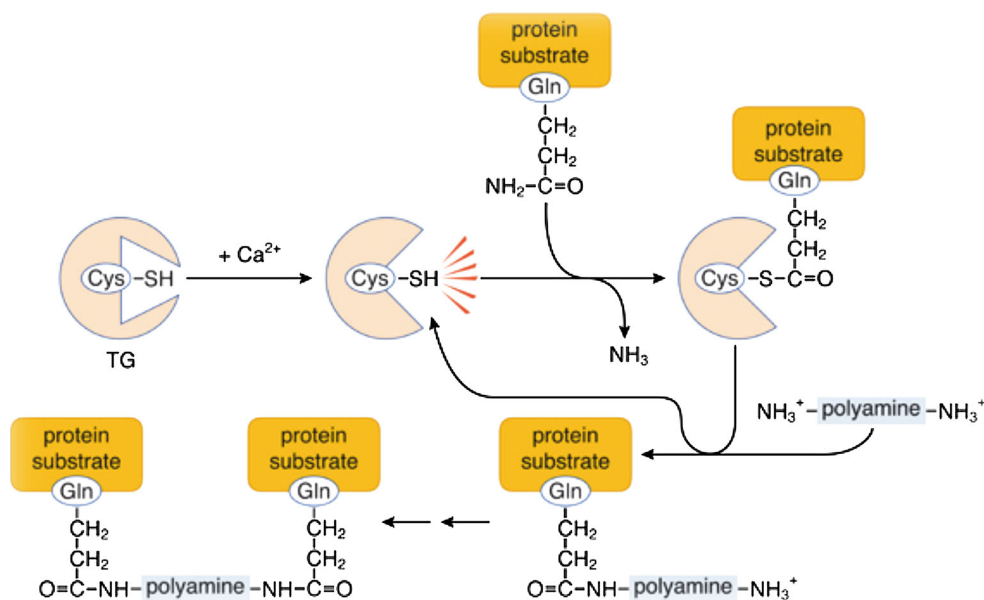


Fig. 2 The two steps of transamidase reaction of TG. In the presence of Ca^{2+} the enzyme could change its conformation and the active site cysteine became ready to catalyse the reaction that involves the formation of a thioester acylenzyme intermediate, with the release of ammonia. Then a molecule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

for protein interactions, i.e., in the immunogenic responses that take place in celiac disease (Fig. 1) (Griffin et al. 2002; Lorand and Graham 2003).

TG is also responsible for esterification (Fig. 1) as in keratinocytes, where TG not only participates in the cross-linking of cell envelope structural proteins, but also forms

the lipid-bound envelope by esterification of long chain ω-hydroxyceramides onto cell envelope proteins.

A particular reaction observed only in vitro (with purified molecules) by Lorand and coworkers (2003) is the isopeptidase reaction, which reverts the crosslinking one; it is, however, unclear whether this reaction takes place in vivo.

Extracellular transglutaminases in mammals

In mammals the transglutaminase (TG) family comprises eight enzymes (FXIIIA and TG1-7) and one structural protein (protein 4.2). Among them, ubiquitous "tissue" TG (TG2), the A subunit of Factor XIII (FXIIIA) and prostate TG (TG4) are externalised from cells, despite the lack of a putative signal sequence required for conventional endoplasmic reticulum/Golgi export (Lorand and Graham 2003).

FXIIIA is present in plasma in a heterotetrameric form and it is best known for the cross-linking of fibrin clots after thrombin activation. However, it is also secreted by osteoblasts in the extracellular matrix (ECM) where it acts as a protein cross-linker participating in wound healing and tissue calcification (Al-Jallad et al. 2011). Its mechanism of secretion into the plasma or the ECM appears to be distinct from that of other leaderless proteins (e.g., interleukin 1beta), and its transport seems to depend on either aspecific release upon cell death and lysis or association with peripheral Golgi proteins (Cordell et al. 2010).

TG4, whose distribution is restricted to the prostate gland, is a highly glycosylated apocrine-secreted protein (Aumuller et al. 1999), whose enzymatic activity leads to the formation of the copulatory plug in the dorsal prostate of rodents (Seitz et al. 1990). In humans, TG4 has been recently attributed a diverse range of effects on prostate cancer cells such as a role in cell growth, migration and invasiveness, and it has also been involved in epithelial-mesenchymal transition (Jiang and Ablin 2011).

Epidermal type TG (TG1) is another TG member recently reported to be exported from cells (Jiang et al. 2010). Anchored in the inner plasma membrane in surface epithelia, TG1 has the important function of catalysing the formation of the cornified envelope.

Among the secreted TG in mammals, TG2 has an intriguing individuality. Extracellular TG2 has enzymatic and non-enzymatic functions, which coexist with enzymatic and non-enzymatic activities inside the cell, all attributed to the same protein. The outside-in function and secretion of TG2 influence several physio-pathological conditions. Several methods and biological tools leading to the identification and visualisation of extracellular TG2 in mammals have been applied to research in plant transglutaminase; therefore, extracellular TG2 is relevant to this discussion.

TG2 is a protein with distinct molecular activities in the intracellular and extracellular milieu. Inside the cell it acts as a GTPase involved in signal transduction (for which role it is named Gh). In the nucleus TG2 is increased under hypoxic conditions and there is evidence for its interaction with transcription factors (Filiano et al. 2008). Outside the cell, TG2 is a calcium-dependent protein crosslinker of ECM proteins, leading to ECM stabilisation in situations of wound healing. It is also a structural matrix protein, capable of inducing outside-in signalling. This action is independent from the classic cross-linking activity and it is modulated/facilitated by extracellular TG2-non covalent binding of ECM proteins, such as fibronectin (FN) and heparan sulphate proteoglycans (HSPGs) (Verderio et al. 2003; Telci et al. 2008; Wang et al. 2010a, 2011a). Therefore, TG2 is a multiple function protein displaying different activities depending on the microenvironment in which it resides. In common with other multiple-function and poly-located proteins found inside and outside the cell, TG2 transits the plasma membrane by unconventional secretion. As discussed by Radisky et al. (2009), absence of a powerful exocytotic signal and a non-canonical secretion pathway may be a nature's *escamotage* to control tissue distribution and allow for flexibility of function. Other examples of dual function proteins are epimorphin/syntaxin 2, amphoterin/HMGB1 which like TG2 are encoded by the same gene (Radisky et al. 2009); however, they do act differently depending on their location, whether this is inside or outside the cell.

TG2 role in the ECM environment

TG2 transamidation finds in the ECM the ideal microenvironment in terms of high calcium/GTP for its activity and numerous ECM proteins, like fibronectin and collagen, acting as TG2 substrates (Fesus and Piacentini 2002). However, for a long time the notion of TG2 presence in the extracellular matrix has been met with scepticism in the scientific community. Early work by Aeschlimann and Thomazy (2000) showed a role for TG2 in the modulation of the ECM, and work by Griffin's group in the nineties imaged and quantified the presence of extracellular TG2 for the first time in cell cultures and demonstrated TG2 transamidating activity at the surface of growing cells (Verderio et al. 1998). Belkin and collaborators proved that TG2 plays a catalytic-independent role in the ECM. They also showed that one important TG2-function outside the cell is to modulate the fibronectin–integrin receptor interaction leading to cell adhesion to the ECM (Akimov et al. 2000). In 2003, a novel RGD (Arg-Gly-Asp)-independent role for fibronectin-bound extracellular TG2 was described.

This adhesive process is relevant in conditions of wound healing/matrix fragmentation, where it is conducive to cell survival by protecting cells from anoikis (Verderio et al. 2003). It was later attributed to the HSPG syndecan 4 the role of acting as a cell surface receptor for FN-bound TG2, mediating cross-talk with β1 integrin via activation of PKC-α (Telci et al. 2008; Scarpellini et al. 2009). Wang et al. (2010b, 2011b) studied this pathway further and disclosed the importance of syndecan-2 in this process by acting as a downstream effector in actin cytoskeleton organization.

It soon became clear that extracellular TG2 plays an important role in vivo. In situations of chronic tissue damage and increased externalisation, TG2 plays a pathogenic role in matrix accumulation/stabilisation leading to tissue fibrosis. This role has been uncovered by work led by Johnson and Griffin using experimental models of kidney fibrosis (Johnson et al. 2003) and treatment of affected rat models with TG2 inhibitors (Huang et al. 2009). The role of extracellular TG2 in cancer progression has been predominantly linked to its role in promoting interactions with the ECM and migration leading to cell survival and metastatic potential of cancer cells (Satpathy et al. 2007; Verma and Mehta 2007). TG2 must be in the ECM to deamidate gluten peptides and turn them into strong T cell antigens, an event which is definitely linked with celiac disease. An anti-TG2 antibody response is escalated in patients on a gluten-containing diet (Shan et al. 2002). Therefore, targeting extracellular TG2 is an area of intense interest for a range of disorders where TG2 plays directly or indirectly a causative role.

