

Transglutaminase as polyamine mediator in plant growth and differentiation

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Abstract Transglutaminases (TGases) are ubiquitous enzymes catalyzing many biological reactions. The best-known TGase activity, namely the transamidation of specific proteins by polyamines (PAs), has been studied in plants to verify if TGase is a mediator of PAs mechanism of action to re-interpret some of PAs effects. Usually, the TGase activity is present at basal level in plant cells, but it can be induced by internal or external events or stresses, like rehydration, wounding, light, developmental differentiation and programmed cell death (PCD). Here, two models of induced growth are presented, namely pollen apical growth and dedifferentiation followed by reacquisition of the pluripotency of already differentiated cells. Moreover, PAs and TGase involvement during the differentiation and the activity of organelles and finally during the terminal organ differentiation or self-incompatibility-induced PCD are reported. In all of these models, TGase plays a role. The enzyme was detected in several cell compartments, like cytosol, chloroplasts and possibly mitochondria, microsomal fraction, cell wall and also extracellularly. The products of TGase catalysis, modified with PAs, mainly consist of high molecular mass complexes. Among the protein substrates until now identified we mention the cytoskeletal proteins, actin and tubulin, whose PA modification also affects their interaction with motor proteins and the dynamic of

cytoskeleton. The most widely studied substrates are component of chloroplast photosystems, in particular light-harvesting complexes, whose modification is light dependent and whose differentiation and size are affected by TGase, thereby conditioning photosynthetic efficiency and photoprotection. Finally, modification of cell wall substrates affects wall growth and reinforcement.

Keywords Polyamines · Plastids · Pluripotent cells · Pollen · Programmed cell death · Transglutaminases

Abbreviations

PAs	Polyamines
PCA	Perchloric acid
PCD	Programmed cell death
LHCII	Light-harvesting complex of photosystem II
PAO	Polyamine oxidase
PNGase	Peptide: <i>N</i> -glycanase
PSII	Photosystem II
Put	Putrescine
Spd	Spermidine
SI	Self-incompatible
Spm	Spermine
TCA	Trichloroacetate
TGase	Transglutaminase

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General features of transglutaminases in plants

Aliphatic polyamines (PAs), essential growth substances in all living organisms, regulate differentiation, organogenesis, reproduction and cell proliferation in higher plants and algae, as well as senescence, PCD, and homeostatic adjustments in response to external stimuli and stresses (Tiburcio et al. 2014; Takahashi and Kakehi 2010). The amount of

PAs positively correlates with normal or tumor cell growth. In plants, the multiple functions of PAs are exerted by different molecular modes of action.

PAs are present in cells as free and bound forms. PAs act as free radical scavengers and some of their derivatives might result cytotoxic. Some products of their catabolism, such as H_2O_2 , or other related signaling molecules (NO), as well as their bound forms, have been found to contribute to the complex signaling pathways that replay to hormonal signaling to modulate stress or growth responses (Wimalasekera et al. 2011). Being polycations, they are able to form linkages of various types and strength with several molecules. In addition to ionic linkages with negatively charged molecules, interactions may occur by electrostatic linkages, causing conformational rearrangements of DNA, RNA, chromatin, and proteins. Covalent bonds give rise to formation of insoluble complexes, and “cationization” or formation of cross-links between proteins, hydroxycinnamic acids (the latter specific of plants) and hypusine. Covalent linkages of PAs with proteins are catalyzed by transglutaminases (TGase) a family of enzymes object of many studies for their roles in bacteria, animals, and mainly in human pathology. Nine genes and eight active forms have been identified, among which TG2 is the best known; it is studied in many laboratories and is widespread in many cell types, in addition to be a multifunctional enzyme (Lorand and Graham 2003; Beninati et al. 2009; Gundemir et al. 2012).

TGases are Ca^{2+} -dependent enzymes that catalyze an acyl-transfer reaction between primary amino groups and protein-bound Gln residues. The γ -carboxamide group of protein-bound Gln is the exclusive acyl-donor substrate of TGases; however, a variety of primary amino groups may act as amine donors. These include the ϵ -amino group of protein-bound Lys residues, which result in the formation of inter- or intra-molecular protein cross-links via ϵ -(γ -glutamyl)-Lys isodi-peptide bonds. Alternatively, the primary amino groups of PAs may be incorporated into Gln, resulting in covalent posttranslational modification of proteins through the formation of *N*-(γ -glutamyl)-polyamine bonds. Some of the roles exerted by PAs could be mediated by their conjugation to specific proteins.

TGase activity has been also studied in plants (Serafini-Fracassini et al. 1995). The research started with the early papers on *Helianthus tuberosus* tuber parenchyma pluripotent cells (below described) and primary meristem of sprout apices (Mossetti et al. 1987; Serafini-Fracassini et al. 1988, 1989) and of *Pisum sativum* sprout apices (Ickson and Apelbaum 1987). Many years later, in the *Helianthus tuberosus* sprout apices, at least three TGase forms of 85, 75 and 58 kDa were identified, the first two showing a great similarity with some animal TGases (Beninati et al. 2013).

Since these first researches, TGase has been detected in algae (*Chlamydomonas reinhardtii*, *Dunaliella salina*) fungi and in different organs and subcellular compartments of other Angiosperms, like Arabidopsis, tobacco, maize, barley, rice, cucumber, lettuce, pea, broad bean, wheat, apple, pear, *Citrus grandis* with chloroplasts being the best-studied organelles (Del Duca et al. 1995; Serafini-Fracassini and Del Duca 2008; Campos et al. 2010; Sobieszuk-Nowicka and Legocka 2014).

