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AtPng1 knockout mutant of _Arabidopsis thaliana_ shows a juvenile phenotype, morpho-functional changes, altered stress response and cell wall modifications

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Abstract

In order to ascertain the role of plant transglutaminases (TGase) in growth and abiotic stress response, the *AtPng1* knock out (KO) line of *A. thaliana* has been analyzed during plant development and under heat and wound stress. Comparing wild type (WT) and KO lines a 58-kDa band was immunodetected by anti-AtPng1p antibody in the cell wall and chloroplasts only in the WT line. A residual TGase activity, not showing correlation with development nor stress response, was still present in the KO line. The KO line was less developed, with a juvenile phenotype characterized by fewer, smaller and less differentiated cells. Chloroplast TGase activity was insensitive to mutation. Data on stressed plants showed that (i) KO plants under heat stress were more juvenile compared to WT, (ii) different responses between WT and KO lines after wounding took place. TGase activity was not completely absent in the KO line, presenting high activity in the plastidial fraction. In general, the mutation affected *A. thaliana* growth and development, causing less differentiated cytological and anatomical features.

Key words: *A. thaliana*, Transglutaminase, AtPng1p, Differentiation, Phenotype, Abiotic stress, Cell Wall, Polyamines.

List of Abbreviations:

AtPng1p: *A. thaliana* peptide N-glycanase 1 protein

PAs: Polyamines

KO: knock out

TGase: Transglutaminase

WT: Wild Type.

1. Introduction

Plant growth is a process regulated by a cohort of environmental and internal factors, which tune a coordinated morphogenesis. Several recent reviews summarize the increasing relevance of the main aliphatic polyamines (PAs), in cell differentiation and wound stress response (Handa et al. 2018; Aloisi et al. 2016a; Chen et al. 2019). Due to their polycationic nature, PAs easily react with other biological molecules, *i.e.* DNA/RNA and proteins (Iacomino et al. 2012; Antognoni et al. 1999). In plants, the formation of cross-links with hydroxycinnamic acids might also occur in cell walls (Bassard et al. 2010) and in addition, by forming covalent linkages, PAs make cross-links between proteins, forming supramolecular nets (Serafini-Fracassini and Del Duca 2008).

Transglutaminases (TGase: E.C. 2.3.2.13), a family of enzymes present in all living organisms, catalyze the covalent linkages of PAs with proteins (Griffin et al. 2002; Beninati et al. 2013; Lorand and Graham 2003). Plants TGases are Ca^{2+}-dependent enzymes that catalyze an acyl-transfer reaction between primary amino groups and specific protein-bound Gln residues. A variety of primary amino groups may act as amine donors, including the ε-amino group of protein-bound Lys residues, which result in the formation of inter- or intra-molecular protein cross-links, or one or two primary amino groups of PAs to specific protein-bound Gln residues giving rise to N-(γ-glutamyl)-polyamine bonds. In case of PAs, the linkage with one of the two terminal amino groups increases the positive charge on the protein surface (cationization), while two linkages with both terminal amino groups result in covalent crosslinking between the proteins.

In plants the enzyme is also present with the same mechanism of action (Aloisi et al. 2016b; Del Duca and Serafini-Fracassini 2005). Several isoforms of TGases with a broad range of molecular mass have been found in different organisms (Del Duca and Serafini-Fracassini 2005) and among angiosperms in different organs, such as seeds, pollen, meristems and vegetative organs (Del Duca et al. 2013; Campos et al. 2013; Falcone et al. 1993). TGases localize in distinct subcellular compartments, *e.g.* cytoplasm, chloroplast, microsome fraction, nuclei and cell wall, suggesting either different roles or different regulatory mechanisms of enzyme activity (Campos et al. 2013; Campos et al. 2010; Ioannidis et al. 2012; Della Mea et al. 2007; Mandrone et al. 2019). Cytosolic enzyme is translocated to the cell wall by probably two different mechanisms, both involving and secretion (Del Duca et al. 2013). In mature plants, the enzyme activity is extremely low, while it is enhanced during growth and development, and as response to external stimuli such stresses (Aloisi et al. 2020; Sobieszczuk-Nowicka et al. 2015; Del Duca et al. 2014; Shu et al. 2020; Zhong et al. 2019).