TG2 secretion in mammals

TG2 follows an unconventional way of secretion, which is different from the classical ER-Golgi route, through which most secreted proteins are exported. Unravelling the mechanism of TG2 externalisation is a priority area because of its unicity and potential exploitation in therapy. Although progress has been made in this direction, there is still a wide debate on the molecular events leading to TG2 secretion. Belkin's group recently proposed that TG2 is externalised to the extracellular space from the recycling endosomes in complex with β1integrin, to which it is targeted via binding to phosphoinositides on endosomal membranes (Zemskov et al. 2011). They also showed that the endogenous second messenger nitric oxide, a potent vasodilator, attenuates TG2 externalisation in a model of human aortic endothelial cells (Santhanam et al. 2011). Trafficking of TG2 has also been attributed to its association with HSPG because of the affinity of TG2 for heparan sulphate and the accumulation of cytosolic TG2 in the absence of HSPG syndecan-4 (Scarpellini et al. 2009; Wang et al. 2012). Enhancers and inhibitors of HSPG shedding (PMA and Go6976, respectively) modulated the amount of matrix TG2 in a model of Swiss 3T3 fibroblasts, suggesting that syndecan-4 ectodomain-shedding leads to the release of TG2 in the matrix (Wang et al. 2012). Furthermore, the heparin-binding site of TG2 has been recently characterised by site directed mutagenesis (Wang et al. 2012; Lortat-Jacob et al. 2012; Teesalu et al. 2012); although there is no consensus on this in the literature, there is general agreement that TG2 binding to heparin requires the folded "closed" protein conformation (Wang et al. 2012; Lortat-Jacob et al. 2012). HSPG are also known to affect the secretion of FGF (fibroblast growth factor) through a similar but yet-to-be-elucidated "molecular trapping" event, involving a concentration gradient of the growth factor towards the cell surface (Zehe et al. 2006). Despite all this effort, how TG2 can enter the endosomes on one the hand and how can HSPG facilitate TG2-crossing of the cell membrane on the other hand, are still open questions. Recently, an N-terminal fragment of TG2 (88WTATVVDQQDCTLSLQLTT106) within the beta-sandwich domain was proposed to be critical to its export in tubular epithelial cell lines (Chou et al. 2011).

A variety of players, modulators and sequence peptides responsible for TG2 secretion have been proposed but not a unique clear mechanism. It is tempting to hypothesise that TG2-unconventional secretion across the plasma membrane may be influenced by the cellular context and the extracellular microenvironment, not only by its sequence features; the mode of export may depend on whether it occurs as a result of wounding or as part of an inflammatory response, or for cell survival via outside-in signalling of healthy cells and tissue.

Extracellular TGs in plants

Extracellular TGs have been detected in fungi, algae and, recently, new data have been made available in higher plants. Plant cells possess a continuous cell wall which has both a protective and structural role, and in pluricellular organisms, cells connect to each-other by the middle lamella and intercellular spaces. Cell wall structure and composition appear to be quite conserved among tissues of higher plants, with a common composition of cellulose microfibrils linked with other non-fibrillar lubricant polysaccharides, known as hemicelluloses. These cellulose–hemicellulose fibrils constitute a net, embedded in a complex gel-like matrix, which determines the cell wall porosity and its pH. Major components of the cell wall matrix are highly hydrated pectins, structural proteins (among which are glycoproteins) and diverse soluble proteins such as enzymes. Thus, intercellular communication in plants is mediated by cell walls and

ECMs, in addition to plasmodesmata. The composition and structure of fungi and algae cell walls are, with some exception, different according to the taxonomic group and from that of higher plants.

Extracellular TG in "lower" organisms

TG activity has been shown to affect cell wall glycoproteins of the alga *Chlamydomonas reinhardtii*, (Waffenschmidt et al. 1999). During its life cycle, the alga elaborates two distinct cell walls. The vegetative wall, which surrounds both vegetative and gamete cell displays salt-soluble glycoproteins on outer layers and salt- and detergent-insoluble components only in the inner layers. In the early zygotes, this wall is substituted by a desiccation-resistant, insoluble zygote wall. As both walls lack abundant complex polysaccharides typical of higher plants ECMs (cellulose, hemicellulose and pectins), the investigation of the assembly of the structural wall proteins is simpler. Three distinct vegetative-wall assembly events have been identified by these authors: (a) an early TG catalyses cross-linking reactions which lead to the formation of a "soft envelope"; (b) this organises the self-assembly of glycoproteins; (c) finally, an oxidative cross-linking reaction (isodityrosine cross-linking) renders the wall insoluble (Waffenschmidt et al. 1999). *Chlamydomonas reinhardtii* secretes an extracellular 72 kDa-TG, the maximal activity of which precedes the insolubilization of the assembled Hyp-rich glycoprotein. The addition of cadaverine, spermine (SM), spermidine (SD) and putrescine (PU) at high concentrations disrupts the normal process of wall assembly. In the cell wall, PAs are linked to several proteins and salt-soluble glycoproteins; two glycoproteins carry potential sites for the covalent attachment of PU by TG, but these sites are normally inaccessible. By contrast, the labelling of inner-wall components is likely to be significant because TG activity appears responsible for nucleating the assembly of the wall.

TG activity was suggested to exert an important role in the structural organization of the cell wall of the fungus *Candida albicans,* possibly by establishing cross-links among structural glycoproteins. The activity was detected by incorporation of radioactive PU into TCA-precipitable material and most of the activity was present in the cell wall (Ruiz-Herrera et al. 1995). Cystamine, an inhibitor of TG was used to analyse the role of TG in the organisation of the fungus-cell wall: it delayed protoplast regeneration, increased the sensitivity of the protoplast to the osmotic shock and inhibited the yeast-to-mycelium transition. Cystamine also affected the cell morphology as cells appeared as dwarf mycelia, pseudomycelia and budding yeast, whilst the incorporation of covalently bound high-molecular-weight proteins into the cell wall was inhibited.

In *Saccharomyces cerevisiae*, the interpeptidic cross-link *N*-ε-(γ-glutamyl)-lysine was shown to occur prevalently in cell walls, by the incorporation of [^{14}C]lysine (Iranzo et al. 2002). Like in *Candida albicans,* cystamine inhibited TG activity and cell growth, altered cell morphology and sensitivity to cell wall degrading enzymes. In *Phytophthora* sp. (Oomycete), a destructive plant pathogen, a cell wall glycoprotein was identified as a Ca^{2+}-dependent TG. A surface-exposed peptide fragment of this protein acted as an elicitor of defence responses in parsley and potato (Brunner et al. 2002). *Phytophthora sojae*, a pathogen of soybean, secretes a Ca^{2+}-dependent TG (GP42) which activates defence responses in both host and non-host plants. GP42 even though does not share significant primary sequence similarity with known TGs from mammals or bacteria, possesses a core region that displays significant similarity to that of Mac-1 cysteine protease from Group A Streptococcus, a member of the papain-like superfamily of cysteine protease, suggesting that a lateral gene transfer event may have occurred between bacteria and oomycetes (Reiss et al. 2011). In *Phytophthora ramorum* two cell wall-associated TGs were suggested to be important for pathogenicity (Meijer et al. 2006). The presence of TG, in the cell wall of "lower" eukaryotes having different chemical composition, and pertaining to organisms taxonomically very distant, suggests that this active component has a function conserved by the evolution for both the cell wall structural organisation and cell defence.

Extracellular TG in "higher" plants

Cell wall-associated TG

Similarly to animal TGs, "higher" plant enzymes are Ca^{2+}-dependent and able to produce glutamyl-polyamine derivatives; most of them respond to animal TG antibodies (Serafini-Fracassini and Del Duca 2008). One of them, (AtPng1p) (Q9FGY9, PNG1_ARATH), was cloned showing in its sequence the TG catalytic triad. It was sequenced from *Arabidopsis thaliana* and presented all the biochemical characteristics of the typical TGs, but showed little sequence homology with the best-known animal enzymes (Della Mea et al. 2004; Serafini-Fracassini et al. 2009; Beninati et al. 2013). Antibodies raised against this enzyme were used to immunodetect extracellular TG, in addition to other antibodies against TGs of animal species.