More than 10 years ago, the first plant protein showing TGase activity was characterized (Della Mea et al. 2004a); the identification of the plant TGase derived from a computational analysis that allowed the identification of the *AtPng1* gene in *Arabidopsis*, which codes for a putative peptide *N*-glycanase. The *AtPng1* gene product had the Cys-His-Asp triad, which is typically present in the catalytic domain of TGase; the regions outside the catalytic domain are quite unique and show no apparent homology with the primary structures of animal/fungal orthologues.

When *AtPng1* coding sequence was overexpressed in *Escherichia coli*, the purified recombinant protein showed typical TGase features the principal being the capacity to conjugate PAs to glutamyl residues of known TGase substrates, such as the dipeptide Z-L-Gln-L-Leu, fibronectin and dimethylcasein (Beninati et al. 2013). This protein cross-linking activity showed a typical Ca^{2+} -, pH-, and GTP-dependency (Della Mea et al. 2004a). Interestingly, *AtPng1p* showed TGase activity in vitro (Della Mea et al. 2004a), in addition to PNGase activity (Diepold et al. 2007). Recent report clearly indicates that in yeast *AtPng1p* functions as a PNGase also in vivo (Masahara-Negishi et al. 2012). An antibody raised against *AtPng1p* recombinant protein cross reacts with proteins of other plants also reacting with antibodies raised against TGases of animal origin (Della Mea et al. 2007; Di Sandro et al. 2010).

The widespread occurrence of TGase activity in all organs and cell compartments suggests that these proteins are relevant for plants even if their physiological roles remain incompletely defined (Serafini-Fracassini and Del Duca 2008). The TGase activity frequently parallels the amount of PAs suggesting a not occasional concomitance.

In the present mini-review, we have discussed only a particular aspect of the multitude of PA actions, namely the one mediated by the conjugation of PAs to proteins via transglutaminase. Even though the number of labs continuously involved in this study consists only of few units, nevertheless a consistent corpus of researches on TGases is available dealing with plant growth and differentiation, which can be grouped in the four main models here presented. The very large literature dealing with many other aspects of the biology of PAs (and of their derivatives) is analyzed in detail in other recent reviews to which readers are addressed.

We start from a model of apical growth, such as pollen tube in Angiosperms, in which growth occurs unidirectionally from a rehydrated pollen grain according to a pre-determined stereotypical model, in order transport the male gametes and achieve fertilization. Development of pollen tubes is strictly dependent on complex macromolecules, like the cytoskeleton and cell wall, and on interaction with pistils. Macromolecules modified by PAs and, more generally, the process of fertilization are both affected by TGase, which either triggers or controls changes of developmental pathway.

The second model (*Helianthus tuberosus* dormant storage parenchyma) is an example of a dedifferentiation induced by wounding (explantation) of a tissue determined to die. It becomes able to reacquire its pluripotency, that is differently expressed in its cells according to the new “environmental” conditions: position in the space and contact with other cells or air or medium, namely with different mechanical tensions, molecular signals, light, oxygen, temperature, water, hormones, salts, nutrients, etc. Accordingly, some cells directly re-differentiate and others become meristematic generating xylem and phloem.

A model of differentiation, peculiar of plants, is the chloroplast of photosynthetic cells, whose morpho/functional aspects and development, either related to PAs or to their conjugation by TGase, were the object of study of different specialized scientists along many years. PAs are present and synthesized in these organelles. Their binding to light-harvesting components of photosystems by TGase presents the novel characteristic to be activated by light; this occurs *in vivo* and also *in vitro* but only if enzyme and substrate are of plant origin, whereas if one of them is of animal origin light has no effect. TGase activity is in fact strictly correlated with chloroplast functionality and also to its photoprotection as detailed below. Another organelle, mitochondrion, is also reported but data are really very poor.

Last but not least, we discussed the role of PAs, and of their conjugating enzyme TGase, during PCD, which is the last stage of cell development or a mechanism to favor biodiversity during self-incompatibility response.

Transglutaminase in tip-growing cells: the pollen tube model

It is clear that TGase is a fundamental enzyme in plant cells and takes part in several cellular processes where cross-linking of amines with macromolecules is necessary. In this context, pollen tubes are not an exception. A number of experimental evidence over the years shed light on the role of TGase during reproduction of seed plants, more particularly of Angiosperms. Because TGase is a

multifunctional enzyme, its precise role is not easily deciphered. Currently, several evidences indicate that TGase participates in processes related to pollen tube growth, including strengthening of cell wall and dynamics of the cytoskeleton. Most notably, TGase is likely to take part in rejection of pollen tubes during self-incompatibility (SI), the mechanism by which Angiosperms reject self-pollination and therefore maintain genetic variability. Identification of polyamine-based cross-linking coupled to immuno-detection of TGase epitopes suggested that pollen tubes of apple trees *Malus domestica* possess proteins related to TGase. Two further lines of evidence indicated that TGase was activated by Ca^{2+} and that it might incorporate PAs in actin and tubulin (Del Duca et al. 1997). Therefore, the two major cytoskeletal proteins could be target of the enzymatic activity of TGase and modification of the cytoskeleton appeared as an activity of TGase in pollen tubes. This hypothesis was later corroborated by experimental data showing that both actin and tubulin are target of TGase in SI crossings in pear suggesting that control of cytoskeletal functionality is essential for proper development of pollen tubes during compatible crossings (Di Sandro et al. 2010). The presence of enzymatically active TGase at the pollen tube apex, as shown by incorporation assays of fluorescent analogs (Di Sandro et al. 2010), suggests a physiological role of TGase in the regulation of pollen tube growth. Interestingly, the apical region of pollen tubes is characterized by intense dynamics of cytoskeletal elements. Currently available data indicate that actin filaments in the apical region are extremely dynamic and that they undergo three different conformations, whose balance is crucial for proper growth of pollen tubes; high dynamic short actin filaments at the pollen tube tip, a collar of parallel-arranged actin filaments (the so-called actin fringe, whose extension is related to tube growth rate), and long actin bundles parallel to the pollen tube axis (Qu et al. 2015). If TGase is present and enzymatically active at the pollen tube apex, it might then participate in the regulation of actin filament dynamics at the pollen tube apex by post-translationally modifying cytoskeletal proteins.