There is no evidence whether these enzymes result from posttranslational modifications of a single gene or whether multiple genes are present, as reported for mammals in which 9 genes are known. In *A. thaliana* a single *AtPng1* gene was found, containing the catalytic domain with the Cys–His–Asp triad typical of the TGases superfamily (Suzuki et al. 2000; Della Mea et al. 2004a). The encoded protein, the first TGase identified in plants, was shown to have a Ca^{2+}- and GTP-dependent transamidase activity and to catalyze the formation of glutamyl-PA derivatives in several organs of *A. thaliana* (Della Mea et al. 2004a). As some of the TGase substrates are, in addition to plastid proteins, also structural proteins, like cytoskeleton and cell wall components, the activity of the enzyme could be relevant for the cell structure and consequently for the tissues and the general plant structure.

In an attempt to clarify the role of TGase on plant growth by identifying the cell component eventually affected, a TGase knock-out (KO) *A. thaliana* line was phenotypically and physiologically analyzed, compared to WT. Thus, the TGase 5' UTR loss-of-function line was analyzed, verifying the expression, localization and activity of TGase by phenotypic observations, cyto- and histological analysis of plant tissues, and quantitation of various parameters. To highlight differences in KO and WT lines, TGase activity has also been stimulated by abiotic stresses, *i.e*. heat and wound treatments. To our knowledge, this is the firt paper in which a *AtPng1* mutant was phenotypically and physiologically analyzed.

2. Materials and methods

2.1. Chemicals, plant material and stress conditions

All chemicals (unless otherwise indicated) were obtained from Sigma–Aldrich (Milan, Italy). Mutant (KO) and WT lines were in the Columbia (Col-0) background. T-DNA insertional mutants SALK_076538 was identified from the SALK collection (Alonso et al. 2003). All phenotypic characterizations were performed with homozygous plants (http://natural.salk.edu/geno/sum.txt). A map of the gene structure of the three T-DNA insertion mutant lines is reported in Supplementary material.

All plants were grown in greenhouse conditions (23°C) with 16 h light /8 h dark (light intensity, 55 μmol photons m^{-2} s^{-1}). Heat stress treatment was performed on surface-sterilized seeds plated on half-strength MS (Murashige and Skoog 1962) medium (2.15g/L), sown at 30 °C and allowed to develop for 5 and 10 days.

With a razor blade, wound stress was performed by a longitudinal radial cut of about 2 mm deep until the medullary parenchyma on stems of two-months old plants. Transverse slices cut and stained as reported below, were microscopically observed at different days (day 0, namely immediately -1-5-9-14 until day 21) in the region between the basal rosette leaves and the first node. Leaves were injured with 5 cuts perpendicular to the midrib and analysed at different times. The other leaf half symmetrically opposite to the wounded half was left unscathed. Controls are represented by not-wounded leaves of the same age and taken from the same plant.

2.2. mRNA extraction and TGase expression analysis

mRNA was isolated by the kit Nucleospin RNA plant (Macherey-Nagel) starting from 100 mg of leaf tissue and following manufacturer's instruction. mRNA quality was checked spectrophotometrically and by gel electrophoresis. Ten micrograms of RNA were used to synthesize cDNAs using oligo(dT) primers according to manufacturer's instructions (Invitrogen, San Giuliano Milanese, Italy). TGase and actin (housekeeping gene) amplification was performed as previously described (Della Mea et al. 2004a). Amplification products were checked on 1% agarose gel and the density of the bands was analysed by the AIDA software (Fuji Inc., Tokyo, Japan). Primers used are listed below: AtPng1 FW: TTGTCGCTGCACGAGATAACG; AtPng1 REV: CTTCTGTACAGATCGATG CTCCC; Actin FW: GACTCTGGAGATGGTGTG; Actin REV: ATCTGCTGGAAGGTACTGAG

2.3. Preparation of cellular fractions, protein extraction and Western-blot analyses

Cell wall, microsomal, soluble and plastidial fractions were obtained from 21-d-old *A. thaliana* leaves according to previous literature with some modifications (Bregoli et al. 1997). Nuclei enriched fraction was prepared as previously described (Zhao et al. 2001).

Total proteins were extracted as previously described (Parrotta et al. 2020), from 21-d-old plants. In detail, entire plants were grinded in liquid nitrogen and proteins were extracted in agitation for 30 min at 4 °C in extraction buffer (60 mg/mL) containing 100 mM Tris–HCl pH 8.5, 10 mM 2-Mercaptoethanol, 0.2% Triton X-100 and protease inhibitor cocktail. Large cell debris were removed from the total homogenate by centrifugation at 12,000 rpm for 10 min at 4 °C. Protein concentration was estimated on the supernatant by the Bradford method with bovine serum albumin (BSA) as the standard protein. Extraction was repeated in triplicate. 30 μg of protein from each enriched fraction were loaded onto a denaturing 10% (w/v) SDS-PAGE gel and migrated using a Mini-protean II apparatus (Bio-Rad, Italy). Protein were blotted to a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) using a semidry Trans-Blot system (Bio-Rad, Italy). Western blotting was performed as previously described, using an anti AtPNG1 antibody as primary antibody (1:2000 dilution) (Della Mea et al. 2007). An anti-chicken antibody conjugated to alkaline phosphatase was used as a secondary antibody (1:3000 dilution).