The presence of TG in the cell wall of higher plants was hypothesised based on data showing that the digestion of parenchymal cell walls by cellulase and pectinase caused the disaggregation of PA-conjugated proteins of apparent high mass, an event which suggests the existence of an interconnection between these proteins and some wall polysaccharides (Dinnella et al. 1992). The presence of

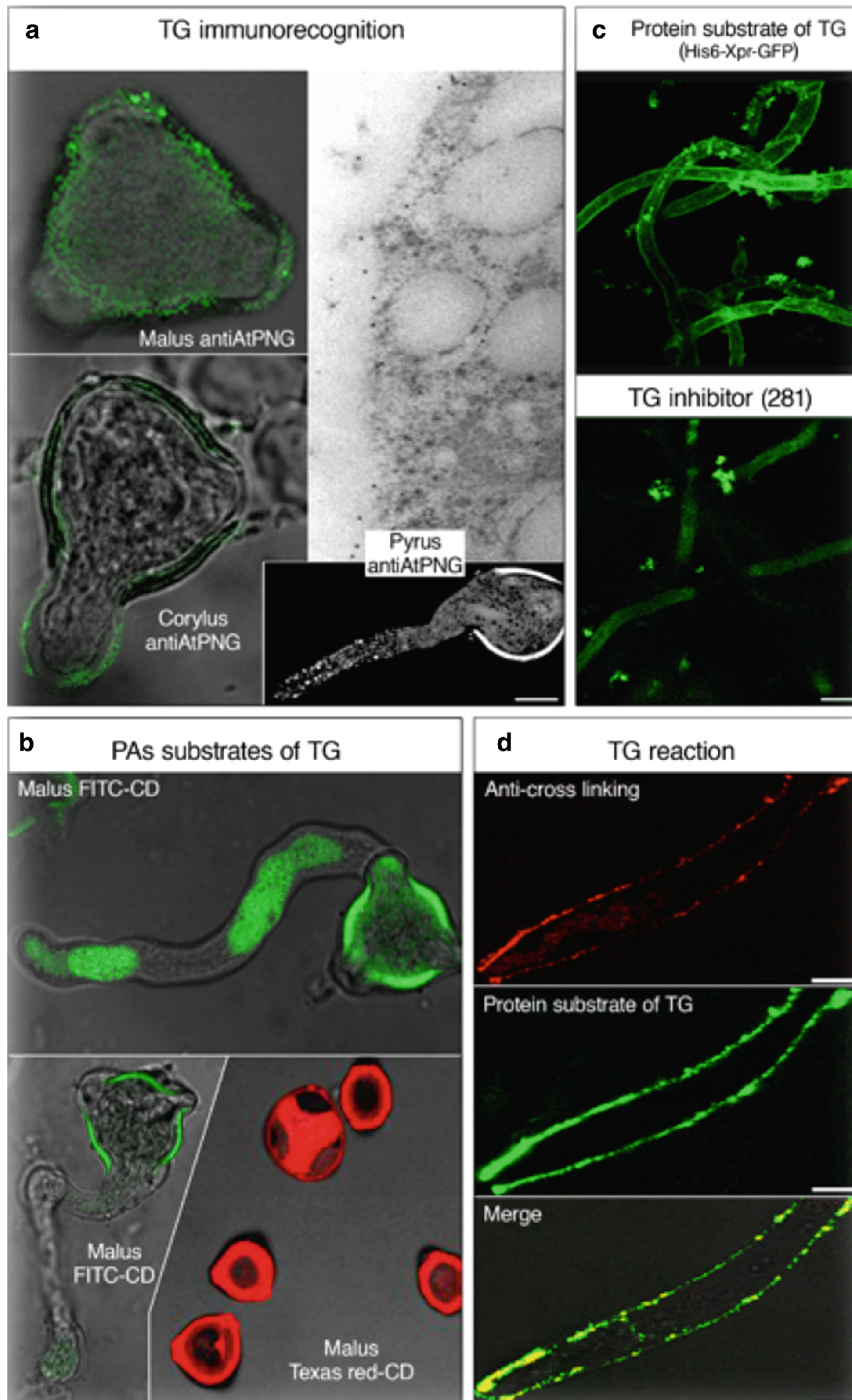
PAs in the cell wall is well documented (Berta et al. 1997). All the three main aliphatic polyamines (PU, SD and SM) have been detected in the cell wall of higher plants and SM can access the high-affinity sites of the cell wall even more easily than other cations. It has been hypothesised that PAs could be a regulatory factor of cell wall expansion (D'Orazi and Bagni 1987). PAs interact with pectic substances and are responsible for the ordered packing of polygalacturonic chains by changing the cell wall pH, and they could also be involved in the synthesis of cellulose (D'Orazi and Bagni 1987). Thus, one of the substrates of TG is normally present in the cell wall. The inhibition of PAs biosynthesis induces modification of the structure, shape and size of the primary cell walls of *Nicotiana* thin layers, with loosening of the fibrillar components, detachment of contiguous cells and lysis of wall components (Berta et al. 1997). PA oxidases of the cell wall (enzymes involved in lignification, cross-linking extensine and polysaccharide-bound phenols) are particularly active during programmed cell death (PCD) (Angelini et al. 2010). The strength of the links among cell wall components by PAs could be due to their ionic interaction with pectic substances, and/or to covalent TG-mediated interactions with proteins.

TG is localized in the petals-cell wall of *Nicotiana tabacum* flower, where the enzyme was detected by immunoreactivity in western blotting and its catalytic activity was also measured. This plant model allows evaluating the changes occurring in the walls of cells undergoing developmental processes, senescence and cell death, as evidenced by an increased autofluorescence and by the rigid/papyraceous-like aspect of the corolla (Serafini-Fracassini et al. 2002). By isolating a cell wall-enriched fraction, an active 58-kDa TG form was detected; the enzyme activity prevailed in the distal part of the corolla and progressively increased with the development of differentiation and senescence during the flower life span stages. The maximum activity shifted from the proximal to the distal part, preceding the PCD acropetal pattern, suggesting a relationship between them. The contents of *bis*-glutamyl-putrescine and of bis-glutamyl-spermidine, whose presence are indicative of linkages among proteins, were highest during complete differentiation (Serafini-Fracassini et al. 2002). Data suggested that in this system, TG could be responsible for the corolla strengthening, by forming high-molecular nets among the proteins/glycoproteins, especially at the abscission zone and during corolla shape change, possibly finalized to the protection of the ovary containing the developing embryo (Della Mea et al. 2007).

Another model in which cell wall-associated TGs are supposed to regulate growth is pollen, whose developing tube is an excellent haploid cellular example of apical growth. TG is widely distributed in the pollen of pear and apple, as well as of hazel (*Corylus avellana)* and pomelo (*Citrus grandis*), and it occurs as both a cytosolic and a membrane/cell wall form (Iorio et al. 2012a; Gentile et al. 2012).

In apple pollen, monoclonal and polyclonal antibodies against TG2 cross-react with two main immunoreactive bands of 70 and 75 kDa, the latter more evident in crude extracts of germinating pollen, which were detected in germination medium and in the cell wall (Di Sandro et al. 2010). A more detailed analysis of pear pollen showed that TG antibodies cross-reacted with a 70-kDa TG-related polypeptide in the cytoplasmic protein pool, in the membrane (microsomal) fraction and in both Triton- and sodium dodecyl sulphate (SDS)-extracted cell wall proteins. This form of TG might not be identical to the cytosolic one, probably being the result of post-translational modifications, as suggested by their different isoelectric point in western blots (Del Duca et al. 2013a). The presence of different forms of TG has been reported recently also in sprout apices of *Helianthus tuberosus* (Beninati et al. 2013).