TGase may also control the interaction of actin filaments with the motor protein myosin. *In vitro* incorporation of putrescine with pollen actin showed that modified actin did not bind efficiently to myosin (Del Duca et al. 2009) suggesting that the processes based on the interaction between actin and myosin are compromised. In pollen tubes, transport of organelles and accumulation of secretory vesicles at the tube apex depends on the movement of myosin along actin filaments (Lovv-Wheeler et al. 2007). As a result, the activity of TGase at the pollen tube apex could regulate the motor activity of myosin through the enzymatic modification of actin.

The pollen tube apex is also characterized by a steep Ca^{2+} gradient, whose oscillation is absolutely necessary for correct growth of pollen tubes (Hepler et al. 2012). TGase is a Ca^{2+} -dependent enzyme and experimental data indicate that TGase of pollen tubes are also controlled by Ca^{2+} (Del Duca et al. 1997). Perturbations of Ca^{2+} concentration at the pollen tube apex may therefore deregulate the enzymatic activity of TGase leading to formation of actin aggregates and subsequent block of pollen tube growth. Since pollen tubulin is another target of TGase (Del Duca et al. 2009), deregulation of its enzymatic activity could significantly affect the dynamics of microtubules, possibly leading to damage of cell wall assembly.

Pollen tubes also possess a cell wall-associated TGase as resulting from antibody screening and incorporation assay of fluorescent substrates (Di Sandro et al. 2010). TGase of cell wall was identified in the form of aggregates unevenly localized and antibodies to TGase, as well as specific inhibitors, can inhibit pollen tube growth, thereby suggesting that TGase activity is necessary at the level of cell wall. The presence of TGase in the cell wall allows speculating some possible scenarios (Del Duca et al. 2013a, b, 2014b). An extracellular form of TGase (likely associated with the cell wall) is released in the growth medium during in vitro pollen germination, together with PAs and other proteins, and it could hypothetically be involved in mediating the in vivo interactions or the signaling mechanisms between pollen tubes and pistils. In analogy with animals (Spurlin et al. 2009), TGase may change the molecular structure of the extracellular matrix affecting the growth response of pollen tubes because a more or less stiff cell wall could be required to counteract the physical resistance exerted by pistil cells. Unfortunately, it is not clear whether the extracellular matrix-secreted TGase acts either on the cell wall polysaccharides of pollen tubes or on cell wall-associated proteins or on molecules of the pistil cell walls. Pectins may represent a theoretical target of the enzymatic activity of extracellular TGase because interactions between pectins and PAs are known since long time (D'Orazi and Bagni 1987). Unfortunately, an exact correlation between TGase and pectins was not demonstrated in pollen tubes, except for co-localization. TGase was also found to co-localize with MAC207-labeled AGPs in the growing region of pollen tubes, suggesting possible interaction between TGase and polysaccharide-protein complex (Del Duca et al. 2013a).

Although the function of cell wall TGase is unclear, some observations suggest that TGase might play a critical role in the pollination process by covalently modifying proteins. Under self-incompatibility, the activity of TGase increases while the enzyme activity decreases in the case of compatible pollination. It therefore appears that TGase (presumably mainly the cell wall-associated form) may block pollen tube growth in incompatible systems of *Citrus*

grandis (Gentile et al. 2012) and *Pyrus communis* (Del Duca et al. 2010). The way the inhibition of pollen tube growth occurs is not known. We can assume that TGase can either alter some cell wall protein fundamental for signaling between pollen and pistil or increase cell wall strengthening. It could also affect cytoskeleton organization thereby preventing pollen tube growth.

Being present in various cellular compartments, TGase can be synthesized in different sites (for example, on free cytosolic ribosomes or on ribosomes associated with the endoplasmic reticulum). The relationship between different forms of TGase is unfortunately unknown. In particular, we ignore if the relative concentration of different forms (cytosolic, membrane or cell wall-associated) is balanced or if their levels are regulated independently. In any case, the simultaneous presence of cytosolic and cell wall TGases raises questions about the trafficking of TGase towards the extracellular matrix. In addition, TGase is also associated with membrane compartments of pollen tubes, including both Golgi membranes and plasma membrane (Del Duca et al. 1997, 2013a). These data indicate that TGase can be transported to the plasma membrane (and hence to the extracellular matrix) through a process of exocytosis dependent on cytoskeleton and membrane dynamics. In pollen tubes, this process could take place in the apical region. Bidimensional electrophoresis assays have shown that different isoforms of TGase are present in distinct subcellular compartments, such as Golgi and plasma membrane-enriched fractions and mitochondria-enriched fractions (Del Duca et al. 2013a). These findings seem to indicate that different TGases (produced either by different genes or as result of posttranslational modifications) may have distinct roles or be differentially regulated.

Transglutaminase in the transition from dormant to active pluripotent cells

In 2015 some reviews were published evidencing a renewed interest for pluripotency and dedifferentiation of cells either of plants or animals (Gaillochet and Lohmann 2015; De Los Angeles et al. 2015). Stem cell-related genes play important roles in dedifferentiation, which exhibits similar histone modification and DNA methylation features to stem cell maintenance (Jiang et al. 2015). Nevertheless, current knowledge still needs to be greatly improved about the genetic and the epigenetic mechanisms underlying the balance between differentiation and dedifferentiation.

Early studies, still of valuable interest, were conducted on *Helianthus tuberosus* model of dedifferentiation and pluripotency in relationship with PAs and their conjugation by TGases. In the present issue only the main references on TGase in *Helianthus tuberosus* are reported. The references

related to free PAs, their metabolism and their not-TGase-mediated bindings are not reported due to their large number; they can be found in the report of Tassoni et al. (2010) which also shows a new use of the *Helianthus tuberosus* model to assay the antiproliferative and/or cytotoxic effects of different drugs of clinical use.

The *Helianthus tuberosus* in vitro model provided the first demonstration in plants that PAs act as growth substances. In fact, culture medium containing among other PAs, mainly Spm, activated the same differentiation program of explants grown with hormones (Bertossi et al. 1965). In addition, explants very rapidly synthesised PAs when stimulated by hormones and treatment with inhibitors of PA biosynthesis confirmed the PA requirement (Tassoni et al. 2010). This growth model, which benefits from the possibility of using different labeled tracers or precursors, has allowed developing many further researches.

This *Helianthus tuberosus* model presents two important characteristics: it consists of a totally homogeneous storage parenchyma poorly differentiated and in winter its cells are naturally dormant with a low metabolism. This medullary parenchyma is located in a subterranean stem (tuber) surviving winter climate, and whose fate is to release, in springtime, the stored substances (mainly inulin, arginine and glutamine) necessary for the tuber buds developing into sprouts and then to die. Therefore, this parenchyma can be considered a model of terminal differentiation and PCD (Del Duca et al. 2014a; Cai et al. 2015b).

The tuber homogeneous parenchyma cells are characterized by a thin layer of cytoplasm, which includes a heavily stainable nucleus with small nucleoli, adherent to the thin cell wall, a large storage vacuole and non-photosynthetically competent small proplastids. Being dormant respiration rate and protein synthesis are at low levels and these cells contain very low amounts of several growth substances and a considerable amount of growth inhibitors; moreover, PAs are insufficient to sustain growth. Endoplasmic reticulum and polysomes are scarce; the latter being unable to incorporate amino acids in a cell-free extract if exogenous PAs are not supplied. Many PA biosynthetic and oxidative enzyme activities as well as TGase are low or practically absent.

When the dormancy of these large storage cells (considered in G0 phase of the cell cycle) is broken, they dedifferentiate and manifest their pluripotency, re-acquiring the capacity to divide and/or differentiate. This occurs when cylindrical slices of parenchyma are excised and put in an in vitro sterile culture containing precise auxin concentration. These cells suddenly acquire a very active metabolism; an abrupt synthesis of all the substances necessary for the new program of cell division and differentiation (PAs, TGase, nucleic acids, enzymatic/structural proteins, hormones, etc.) takes place.

When the parenchyma slices are explanted and cultured with auxins or PAs, their cells undergo the same morphologic and physiologic program, according to the cell position in the space and contact with other cells or air or medium, namely with different mechanical tensions, molecular signals, light, oxygen, temperature, water, hormones, salts, nutrients, etc. The external cells of the explant start to form a protective layer within 1 day of culture; the internal cells located in a ring 4–5 cell layers beneath the surface, enter immediately the G1 phase. In few hours they start to divide synchronously for two to three cycles, assuming a meristematic capacity and then forming a regular cambium according to the positional information that plays a dominant role in plant cell fate acquisition. During the first cycle, the nuclei divide amitotically and then mitotic features can be observed. This meristem generates vascular bundles and then layers of xylem and phloem regularly disposed, recalling the dicotyledonous stem anatomy. In other hormonal conditions, the explant can actively divide giving rise to a disorganized callus.

The G1 phase is rather long and its first half appears to be the most interesting, as it shows the abrupt changes undergoing the cells. One of the first detected events (in few minutes) is the increase in PA content and, after 1 h, the PA level is already sufficient to sustain protein synthesis. Free PAs continue to increase throughout the G1 phase, in part as consequence of the very low activities of enzymes that remove free PAs either by oxidation or by covalently binding to proteins via TGase-like activity (Serafini-Fraccassini et al. 1989). Storage substances are rapidly utilized. Extracted ribosomes are active, since they already contain PAs in sufficient amount.

The synthesis of RNA, mitochondrial and plastidial DNA become detectable after 20–30 min and is independent from the nuclear DNA synthesis (S phase) that peaks after 12 h. Thus, organelles immediately react and if the explant is maintained in culture, without hormones but in the light conditions, it become green as the proplastids develop into chloroplasts and a new TGase activity can be detected (Del Duca et al. 1993).

In the early G1 phase PAs have been found tightly bound and non-tightly bound to tRNA, rRNA and to a poly(A)RNA, and the molar ratio PAs/RNA increases in early G1 phase (particularly Put/RNA); this suggests that PAs can be directly involved in the activation of RNAs or in protecting them from nucleases. TCA-soluble PAs bound to low molecular mass compounds and peptide-PA conjugates are present, whereas hydroxycinnamoyl-PAs are not detectable.