In the pollen tube, the presence of TG in association with the cell wall is documented by several cytological and biochemical data, both direct and indirect. Preliminary data obtained at the end of the 90 s revealed the presence of TG activity in association with the Triton X-100 fraction obtained from apple pollen tubes, suggesting that a possible pollen TG might exist in association with the endomembrane system, thus also including the plasma membrane and vesicles (Del Duca et al. 1997). Direct data obtained via immunolocalisation by using a polyclonal antibody raised against a plant TG (AtPng1p) gave positive indications of its presence in cell walls, when tested on apple, pear and *Corylus* pollen (Fig. 3a). In apple pollen, TG was also immunorecognized by a monoclonal antibody directed against the mammalian TG2 by confocal fluorescence microscopy (Di Sandro et al. 2010). The TG staining was present in a finely punctuate pattern (apparently in the intine) in the emerging tube. During germination, with the increase in pollen tube length, the fluorescent signal became widespread in a continuous finely punctuate pattern inside the tube: some fluorescent spots were detected predominantly in the proximal region of the tube, whose shanks, in some tract, were strongly labelled, and at the apical tip. The presence of TG in the cell walls of either apple or pear pollen was also observed by immunogold transmission electron microscopy, and TG was found associated with membrane-bound organelles or vesicles and with both the inner amorphous layer and the outer fibrillar layer of the cell wall. TG was often detected aligned along the plasma membrane, but occasionally in association with unclassified membrane structures, very close to the plasma membrane (Di Sandro et al. 2010; Del Duca et al. 2013a) (Fig. 3a).


a
TG immunorecognition
Malus antiAtPNG
Corylus antiAtPNG
Pyrus antiAtPNG
c
Protein substrate of TG
(His6-Xpr-GFP)
TG inhibitor (281)
b
PAs substrates of TG
Malus FITC-CD
Malus FITC-CD
Malus Texas red-CD
d
TG reaction
Anti-cross linking
Protein substrate of TG
Merge

◂**Fig. 3** Transglutaminase enzyme, its substrates and activity in different pollens. **a** The enzyme was immunolocalized by laser-scanning confocal and fluorescent microscopy using antibodies against a plant TG (AtPng1p), on pollen of apple (*Malus domestica*), hazelnut (*Corylus avellana*) (Iorio et al. 2012a) and pear (*Pyrus communis*) (original Dr. Faleri) as indicated. In the latter pollen, TG was observed by immunogold transmission electron microscopy in association with membrane-bound organelles or vesicles and with both the inner amorphous layer (the clear layer) and the outer fibrillar layer of the cell wall (original Dr Faleri). **b** TG substrate localisation. Fluorescein cadaverine (FITC-CD) (Iorio et al. 2008, 2012a) and texas red cadaverine (CD) (original Dr Verderio) TG-catalysed conjugation to apple pollen substrates observed by laser-scanning confocal microscopy. In the lower picture on the left, a germinating apple pollen pre-hydrated in stressing environmental conditions (Iorio et al. 2012a). **c** Specific TG substrate conjugation. Laser-scanning confocal microscopy of the tube cell wall of non-digested and non-permeabilized apple pollen. The fluorescent-specific TG substrate (His6-Xpr-GFP) was incubated with germinating pollen (*upper panel*). The specific TG inhibitor 281 was incubated together with His6-Xpr-GFP, as above (*lower panel*) (Di Sandro et al. 2010). **d** Co-association of TG-mediated cross-linking and protein products. Conjugating TG activity on the tube cell wall of non-digested and non-permeabilized apple pollen was observed by laser-scanning confocal microscopy of germinating pollen tubes incubated with His6–Xpr–GFP prior to fixing (*upper panel*) and incubated with 81D4 [monoclonal antibody against glutamyl-lysine and glutamyl -PAs] followed by TRITC-conjugated secondary antibody (*middle panel*) and merge (Di Sandro et al. 2010)

The indirect evidence of the localization of the enzyme is based on the TG-catalysed conjugation of fluorescent derivatized PAs to pollen substrates (Iorio et al. 2008; Di Sandro et al. 2010). Specifically, fluorescein cadaverine (FITC-CD) and texas red cadaverine were localized both in actively growing regions of the tubes (the apex) and in regions (such as the grain) characterised by no growth activity (Fig. 3b). These derivatized PAs, supplied at relatively high concentration, acted as competitors of endogenous amine-donor substrates (ε-amino group of Lys residues or polyamines) and limited or blocked tube elongation of pollens. A similar pattern was observed by incubating germinating pollen with a fluorescent specific TG substrate (His6-Xpr-GFP) that appeared to be linked to the tube cell wall of non-digested and non-permeabilized pollen. The role of pollen TG in this catalysis was confirmed by inhibiting the enzyme activity with the TG inhibitors (281 and 283): the tube cell wall was devoid of labelling (Fig. 3c). By omitting cell wall-digestion and permeabilization of pollen prior to fixation, it was showed that TG was concentrated in clusters along the pollen tube, but the enzyme could be removed by cell wall-digestion (Di Sandro et al. 2010).

The enzymatic activity of an extracellular TG in various pollens was confirmed by measuring it during pollen germination in in vivo. Addition of biotin-cadaverine (BC) to the germinating medium allowed measurement of TG activity via the conjugation of BC into *N*, *N*′-dimethylcasein (substrate of animal TGs) immobilised on the germination wells. The enzyme was found to be mainly expressed at the beginning of germination, not only when it was particularly active, but also throughout the germination period (Iorio et al. 2012a). TG activity was specifically inhibited by TG inhibitor 281, cystamine, EGTA and competitive substrates such as iodoacetamide and putrescine, which also inhibited the apical growth of the pollen tube, demonstrating that the extracellular TG is involved in this event (Di Sandro et al. 2010; Iorio et al. 2012a).

The occurrence of this conjugating activity in pollen was confirmed by the co-localisation of the TG activity (revealed by antibody directed against the cross-links Gln-Lys or Gln-PAs (81D4)) in cell wall with its substrates. This was also evidenced by the conjugation of His6–Xpr–GFP, a specific substrate of animal TG (Fig. 3d) (Di Sandro et al. 2010a).

In conclusion, these data have shown that the enzyme-distribution overlaps with that of the substrates and of the cross-linked products. The immuno-histochemical analysis showed that the cross-reactive proteins of pollen were significantly concentrated in the apical region of the tube, suggesting that the putative TG is related to the process of tube extension (Fig. 3a, b); it would be interesting to speculate that the cross-linked products may provide strength to the pollen tube migrating through the style in planta. These immunological and biochemical data also indicate that the extracellular TG is not a specific enzyme of cell walls, but it shares many characteristics with the intracellular one.

The different composition and organisation of the cell walls of anemophilous (i.e., *Corylus avellana)* or entomophilous (i.e., *Malus domestica*) pollen grains is also reflected by the different extent of TG distribution (*Malus domestica* cell wall was more intensely immuno-labelled than that of *Corylus avellana*), and by the extent of the release of various molecules. The latter include allergens, and probably extracellular TG released during germination and indeed dispersed into the germination/assay medium (Iorio et al. 2012a). Simulated adverse environmental conditions (such as climate change conditions) and pollution caused an increase in extracellular TG activity, which was much more marked in the allergenic *C. avellana* pollen in comparison with *M. domestica.* An example of the damage exerted by environmental criticalities on apple pollen germination is shown in Fig. 3b. The fluorescent signals detected in vivo with the polyclonal antibody anti AtPng1p showed that the enzyme was released into the medium, probably due to a leakage from pollen cell wall or membrane. Due to the potential of externalised pollen TG to modify pollen antigens, these data suggest that TG may be one new factor which links environmental conditions and pollution to respiratory pollen allergies (Iorio et al. 2012a).