The covalent linkage of PAs to proteins by TGase activity increases significantly only after mid-G1 and conjugates with Put, Spm and mainly Spd, are formed (Del Duca et al. 2000a). The involvement of TGases in this conjugation was

supported by the immunodetection, of two neo-synthesized SDS-protein bands of 90 and 58 kDa starting to be visible in early G1 phase and continuing to increase during the entire first cell cycle (Del Duca et al. 2000a). TGases having these molecular weights were later on found in other plants. The presence of a TGase with the same mechanism of action and Ca^{2+} requirement of animal TGases was demonstrated by the identification of glutamyl-PAs in *Helianthus tuberosus* chloroplasts (Del Duca et al. 2000b). During the S phase, the TGase activity continues to increase becoming 7–8 folds higher than its initial value (Serafini-Fracassini et al. 1989). Protein accumulation begins to decline or proteins become insoluble and more difficult to analyze. Different protein-conjugated PAs have been found in vivo during the *Helianthus tuberosus* cell cycle: low molecular-mass conjugates appear in early G1 phase (18, 20 kDa ones, for example, which then decreased), while very high molecular-mass conjugates (which cannot enter in the SDS-running gel) were mainly formed during the S phase until cell division (Del Duca et al. 2000a). PAs have been found incorporated in supramolecular cell structures among which possibly the cytoskeletal ones, as demonstrated in pollen (Del Duca et al. 2009). A preliminary indication of the presence of TGase products in *Helianthus tuberosus* cell wall was provided by the digestion of cell wall polysaccharides, which caused the disaggregation of a complex of PA-conjugated proteins of high mass from polysaccharide net.

In summary, TGase activity is fairly absent in steady state cells but it is required by activated cells, which express their pluripotency, organizing their new programs, mediated by differentiation of organelles, new cell walls and cytoskeleton complexes.

Transglutaminase in differentiating and mature chloroplasts

PAs as well as PAs biosynthetic and oxidative pathways are present in chloroplasts, where PAs play a role in the formation and functioning of the photosynthetic apparatus (Sindhu and Cohen 1984; Torrigiani et al. 1986; Andreadakis and Kotzabasis 1996; Bortolotti et al. 2004). Biosynthesis of PAs is controlled by white light and their concentration is correlated to chlorophyll biosynthesis and photosynthetic rate. PAs are thought to affect regulatory mechanisms of the photosynthetic apparatus during photoadaptation (Navakoudis et al. 2007).

Also in chloroplast, PA action is mediated at least in part by TGase. In fact incubation of chloroplasts of *Helianthus tuberosus* leaves with labeled Put and/or Spd and proteolytic digestion of TCA-soluble and -insoluble plastidial proteins revealed the presence of *mono*- and

bis-(γ -glutamyl) derivatives of PAs, an unequivocal proof of the presence of TGase activity. In addition, recovery of Spd or Put and acetyl-Spd from chloroplasts incubated with [^3H]Put or [^3H]Spd, respectively, indicated the presence of biosynthetic and, to our knowledge for the first time in chloroplasts, of a back conversion oxidative pathways involving acetyl-PAs (Del Duca et al. 1995). In chloroplasts of *Helianthus tuberosus*, TGase was able to incorporate PAs into Rubisco and into light-harvesting complex of photosystem II (namely LHCII oligomeric form, CP 29, CP 24, CP 26) following light stimulation and TGase was supposed to stabilize these substrates (Del Duca et al. 1994; Dondini et al. 2003; Serafini-Fracassini and Del Duca 2008). On the base of the enzyme isolated from thylakoids of *Helianthus tuberosus*, a cDNA has been obtained in Torné lab to transform maize genome and to express two plant TGase sequences that have been named TGZ15 and TGZ21 (Villalobos et al. 2004).

These results in *Helianthus tuberosus* chloroplasts were supported by data obtained in chloroplasts of other plants, i.e., *Zea mays* where Put, Spd and Spm were conjugated to isolated LHCII with different degrees of efficiency, Spm being the PA most efficiently conjugated. A TGase with a light-sensitive activity was identified in the photosystem II-enriched fraction and its partial purification allowed the separation of a 39-kDa band, a molecular mass similar to that of microbial TGase (Della Mea et al. 2004b). Similarly, when Spm was sprayed on the leaves of *Lactuca sativa*, it was found conjugated in vivo both to chloroplast LHCII and to LHCI, preventing leaf senescence. Both fractions contained TGases of multiple molecular mass in young leaves, whereas in senescing leaves a unique high molecular mass TGase was immunodetected. The supply of Spm and exogenous TGase exerted a clear protective effect on chloroplasts of aging *Lactuca sativa* leaf disks (Serafini-Fracassini et al. 2010).

Also in the unicellular green alga *Dunaliella salina*, the photosynthetic complexes were found to be TGase substrates. These were severely affected by hyper-saline stress induced to the alga under light conditions and the amount of one TGase isoform increased rapidly in connection with changes of enzyme activity, also affected by the light. The substrates were much more markedly polyaminated in algae already acclimated to hyper-saline conditions (Dondini et al. 2001). In a *Dunaliella salina* variant strain characterized by low growing, deficient in PAs, the supply of Put or ornithine induced the resumption of growth and stimulation of chloroplast TGase activity (three times more in the light than in dark assay conditions). The *Dunaliella salina* chloroplast TGases appeared to have features in common with those of higher plant chloroplasts, either as molecular mass and immunological characteristics or substrates (Dondini et al. 2000).