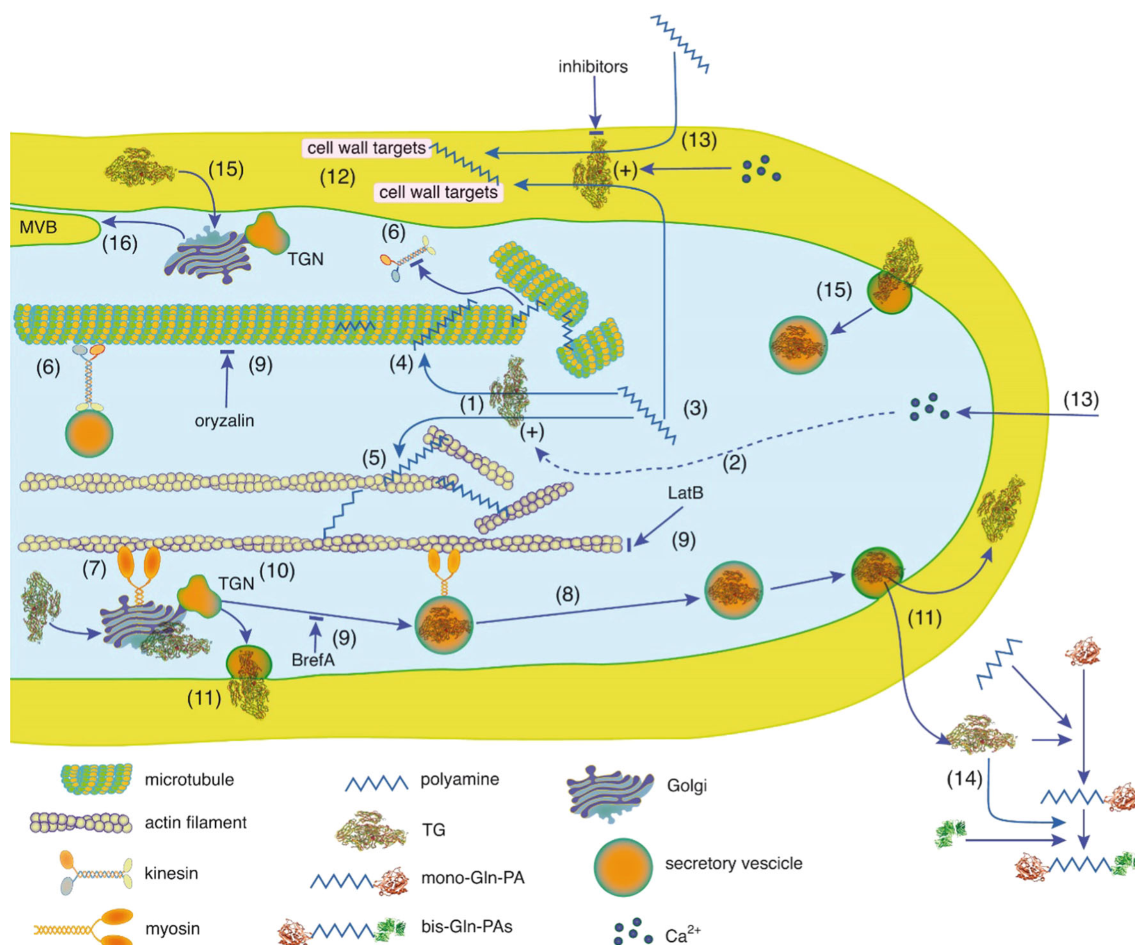


Fig. 4 Schematic diagram of the transport and secretion of TG in the pollen tube. The cytosolic TG (*1*), presumably after activation by calcium ions (*2*) and in the presence of endogenous polyamines (*3*), modifies post-translationally the cell substrates, including microtubules (*4*) and actin filaments (*5*). Alteration of the microtubular structure consequently inhibits the ability of kinesin motors (*6*) thus affecting membrane trafficking. The modifications introduced in the structure of actin filaments affect the enzymatic activity of myosin (*7*) and then the transport of membranes (organelles/vesicles) dependent on this motor protein (*8*). Substantial changes in the distribution of TG (both cytosolic and membrane bound) can be induced through the use of cytoskeleton inhibitors, such as oryzalin for microtubules and Latrunculin B (LatB) for actin filaments, and of membrane trafficking, such as Brefeldin A (BrefA) (*9*). Membrane TG is supposed to be transported through vesicular intermediates probably produced by the Golgi apparatus and/or by the Trans-Golgi-Network (TGN) (*10*) and to be secreted externally (*11*). In the cell wall, TG could conjugate polyamines to specific molecular targets (*12*) in the presence of calcium ions, which are also known to be able to enter from the tip (*13*); TG might catalyse the conjugation of PAs to pollen-secreted proteins thus forming mono- or bis-PA derivatives (*14*). It is not to be excluded that part of the cell wall TG is recovered through endocytotic mechanisms (*15*), which may deliver it to the TGN and from here to the multi vesicular body (MVB) for degradative processes (*16*)

Transglutaminase secretion

In plants, the way by which extracellular TG is likely to be secreted outside the plasma membrane is not known, although a mechanism including fusion of secretory vesicles is likely. No data clearly show the association between TG and secretory vesicles or that the transport of TG to the plasma membrane and cell wall is vesicle-based. Figure 4 summarizes some of the data recently published that are described here below and also suggests some working hypotheses. A more detailed analysis of the cellular localisation of TG, particularly in relation to the dynamics of the cytoskeleton and of the endomembrane system, recently showed that the pollen TG is distributed in a way dependent on the integrity of actin filaments (Del Duca et al. 2013a). The actin filament-based membrane trafficking is thus required for the proper distribution of TG, as also proven by the use of membrane trafficking inhibitors (specifically, Brefeldin A) (Fig. 4); in fact, when the accumulation of secretory vesicles was disturbed, the distribution of TG was also altered (Del Duca et al. 2013a). Damages due to the use of microtubule inhibitors were more limited, suggesting that this cytoskeletal system is much less important in determining the localisation of TG (Fig. 4). The pattern of TG

isoforms was not identical in the different compartments of the pollen tube, namely the cytosol, the membrane compartment and the cell wall. The plant TG family is thought to be small compared with the mammalian family of enzymes, e.g., only one gene (*AtPng1p*), encoding for a protein with a catalytic triad typical of the TG family and with TG activity, has been indentified in Arabidopsis; thus it seems likely that the different TG isoform pattern results from a post-translational modification process of TG itself, in its route from the cytosol to the cell wall. A well-known example of post-translational modifications that modifies the cellular localization of proteins is represented by sucrose synthase, which oscillates between a cytosolic and a plasma membrane-associated form, according to the level of post-translational phosphorylation (Hardin et al. 2004). A similar mechanism might apply to TG.

The site of TG secretion remains to be clarified. Classically, the apex of the pollen tube has always been considered as the area of active secretion, but at least two aspects remain to be elucidated. First, the exact site of secretion needs to be revealed, i.e., whether it is exactly at the apex of pollen tubes or at an intermediate zone between the apex and the subapex (Fig. 4) (Geitmann and Dumais 2009). Second, whether the secretion occurs only in the apical area or in other areas of the pollen tube also need to be elucidated. Indeed, although most of the pollen proteins are secreted at the apex of pollen tubes through the activity of secretory vesicles, such as the enzyme pectin-methylesterase (Bosch and Hepler 2006), in recent years some data have indicated the existence of alternative mechanisms to the apical secretion.

For example, an H^+-ATPase was localized and secreted directly in the regions of pollen tubes immediately close to the subapex (Certal et al. 2008). This alternative secretion would be consistent with the function of the protein, which is involved in the regulation of intracellular pH necessary for the proper growth of pollen tubes. Another example is the distribution of the vacuolar sorting receptor (VSR), a receptor protein that mediates the transport to the vacuoles through the multivesicular body. The VSR protein is localized in the plasma membrane of pollen tubes but not at the apex. This differential localisation can be either the result of the particular mode of secretion (the so-called "kiss-and-run") or of a different secretory process that might deliver the protein directly from the Trans-Golgi-Network (TGN) to the plasma membrane (Wang et al. 2011a) (Fig. 4). Presently, data available to us do not show the exact site from which TG is secreted.

Differences in localisation between plants and mammalian protein counterparts are not surprising. For instance, calmodulin (CaM) is a small protein usually described in the cytosol of eukaryotic cells, but also present in the cell wall of plant cells. In the case of pollen tubes, the extracellular CaM stimulates the tube growth (Ma and Sun 1997) acting as a signal molecule. Sucrose synthase was also found in the cell wall of pollen tubes (Persia et al. 2008) and of somatic plant cells (Salnikov et al. 2003). Since sucrose synthase cooperates with cellulose synthase and callose synthase, these data suggest that the cell wall-sucrose synthase is correlated with the synthesis of cellulose and callose (Amor et al. 1995) and might be an alternative system for the extracellular synthesis of cell wall polymers (Salnikov et al. 2003). Similarly to apple pollen TG, that shows a different value of isoelectric point in western blots, possibly being the result of post-translational modifications (Del Duca et al. 2013a), sucrose synthase also exhibits an isoform pattern resulting from specific post-translational modifications, dependent on the type of localisation (Persia et al. 2008).

To explain the presence of TG extracellularly, we can hypothesise two different mechanisms of export. First, TG could express a signal peptide that may possibly target part of it towards the membrane compartments of the cell. Once in place, TG could be post-translationally modified for the accomplishment of its functions. Alternatively, cytosolic TG may be "captured" by the vesicular system of the pollen tube using a mechanism similar to mitochondria or chloroplast import-system, i.e. through the direct crossing of membranes. In the latter case, any post-translational modification of TG may serve to stabilise the end position of TG or to mark the TG molecules to be allocated to different compartments.

The hypothesis of an unconventional way for secretion of transglutaminase in pollen tubes has been recently reported (Del Duca et al. 2013b). Assuming that TG follows a secretory pathway based on membrane trafficking, it would be necessary to demonstrate the association of the enzyme with the inner membranes of plant cells. This finding was recently confirmed by the immunological and enzymatic identification of TG in association with the pollen tube membranes isolated by fractionation on sucrose gradients (Del Duca et al. 2013a). The pollen TG was localized in fractions containing markers of the plasma membrane and Golgi apparatus. However, while the association of TG with the plasma membrane is documented, the intracellular transport of TG through the standard ER-Golgi route is not yet a demonstrated feature. For example, as reported above, in animal cells the secretion of extracellular TG can occur through the recycling endosomes and not through the ER-Golgi system (Zemskov et al. 2011). There are no comparable data in plant cells but this finding sheds light on possible new scenarios for the secretion of extracellular TG (Fig. 4). The hypothesis of TG travelling through the internal membrane system (Golgi apparatus and TGN) to be secreted externally by secretory vesicles

(Fig. 4) is an attractive one. This part of TG may be labelled by a specific post-translational modification while the cell wall TG would be characterised by a different type of modification. TG could also reach the plasma membrane and/or the cell wall through an alternative mechanism of secretion that directly starts from the TGN. The latter membrane system (in analogy to what is known in the pollen tube) might also be involved in the endocytotic recycling of excess TG. The endocytotic recovery of TG could be essential to maintain a constantly appropriate level of the enzyme. In animal cells, endocytotic processes and lysosomal degradation were proved as necessary to maintain adequate levels of membrane TG (Zemskov et al. 2007). Since several immunofluorescence images have shown that TG accumulates in the apical region of the pollen tube (Fig. 3a), it is reasonable to hypothesise that this distribution is implemented by a balanced process of exocytosis/secretion and endocytosis/recovery. This mechanism has been currently proposed in the pollen tube and it would be necessary to maintain both a differential distribution of apical proteins and an adequate protein level. It is not known where TG carries out its functionality, but immunolocalization data suggest that the enzyme is active in the apical or subapical region of the pollen tube. This working hypothesis is plausible because this region is actively growing and most active processes of growth regulation occur at this point.

As TG is detected also in the walls of no more growing portion of tubes (Di Sandro et al. 2010), it could suggest that TG once exerted his role as catalysing enzyme, could remain "embedded" along the mature wall, as it occurs for TG2 in some animal cells, as above reported.

TG secreted in the extracellular matrix

In addition to the presence and role of TG in the cell wall, the enzyme has been shown to be released in the extracellular environment. In fact, in apple pollen germinating in vitro, TG activity has been found on the cell wall surface and in the germinating medium (Di Sandro et al. 2010). The significance of this finding in vitro is to be related to flower biology and reproduction. In fact pollen germination in the pistil tissues represents one of the rare events of plant cell migration, since usually cells are blocked from moving by the contact of their cell walls.

Flower receptivity plays a crucial part in pollination dynamics, reproductive success and plant productivity. The stigma is the first surface of the pistil to allow adhesion with the pollen grains, followed by pollen hydration and germination, providing an adequate environment for pollen germination. Even though little is known about its capability to be receptive for pollen grain germination, pollen adhesion and hydration (with the stigma) appear to be mediated by lipids, pectins or peptides accumulated in the stigmatic exudates (Wolters-Arts et al. 2002), some of which can be released by pollen and interact with stylar glycoproteins to anchor the pollen tube to the pollen-stylar extracellular matrix (psECM) (Lord and Russell 2002). The molecular mechanism underlying this process is not fully understood, but it involves adhesion, nutrient delivery and pollen tube guidance (Lord 2003; Sanchez et al. 2004). psECM includes polysaccharides, free sugars, proteins, proteoglycans, lipids, phenolic compounds and glycoproteins (Cheung and Wu 2008). Arabinogalactan proteins (AGPs) exhibit an increasing gradient of glycosylation towards the basal parts of the style possibly exerting a nutritive role and being chemotropic factors for the growing pollen tube (Cheung and Wu 2008). Pectinolytic enzymes identified in pollen tubes probably have a role in facilitating their penetration of pectocellulose papillae cell walls and to deliver the precursors of pollen tube cell wall biosynthesis (Bosch et al. 2005; Bosch and Hepler 2006).

In this context, we can speculate that one modulator of pollen tube/psECM interactions could be TG as it plays an essential role in pollen tube growth, for instance by strengthening its cell wall scaffold due to protein cross-linking and amine-protein conjugation. Moreover, the released enzyme could be involved in connecting the pollen tube to surrounding stylar cells through the psECM, thus allowing style anchorage and subsequent tube migration to reach the female gamete inside the ovule to perform fertilisation.

Self-incompatibility (SI) is the most important system to prevent inbreeding and promote outbreeding; from an evolutive point of view, it is thought that the wide success of the Angiosperms is due, at least in part, to the selection of the SI mechanism. It involves a pollen–pistil interaction and a cell–cell recognition system, which regulates the acceptance or rejection of pollen of the same species, preventing self-fertilisation. SI is a species-specific and genetically controlled mechanism (McClure et al. 2011).

An increase of PA content and TG activity occurs during incompatible pollinations in pear and citrus in a reverse way to their compatible controls, reaching a maximum when SI phenomena become visible (Gentile et al. 2012; Del Duca et al. 2010). Moreover, TG forms a sort of cup around the apex of incompatible pollen of pear where the tip is embedded in a strict net, counteracting the internal turgor pressure and perhaps causing also callose-accumulation at the tip. The increase of activity was not due to a higher expression of the enzyme (Iorio et al. 2012a, b), but possibly to the increase of intracellular Ca^{2+} concentration during SI response in *Pyrus* pollen tubes (Wang et al. 2010a).

One more supporting piece of evidence for a TG role in SI comes from the finding that the cytoplasmic TG of apple

pollen post-translationally modifies actin and tubulin by conjugating them to PAs (Del Duca et al. 1997). If the activity is very high, it generates high-molecular-weight aggregates (Del Duca et al. 2009) capable of inhibiting the enzyme activity and the binding affinity of myosin and kinesin and, consequently, inhibits motor-dependent dynamic activities. Thus, TG could play a role in the rearrangement of cytoskeleton that occurs during SI, event well described in *Papaver rhoeas* (poppy). This event triggers a cascade of Ca^{2+}-dependent signals, which inhibit tip growth by causing the depolymerisation of actin filaments and determining a caspase-like protease activity (Bosch and Franklin-Tong 2008) which may also involve depolymerisation of microtubules (Poulter et al. 2008). The modification of cytoskeleton of pollen tubes is consequently part of the mechanism that regulates the SI response. In *Rosaceae*, like in apple and pear, the SI response is known to be based on the presence of S-RNases, produced by the pistil and internalized into the pollen tube (McClure et al. 2011). S-RNase might degrade the RNA of incompatible pollen to prevent fertilization; however, it is likely that changes to Ca^{2+} concentration and modifications of the actin filament organization are also involved (Liu et al. 2007). Because of this evidence, the molecular mechanisms of rejection of self-pollen might share common features among different families. Moreover, the molecules that mediate the S-RNase-based SI response are not known, and TGs might be considered a promising candidate. The involvement of TGs in the SI response, which is considered a programmed-cell-death (PCD), is also supported by the evidence that plant TGs participate to PCD of leaves and flower corollas, as reported earlier (Serafini-Fracassini et al. 2010; Della Mea et al. 2007).

It is intriguing to note that genes involved in cell–cell recognition (as allergens, resistance genes and F-boxes) are usually organised in clusters in the maloideae genomes; this phenomenon can represent an evolutive conserved strategy for the cells to recognize pathogens or self/non-self pollen. It was suggested that TG could act as one of the factors that play a role in both mechanisms (Iorio et al. 2012a, b).

In conclusion, the subject of extracellular TGs presents interesting aspects with several facets awaiting further investigations, especially in plants, where available data are very limited.

At the present state of the art, extracellular TG seems to be ubiquitous in “higher” and “lower” plants as well as in mammals and plays an important structural role in cell wall or fluid external medium in both the cell-to-cell interaction and cell interaction with local environment. An interesting working hypothesis is also to evaluate if TG is part of an evolutive conserved strategy for the cell defence to recognize pathogens or self/non-self pollen. Very intriguing is the possibility that the TG function in modulating cell migration reported in animal cells may also exist in plants, e.g., to support pollen tubes movement during fertilisation.