A TGase was also found in chloroplast of barley leaves; this protein is likely involved in photosystem organization, stress response, and oxidation processes. The plastidial barley TGase was computationally identified on the base of cDNA homology with the *Arabidopsis thaliana* *AtPng1* gene, and so it has been called *Hv-Png1-like* (Sobieszczuk-Nowicka et al. 2015).

The LC–MS mass spectrometry identification of protein complexes indicated that the above reported *Zea mays* TGases TGZ15 and TGZ21 (Villalobos et al. 2004), form part of a specific photosystem II protein complex. This complex plays a role in energy-dependent quenching that increases thermal dissipation of excess absorbed light energy in the photosystem (Campos et al. 2010).

Interestingly, an *HvTGZ21*-like sequence was identified in barley and shows a very high degree of similarity to *ZmTGZ21* at the N-terminus; however, the C-termini of both proteins are drastically different. It seems that the two maize cDNAs lack a conserved G in position 747, which results in a frame shift that extends the length of TGZ15/TGZ21 proteins in comparison to other *Poaceae* sequences (Sobieszczuk-Nowicka et al. 2015). According to Villalobos et al. (2004), the TGase catalytic triad in the TGZ15 and TGZ21 proteins is placed in this unique C-terminal region.

Recently, the isolation and characterization of a rice homolog of the TGZ15/TGZ21 proteins named TGO was reported. The TGO cDNA possesses a unique G deletion not observed in the other available rice sequences, including the genomic sequence of rice chromosome 4 (Campos et al. 2013). Both the maize TGZ15/TGZ21 and rice TGO proteins share high sequence homology (70 %), but only upstream of the G deletion positions, while their C-termini differ in length and sequence as well as in catalytic triad presumptive localization. However, overexpression experiments provided evidence that both proteins possess TGase activity (Campos et al. 2010, 2013; Villalobos et al. 2004; Ortigosa et al. 2010; Ioannidis et al. 2012), although their catalytic domains are not conserved. The transgenic rice expressing a mammalian Ca^{2+} -dependent TGase was studied as a vehicle for the production of the enzyme for biotechnological purposes (Claparols et al. 2004).

An interesting contribute concerns the role for TGase in energy production in chloroplasts (Ioannidis et al. 2012). When TGZ is overexpressed in plastids of *Nicotiana tabacum*, bound Spd and Spm significantly increased (80 %). Overexpression of plastid TGase altered photosynthesis via increased polyamination of the antenna of PSII causing the formation of photosystems with a large absorption cross section antenna, doubling the rate constant of light energy

harvested and promoting thylakoid membrane stacking. Polyamination of antenna proteins is a mechanism for modulation of the size (antenna absorption cross section) and sensitivity of PSII to proton motive force (Ioannidis et al. 2012).

Besides affecting the light-harvesting function, TGase is involved also in the differentiation of chloroplasts, in particular during the greening process; this has been observed when explants of *Helianthus tuberosus* dormant tubers were grown in vitro in the presence or absence of hormones for 3 weeks; the untreated explants differentiated chloroplasts deriving from non-photosynthetic pro-plastids with tubular complexes that disappeared when thylakoids began to organize. TGase activity increased 60-fold in greening cells in comparison to dormant parenchyma, whereas in non-greening explants treated with hormones, the increase of TGase activity was about tenfold when compared to dormant tissue. The conjugation of labeled PAs in protein extracts was definitely higher in greening explants compared to non-greening ones suggesting that the maturation of the thylakoids is a process in which TGase seems to participate (Del Duca et al. 1993).

When etiolated cotyledons of cucumber were treated with kinetin and exposed to light, thereby inducing the transformation of etioplasts (a form of inactive plastid present in plants grown in darkness) to chloroplasts, the amounts of Spd bound to etioplast membranes increased in the early greening stage, but when chloroplast membranes were fully organized they decreased. PAs conjugated by TGase could represent a component of the mechanism of etioplast to chloroplast transformation stimulated by kinetin (Sobieszczuk-Nowicka and Legocka 2014).

In the light-dependent differentiation of etioplasts into chloroplasts, the level of intra-plastid PAs decreased; this could be due to metabolism of PAs used as a source of nitrogen during chloroplast development (Andreadakis and Kotzabasis 1996); in detail, PAs may be donors of amine groups taking part in the biosynthesis of amino acids and in the formation of glutamic acid, a chlorophyll precursor. Moreover, PAs are components of etioplast membrane (prolamellar body) required for stabilization of its hexagonal structure and of PSIIa complex. The mechanism by which PAs can affect plastidial membrane assembly was suggested to be, at least in part, mediated by their covalent linkage to these membranes via TGases (Del Duca et al. 1994; Della Mea et al. 2004b; Villalobos et al. 2004; Ioannidis et al. 2012; Sobieszczuk-Nowicka and Legocka 2014). As TGase affect significantly the functionality of key photosynthetic complexes, it is a promising factor also in photosynthesis research.

What it is known about transglutaminase in mitochondria

PAs play a role in functioning of *Nicotiana tabacum* mitochondria, as suggested by the relationship between Spm and mitochondria dysfunction involving the Spm-signal pathway. This Spm signalling pathway involves accumulation of Spm in the apoplast, upregulation of a subset of defence-related genes (such as those encoding pathogenesis related proteins and mitogen-activated protein kinases), and a type of PCD known as the hypersensitive response, in which TGase is involved. This response is triggered by Spm-derived H_2O_2 , produced through the action of apoplastic polyamine oxidase (PAO) (Takahashi and Kakehi 2010).

Plant mitochondria of both potato and mung bean covalently incorporated PAs into acid insoluble material containing membrane bound proteins and the conjugation of PAs to proteins due to TGase activity was hypothesized. Protein sequencing of PA-labelled spots from a 2-DE gel returned a sequence 60 % identical to catalase, whose functionality may be affected by PA covalent binding (Votyakova et al. 1999). Catalase and superoxide dismutase (SOD) are a powerful antioxidant machinery to avoid an excess of ROS that could lead to oxidative stress. These activities have been recently studied in germinating pollen, in which the apical growth mechanism requires mitochondrial activities (Aloisi et al. 2015). Recent evidence also suggests that PAs are major regulators of ROS homeostasis. In *Arabidopsis thaliana*, the back conversion of the PAs is catalyzed by peroxisomal PAOs (AtPAO). Overexpression of AtPAO3 resulted to an increased, but balanced production of both H_2O_2 and $\text{O}_2^{\bullet-}$. The ratio of $\text{O}_2^{\bullet-}/\text{H}_2\text{O}_2$ regulates respiratory chain in mitochondria, with PA-dependent production of $\text{O}_2^{\bullet-}$ by NADPH-oxidase shifting the balance of electron transfer chain in favor of the alternative oxidase pathway (Andronis et al. 2014).

The TGase activity, hypothesized in potato and mung bean mitochondria, was found in fractions enriched in pollen mitochondria (Del Duca et al. 2013a). In these fractions the anti-TGase the commercial antibody ABIII did not cross-react with putative mitochondrial TGase. This is not astonishing considering the prokaryotic origin of these organelles. Pollen mitochondria might thus possess an immunologically unrelated form of TGase hypothetically involved in PCD occurring during SI response (Del Duca et al. 2010, 2013a).

About the presence of TGase in mitochondria as the knowledge in plants is scarce, as a paradigm, animal cells could give some information: under physiological conditions, TG2 localizes in association with the outer membrane and/or with the intermembrane space as well as integral to the inner membranes of mitochondria. This

localization allows the protein to regulate the activity of the ATP/ADP transporter, stabilizes the respiratory complex I and the monomeric/polymeric status of ANT1 (a component of the permeability transition pore complex) induced by a variety of proapoptotic agents (Malorni et al. 2009; Gundemir et al. 2012).

Transglutaminase in programmed cell death

PCD it is a complex process; in fact, besides cellular compartments well known to take part in PCD (e.g., mitochondria and nucleus), plant-specific organelles (such as chloroplasts, vacuoles, and also possibly the cell walls) are involved in the plant cell death network and contribute to unique aspects of plant PCD (Lim et al. 2007). Ca^{2+} is likely one of the key factors in TGase regulation in senescing tissues, because this cation increases in concentration (Ma and Berkowitz 2011), and is released from vacuoles after tonoplast rupture. In mammal cells, under stressful conditions, the Ca^{2+} -dependent increase of TGase activity may favor PCD (Yoo et al. 2012) and a similar mechanism could be hypothesized in plants. Until now, studies have been focused on both reproductive and vegetative organs, mainly on flower petals and leaves during their terminal development as well as on the SI response of pollen (Cai et al. 2015b). Senescence and death of both petal and leaf are highly regulated, in fact, they are controlled by hormones and growth factors (Amasino and Michaels 2010; Rogers 2013) as well as by PAs. The main information on TGase and PA involvement during flower PCD derives from *Nicotiana tabacum* corolla (Serafini-Fracassini et al. 2002; Della Mea et al. 2007; Cai et al. 2015a), while dark-induced senescing barley leaves (Sobieszczuk-Nowicka et al. 2009; Sobieszczuk-Nowicka et al. 2015) or naturally senescing lactuca leaves (Serafini-Fracassini et al. 2010) are the most studied systems for leaf senescence. Both processes are characterized by morpho-physiological parameters, e.g., loss of fresh weight and change of tissues color due to a gradual loss of water, pigments (mostly anthocyanins, chlorophylls) and proteins (as consequence of increased protease activity). The whole process is concomitant with nuclear blebbing (Ambastha et al. 2015), DNA laddering and cell wall modification (Serafini-Fracassini et al. 2002; Ruberti et al. 2014; Cai et al. 2015a).

In *Nicotiana tabacum* corolla, both TCA-soluble bound Put and Spd peaked before DNA laddering and then decreased; however, both maintained a relatively high level until the final stages. Among the insoluble forms, Spd decreased with time, while Put decreased from anthesis onwards (Serafini-Fracassini et al. 2002). A similar trend was found in barley leaves, where levels of Put and Spd bound to thylakoid fractions increased during the early

stages of barley leaf senescence. These data suggested different roles according to the type of PA in thylakoid degradation and thus in aging of plant tissues. A further investigation in barley chloroplasts brought to light that, beside free PAs, also their conjugates could be involved in the specific effect of PAs (Sobieszczuk-Nowicka et al. 2009; Sobieszczuk-Nowicka et al. 2015).

Exogenous supplying of PAs delayed senescence both in leaves and in corolla. While in *Nicotiana tabacum* corolla and *Lactuca sativa* leaves Spm was the most effective PA, in *Avena sativa*, *Petunia hybrida* and barley leaves Spd had the same effect of Spm and the exogenous addition of both PAs inhibited protein degradation and chlorophyll loss stabilizing thylakoid proteins (Mizrahi et al. 1989). In addition, PAs also inhibited RNase activity and degradation of LHCII protein (Legocka and Zajchert 1999). The idea that PAs could act exerting as anti-ethylene factor, the hormone of senescence, was hypothesized and the inhibition of ethylene biosynthesis by PAs has been reported (Lee et al. 1997). However, this idea is still a matter of debate.