Intriguing is also the unconventional way of secretion, which seems to be different from the classical ER-Golgi route, followed by TG2 and probably also by pollen TG. In human pathology the involvement of extracellular TG2 is clearly established (Griffin et al. 2002; Lorand and Graham 2003; Hallstrand et al. 2010; Sorrentino et al. 2012), including its role in respiratory allergy. It is possible that pollen TG (Iorio et al. 2012a) could also be a participating factor in respiratory sensitization of allergenic pollen.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Aeschlimann D, Thomazy V (2000) Protein crosslinking in assembly and remodelling of extracellular matrices: the role of transglutaminases. Connect Tissue Res 41:1–27

Akimov SS, Krylov D, Fleischman LF, Belkin AM (2000) Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin. J Cell Biol 148:825–838

Al-Jallad HF, Myneni VD, Piercy-Kotb SA, Chabot N, Mulani A, Keillor JW, Kaartinen MT (2011) Plasma membrane factor XIIIA transglutaminase activity regulates osteoblast matrix secretion and deposition by affecting microtubule dynamics. PLoS One 6:e15893

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA 92:9353–9357

Angelini R, Cona A, Federico R, Fincato P, Tavladoraki P, Tisi A (2010) Plant amine oxidases “on the move”: an update. Plant Physiol Biochem 48:560–564

Aumuller G, Wilhelm B, Seitz J (1999) Apocrine secretion-fact or artifact? Ann Anat 181:437–446

Beninati S, Iorio RA, Tasco G, Serafini-Fracassini D, Casadio R, Del Duca S (2013) Expression of different forms of

transglutaminases by immature cells of *Helianthus tuberosus* sprout apices. Amino Acids 44:271–283

Berta G, Altamura MM, Fusconi A, Cerruti F, Capitani F, Bagni N (1997) The plant cell wall is altered by inhibition of polyamine biosynthesis. New Phytol 137:569–577

Bosch M, Franklin-Tong VE (2008) Self-incompatibility in *Papaver*: signalling to trigger PCD in incompatible pollen. J Exp Bot 59:481–490

Bosch M, Hepler PK (2006) Silencing of the tobacco pollen pectin methylesterase NtPPME1 results in retarded in vivo pollen tube growth. Planta 223:736–745

Bosch M, Cheung AY, Hepler PK (2005) Pectin methylesterase, a regulator of pollen tube growth. Plant Physiol 138:1334–1346

Brunner F, Rosahl S, Lee J, Rudd JJ, Geiler C, Kauppinen S, Rasmussen G, Scheel D, Nurnberger T (2002) Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora* transglutaminases. EMBO J 21:6681–6688

Certal AC, Almeida RB, Carvalho LM, Wong E, Moreno N, Michard E, Carneiro J, Rodriguez-Leon J, Hm Wu, Cheung AY, Feijo JA (2008) Exclusion of a proton ATPase from the apical membrane is associated with cell polarity and tip growth in *Nicotiana tabacum* pollen tubes. Plant Cell 20:614–634

Cheung A, Wu H (2008) Structural and signaling networks for the polar cell growth machinery in pollen tubes. Annu Rev Plant Biol 59:547–572

Chou CY, Streets AJ, Watson PF, Huang L, Verderio EA, Johnson TS (2011) A crucial sequence for transglutaminase type 2 extracellular trafficking in renal tubular epithelial cells lies in its N-terminal beta-sandwich domain. J Biol Chem 286:27825–27835

Cordell PA, Kile BT, Standeven KF, Josefsson EC, Pease RJ, Grant PJ (2010) Association of coagulation factor XIII-A with Golgi proteins within monocyte-macrophages: implications for subcellular trafficking and secretion. Blood 115:2674–2681

D'Orazi D, Bagni N (1987) In vitro interactions between polyamines and pectic substances. Biochem Biophys Res Commun 148:1259–1263

Del Duca S, Bregoli AM, Bergamini C, Serafini-Fracassini D (1997) Transglutaminase-catalyzed modification of cytoskeletal proteins by polyamines during the germination of *Malus domestica* pollen. Sex Plant Reprod 10:89–95

Del Duca S, Serafini-Fracassini D, Bonner PL, Cresti M, Cai G (2009) Effects of post-translational modifications catalyzed by pollen transglutaminase on the functional properties of microtubules and actin filaments. Biochem J 418:651–664

Del Duca S, Cai G, Di Sandro A, Serafini-Fracassini D (2010) Compatible and self-incompatible pollination in *Pyrus communis* displays different polyamine levels and transglutaminase activity. Amino Acids 38:659–667

Del Duca S, Faleri C, Iorio RA, Cresti M, Serafini-Fracassini D, Cai G (2013a) Distribution of transglutaminase in pear pollen tubes in relation to cytoskeleton and membrane dynamics. Plant Physiol 161:1706–1721

Del Duca S, Serafini-Fracassini D, Cai G (2013b) An unconventional road for the secretion of transglutaminase in pollen tubes? Plant Signal Behav 8:e24446

Della Mea M, Caparrós-Ruiz D, Claparols I, Serafini-Fracassini D, Rigau J (2004) AtPng1p: first cloning and characterisation of a plant transglutaminase. Plant Physiol 135:2046–2054

Della Mea M, De Filippis F, Genovesi V, Serafini-Fracassini D, Del Duca S (2007) The acropetal wave of developmental cell death (DCD) of *Nicotiana tabacum* corolla is preceded by activation of transglutaminase in different cell compartments. Plant Physiol 144:1211–1222

Di Sandro A, Del Duca S, Verderio E, Hargreaves AJ, Scarpellini A, Cai G, Cresti M, Faleri C, Iorio RA, Shigehisa H, Furutani Y, Coutts IGC, Griffin M, Bonner PL, Serafini-Fracassini D (2010) An extracellular transglutaminase is required for apple pollen tube growth. Biochem J 429:261–271

Dinnella C, Serafini-Fracassini D, Grandi B, Del Duca S (1992) The cell cycle in *Helianthus tuberosus*. Analysis of polyamine-endogenous protein conjugates by TG-like activity. Plant Physiol Biochem 30:531–539

Fesus L, Piacentini M (2002) Transglutaminase 2: an enigmatic enzyme with diverse functions. Trends Biochem Sci 27:534–539

Filiano AJ, Bailey CD, Tucholski J, Gundemir S, Johnson GV (2008) Transglutaminase 2 protects against ischemic insult, interacts with HIF1beta, and attenuates HIF1 signaling. FASEB J 22:2662–2675

Folk JE (1980) Transglutaminases. Annu Rev Biochem 49:517–531

Geitmann A, Dumais J (2009) Not-so-tip-growth. Plant Signal Behav 4:136–138

Gentile A, Antognoni F, Iorio RA, Distefano G, Las Casas G, La Malfa S, Serafini-Fracassini D, Del Duca S (2012) Polyamines and transglutaminase activity are involved in compatible and self-incompatible pollination of *Citrus grandis*. Amino Acids 42:1025–1035

Griffin M, Casadio R, Bergamini CM (2002) Transglutaminase: nature's biological glues. Biochem J. 368:377–396

Hallstrand TS, Wurfel MM, Lai Y, Ni Z, Gelb MH, Altemeier WA, Beyer RP, Aitken ML, Henderson WR (2010) Transglutaminase 2, a novel regulator of eicosanoid production in asthma revealed by genome-wide expression profiling of distinct asthma phenotypes. PLoS One 5(1):e8583

Hardin SC, Winter H, Huber SC (2004) Phosphorylation of the amino terminus of maize sucrose synthase in relation to membrane association and enzyme activity. Plant Physiol 134:1427–1438

Huang L, Haylor JL, Hau Z, Jones RA, Vickers ME, Wagner B, Griffin M, Saint RE, Coutts IG, El Nahas AM, Johnson TS (2009) Transglutaminase inhibition ameliorates experimental diabetic nephropathy. Kidney Int 76:383–394

Iorio RA, Di Sandro A, Scarpellini A, Del Duca S, Serafini-Fracassini D, Verderio E (2008) Visualisation of transglutaminase-mediated cross-linking activity in germinating pollen by laser confocal microscopy. Plant Biosyst 142:360–365

Iorio RA, Di Sandro A, Paris R, Pagliarani G, Tartarini S, Ricci G, Serafini-Fracassini D, Verderio E, Del Duca S (2012a) Simulated environmental criticalities affect transglutaminase of Malus and Corylus pollens having different allergenic potential. Amino Acids 42:1007–1024

Iorio RA, Serafini-Fracassini D, Aloisi I, Del Duca S, De Franceschi P, Dondini L, Sansavini S, Cai G, Faleri C (2012b) Post-translational modification by transglutaminase of proteins involved in pear self-incompatibility. Acta Hortic 967:141–148

Iranzo M, Aguado C, Pallotti C, Canizares JV, Mormeneo S (2002) Transglutaminase activity is involved in *Saccharomyces cerevisiae* wall construction. Microbiology 148:1329–1334

Jiang WG, Ablin RJ (2011) Prostate transglutaminase: a unique transglutaminase and its role in prostate cancer. Biomark Med 5:285–291

Jiang H, Jans R, Xu W, Rorke EA, Lin CY, Chen YW, Fang S, Zhong Y, Eckert RL (2010) Type I transglutaminase accumulation in the endoplasmic reticulum may be an underlying cause of autosomal recessive congenital ichthyosis. J Biol Chem 285:31634–31646

Johnson TS, El-Koraie AF, Skill NJ, Baddour NM, El Nahas AM, Njloma M, Adam AG, Griffin M (2003) Tissue transglutaminase and the progression of human renal scarring. J Am Soc Nephrol 14:2052–2062

Liu ZQ, Xu GH, Zhang SL (2007) *Pyrus pyrifolia* stylar S-RNase induces alterations in the actin cytoskeleton in self-pollen and tubes in vitro. Protoplasma 232:61–67

Lorand L, Graham RM (2003) Transglutaminases: crosslinking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol 4:140–156