Exogenous supplied PAs were rapidly metabolized to produce PA-protein conjugates. In *Nicotiana tabacum* the *mono*- and *bis*-Put and *bis*-Spd levels increased in isolated chloroplasts during the progression of corolla senescence (Della Mea et al. 2007), while in Spm-treated senescing lettuce leaves, *mono*- and *bis*-Put and *bis*-Spd (and also *bis*-Spm), were produced in higher amount in respect either to the control or the Spm treated young leaves.

During corolla life of *Nicotiana tabacum* flower, Spm counteracted PCD only when supplied in the narrow developmental window of corolla expansion, whereas before and after these stages, Spm promoted PCD, thus giving an explanation to controversial data of PAs literature. Results indicated that Spm action, is mediated, at least in part, by TGase showing that this enzyme represents an additional factor in petal morphogenesis and PCD. TGase was located mainly in the corolla epidermis cell walls during development and sprayed also in the inner parenchyma rigidifying cell walls when PCD began. By contrast, when Spm was supplied, it caused the precocious accumulation of the enzyme in the inner parenchyma cells that increased their size, intercellular spaces and whose cell walls were not rigidified; it appeared to act as a rejuvenation factor (Cai et al. 2015a).

The evidence that TGase-mediated conjugation PAs is able to delay leaf senescence was directly shown in *Lactuca sativa* during induced cell death using aging leaf disks in vitro, or during the normal developmental senescence of leaves (Serafini-Fracassini et al. 2010). In leaf disks, supplied Spm caused a delay of chlorophyll decay, an increase of endogenous TGase activity, and a threefold increase in chlorophyll content when Spm was supplied together with exogenous TGase. The protective effect of Spm on the

degradation of chlorophyll may be related to its capacity of a direct interaction with chlorophyll rather than to its enzymatic conjugation (Dondini et al. 2003), both in free form and as a *mono*-Put derivative through its primary free amino group. As chlorophyll *b* is also linked to a glutamine of LHCII, this binding could further enhance the protein-chlorophyll stability and delay its degradation. The hypothesis of an involvement of PA-derivatives in the stability of proteins is supported by the enhanced activity of TGase during senescence and PCD when an increase of TGase activity was a constant feature. However, while leaf showed a continuous increase in TGase activity during senescence, *Nicotiana tabacum* corolla showed an acropetal wave of PCD, which was anticipated by the activation of TGase in different cell compartments (Della Mea et al. 2007). Several immuno-detected isoforms of TGase are supposed to take part in this process. In fact, while a soluble 58-kDa TGase (present in the soluble, microsomal, plastidial and cell wall fractions) decreased during corolla life, a 38-kDa TGase, mainly a plastidial form, moved progressively from basal to distal parts of the corolla, where it was exclusively present. This 38-kDa putative enzyme was supposed to be involved in stabilizing photosystems and in sustaining the energy requirements of senescence progression (Del Duca et al. 2014a). Moreover, a soluble 52-kDa TGase decreased with aging, while in late stages of senescence a high (>250 kDa) molecular mass band appeared, suggesting either a binding of TGase with a cytoplasmic component or polymerization of the enzyme as suggested in animals (Lorand and Graham 2003). As for flower corolla, in young lettuce leaves TGase was immuno-detected with molecular mass of circa 39 and 58 kDa while higher bands (>160 kDa) were found in more senescent leaves (Serafini-Fracassini et al. 2010).

These data clearly show that TGase participates to the PCD pathway and that it can influence the fate of the cell depending on the activity state, availability of PAs and localization of the enzyme in agreement to what reported for TG2 in animal tissues (Gundemir et al. 2012).

Conclusion

In all the plant models here presented, in steady-state cells like dormant tuber cells, dry ungerminated pollen or inactive (in dark condition) chloroplasts, or in fully differentiated cells in the absence of stress, TGase activity is very low or latent. This enzyme is activated or synthesized when morpho-functional changes (induced by hydration, wounding, light, PCD, etc.) take place, respectively, in pollen tube apical growth, re-differentiation or cell division of *Helianthus tuberosus* dormant tuber cells, differentiation of chloroplasts and terminal differentiation associated

to senescence of leaves and petals or in incompatibility-induced PCD in pollen tubes. This transamidating activity is functional for acquisition of new characteristics of the cell. PAs, whose content is generally high in the same activated plant models, are substrates of this Ca^{2+} -dependent enzyme; some of the PA roles exerted in different cell compartments appear to be mediated by their conjugation to different specific proteins. The presence of high mass conjugates, resistant to proteases, suggests that TGase catalyzes the formation of supra-molecular complexes with possible structural roles, like rearrangement of cytoskeletal or photosynthetic complexes. Formation of macromolecular complex seems to occur also during cell wall building. A regulatory role of some protein activity by polyamination is also suggested. The present conclusion is in agreement with data in animals. Among the several functions exerted by TG2, usually latent, the elevated transamidating activity is considered to be the main driver of differentiation and modulator of cell death pathway (Gundemir et al. 2012). In plants, possible different functions of the various TGase isoenzymes detected are not explored but some convergences with animal ones (Serafini-Fracassini et al. 2009; Beninati et al. 2013) suggest this as a possible working hypothesis.

Compliance with ethical standards

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

Research did not involve Human Participants and/or Animals.

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