Lord EM (2003) Adhesion and guidance in compatible pollination. J Exp Bot 54:47–54

Lord EM, Russell S (2002) The mechanisms of pollination and fertilisation in plants. Annu Rev Cell Dev Biol 18:81–105

Lortat-Jacob H, Burhan I, Scarpellini A, Thomas A, Imberty A, Vives RR, Johnson T, Gutierrez A, Verderio EA (2012) Transglutaminase-2 interaction with heparin: identification of a heparin binding site that regulates cell adhesion to fibronectin-transglutaminase-2 matrix. J Biol Chem 287:18005–18017

Ma LG, Sun DY (1997) The effects of extracellular calmodulin on initiation of *Hippeastrum rutilum* pollen germination and tube growth. Planta 202:336–340

McClure B, Cruz-Garcia F, Romero C (2011) Compatibility and incompatibility in S-RNase-based systems. Ann Bot 108:647–658

Meijer HJ, van de Vondervoort PJ, Yin QY, de Koster CG, Klis FM, Govers F, de Groot PW (2006) Identification of cell wall-associated proteins from *Phytophthora ramorum*. Mol Plant Microbe Interact 19:1348–1358

Persia D, Cai G, Del Casino C, Faleri C, Willemse MTM, Cresti M (2008) Sucrose synthase is associated with the cell wall of tobacco pollen tubes. Plant Physiol 147:1603–1618

Poulter NS, Vatovec S, Franklin-Tong VE (2008) Microtubules are a target for self-incompatibility signaling in *Papaver* pollen. Plant Physiol 146:1358–1367

Radisky DC, Stallings-Mann M, Hirai Y, Bissell MJ (2009) Single proteins might have dual but related functions in intracellular and extracellular microenvironments. Nat Rev Mol Cell Biol 10:228–234

Reiss K, Kirchner E, Gijzen M, Zocher G, Löffelhardt B, Nürnberger T, Stehle T, Brunner F (2011) Structural and phylogenetic analyses of the GP42 transglutaminase from *Phytophthora sojae* reveal an evolutionary relationship between oomycetes and marine Vibrio bacteria. J Biol Chem 286:42585–42593

Ruiz-Herrera J, Iranzo M, Elorza MV, Sentandreu R, Mormeneo S (1995) Involvement of transglutaminase in the formation of covalent cross-links in the cell wall of *Candida albicans*. Arch Microbiol 164:186–193

Salnikov VV, Grimson MJ, Seagull RW, Haigler CH (2003) Localization of sucrose synthase and callose in freeze-substituted secondary-wall-stage cotton fibers. Protoplasma 221:175–184

Sanchez AM, Bosch M, Bots M, Nieuwland J, Feron R, Mariani C (2004) Pistil factors controlling pollination. Plant Cell 16:98–106

Santhanam L, Berkowitz DE, Belkin AM (2011) Nitric oxide regulates non-classical secretion of tissue transglutaminase. Commun Integr Biol 4:584–586

Satpathy M, Cao L, Pincheira R, Emerson R, Bigsby R, Nakshatri H, Matei D (2007) Enhanced peritoneal ovarian tumor dissemination by tissue transglutaminase. Cancer Res 67:7194–7202

Scarpellini A, Germack R, Lortat-Jacob H, Muramatsu T, Billett E, Johnson T, Verderio EA (2009) Heparan sulfate proteoglycans are receptors for the cell-surface trafficking and biological activity of transglutaminase-2. J Biol Chem 284:18411–18423

Seitz J, Keppler C, Rausch U, Aumuller G (1990) Immunohistochemistry of secretory transglutaminase from rodent prostate. Histochemistry 93:525–530

Serafini-Fracassini D, Del Duca S (2008) Tranglutaminases: widespread cross-linking enzymes in plants. Ann Bot 102:145–152

Serafini-Fracassini D, Del Duca S, Monti F, Poli F, Sacchetti G, Bregoli AM, Biondi S, Della Mea M (2002) Transglutaminase activity during senescence and programmed cell death in the corolla of tobacco (*Nicotiana tabacum*) flowers. Cell Death Differ 9:309–321

Serafini-Fracassini D, Della Mea M, Tasco G, Casadio R, Del Duca S (2009) Plant and animal transglutaminases: do similar functions imply similar structures? Amino Acids 36:643–657

Serafini-Fracassini D, Di Sandro A, Del Duca S (2010) Spermine delays leaf senescence in *Lactuca sativa* and prevents the decay of chloroplast photosystems. Plant Physiol Biochem 48:602–611 (special issue polyamines)

Shan L, Molberg O, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C (2002) Structural basis for gluten intolerance in celiac sprue. Science 297:2275–2279

Sorrentino A, Giosafatto CV, Sirangelo I, De Simone C, Di Pierro P, Porta R, Mariniello L (2012) Higher susceptibility to amyloid fibril formation of the recombinant ovine prion protein modified by transglutaminase. Biochim Biophys Acta 1822:1509–1515

Teesalu K, Uibo O, Uibo R, Utt M (2012) Kinetic and functional characterisation of the heparin-binding peptides from human transglutaminase 2. J Pept Sci 18:350–356

Telci D, Wang Z, Li X, Verderio EA, Humphries MJ, Baccarini M, Basaga H, Griffin M (2008) Fibronectin-tissue transglutaminase matrix rescues RGD-impaired cell adhesion through syndecan-4 and beta1 integrin co-signaling. J Biol Chem 283:20937–20947

Verderio E, Nicholas B, Gross S, Griffin M (1998) Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: effects on the processing of fibronectin, cell attachment, and cell death. Exp Cell Res 239:119–138

Verderio EA, Telci D, Okoye A, Melino G, Griffin M (2003) A novel RGD-independent cell adhesion pathway mediated by fibronectin-bound tissue transglutaminase rescues cells from anoikis. J Biol Chem 278:42604–42614

Verma A, Mehta K (2007) Tissue transglutaminase-mediated chemoresistance in cancer cells. Drug Resist Update 10:144–151

Villalobos E, Santos M, Talavera D, Rodriguez-Falcon M, Torné JM (2004) Molecular cloning and characterization of a maize transglutaminase complementary DNA. Gene 336:93–104

Waffenschmidt S, Kusch T, Woessner JP (1999) A transglutaminase immunologically related to tissue transglutaminase catalyzes cross-linking of cell wall proteins in *Chlamydomonas reinhardtii*. Plant Physiol 121:1003–1015

Wang Z, Collighan RJ, Gross SR, Danen EH, Orend G, Telci D, Griffin M (2010a) RGD-independent cell adhesion via a tissue transglutaminase-fibronectin matrix promotes fibronectin fibril deposition and requires syndecan-4/2 and {alpha}5{beta}1 integrin co-signaling. J Biol Chem 285:40212–44029

Wang CL, Wu J, Xu GH, Gao YB, Chen G, Wu JY, Wu HQ, Zhang SL (2010b) S-RNase disrupts tip localized reactive oxygen species and induces nuclear DNA degradation in incompatible pollen tubes of *Pyrus pyrifolia*. J Cell Sci 123:4301–4309

Wang Z, Telci D, Griffin M (2011a) Importance of syndecan-4 and syndecan -2 in osteoblast cell adhesion and survival mediated by a tissue transglutaminase-fibronectin complex. Exp Cell Res 317:367–381

Wang H, Zhuang XH, Hillmer S, Robinson DG, Jiang LW (2011b) Vacuolar sorting receptor (VSR) proteins reach the plasma membrane in germinating pollen tubes. Mol Plant 4:845–853

Wang Z, Collighan RJ, Pytel K, Rathbone DL, Li X, Griffin M (2012) Characterization of heparin-binding site of tissue transglutaminase: its importance in cell surface targeting, matrix deposition, and cell signaling. J Biol Chem 287:13063–13083

Wolters-Arts M, Van der Weerd L, Van Aelst AC, Van der Weerd J, Van As H, Mariani C (2002) Water-conducting properties of lipids during pollen hydration. Plant Cell Environ 25:513–519

Zehe C, Engling A, Wegehingel S, Schafer T, Nickel W (2006) Cell-surface heparan sulfate proteoglycans are essential components

of the unconventional export machinery of FGF-2. Proc Natl Acad Sci USA 103:15479–15484

Zemskov EA, Mikhailenko I, Strickland DK, Belkin AM (2007) Cell-surface transglutaminase undergoes internalization and lysosomal degradation: an essential role for LRP1. J Cell Sci 120:3188–3199

Zemskov EA, Mikhailenko I, Hsia RC, Zaritskaya L, Belkin AM (2011) Unconventional secretion of tissue transglutaminase involves phospholipid-dependent delivery into recycling endosomes. PLoS One 6:e19414