2.4. Nε-(γ glutamyl)-lysine quantification

The presence of the TGase activity product, the Nε-(γ glutamyl)-lysine was analysed by immunodetection with 81D4 antibody (Covalab, Bron, France) in an ELISA assay. ELISA was carried out as described previously (Mandrone et al. 2019). As positive control X-linked casein was tested in the same conditions. Casein (final concentration 2 mg/mL) X-linking was induced for 2 hours in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 10 mM DTT in presence of 5 μg of Guinea pig liver (gpl) TGase. The final volume of the reaction was 250 μL. For negative control, casein was incubated in the same conditions with the omission of gpl TGase.

2.5. Analysis of TGase activity: microplate-based and radioactivity assays

The *in vitro* TGase activity was measured by the conjugation of biotinylated cadaverine to N, N'-dimethylcasein as previously described (Paris et al. 2017; Del Duca et al. 2018). Specific activity was determined as a change in A450 of 0.1 per hour per mg of protein after subtraction of the value of the controls treated with 20mM EGTA. Alternatively, TGase activity was radioactively determined (Del Duca et al. 2018; Della Mea et al. 2004a).

2.6. Chlorophyll and carotenoids determination

Chlorophyll *a* and *b*, as well as total carotenoids (xanthophylls and carotenes) were extracted with cold 80% acetone then centrifuged at 4000 g for 10 min at 4 °C. Their levels were determined according to the literature (Porra et al. 1989).

2.7. Microscopy analysis and immunolocalization of TGase

Fresh inflorescence stems were transversely cut between the basal rosette leaves and the first node and observed unfixed. Slices were observed by optical microscopy either by white light or by fluorescence microscopy at 380 or at 450 nm. Observations were made using a Zeiss Axio Imager microscope. Images were acquired with an AxioCam MRm camera using the software AxioVision. Specific staining for lignin and suberin were performed by acid floroglucine and Sudan III, respectively (Angelini et al. 2008). The measures of the cells were performed by NIS Element program (Nikon). Measures of the major cell diameters or of the wall thickness were repeated ten times and the mean calculated and reported as frequency classes.

For TGase immunolocalization, inflorescence was directly thawed in a buffer solution (100 mM Pipes pH 6.8, 10 mM EGTA, 10 mM $MgCl_2$, 0.1% NaN_3) plus detergent and fixative (0.05% Triton X-100, 1.5% paraformaldehyde, 0.05% glutaraldehyde) for 30 min on ice and then at 4 °C for an additional 30 min. For the localization of TGase, samples were cut along their length and placed in the buffer solution containing 0.75% cellulysin and 0.75% pectinase for 7 min. For immunofluorescence microscopy, samples were washed in the above buffer and incubated with the anti AtPNG1 antibody diluted 1:20 in the buffer; incubation was 1 h at 37 °C according to previous literature (Del Duca et al. 2013). After washing with buffer, samples were incubated with The secondary antibody was Alexa Fluor 488-conjugated goat anti-chicken (Thermo Fisher Scientific) secondary antibody diluted 1:50 in the buffer solution, for 45 min at 37 °C in the dark. Samples were observed with a Zeiss Axiophot fluorescence microscope (Ex-Max 490 nm/Em-Max 525 nm) equipped with an MRm video camera and a 63× oil immersion objective.

2.8. Data and statistics

Each experiment was repeated at least three times. Differences between samples sets were determined by analysis of variance (one-way ANOVA, with a threshold P-value of 0.05) using GraphPad Prism.

3. Results

3.1. The enzyme TGase is present in different cellular compartments in *A. thaliana*.

To investigate the presence and distribution of TGase in the *A. thaliana* WT line, TGase activity was investigated in several cellular compartments, *i.e.* cell wall, nuclei, chloroplasts as well as in the microsomal and soluble fractions (Fig. 1A). Apart from the soluble fraction, where enzymatic activity was under the detection limit, all other subcellular compartments showed TGase activity, with the cell wall fraction having about 5 times more activity in comparison to nuclei, and the microsomal fraction and 3 times in comparison to chloroplasts. The higher activities in cell walls and chloroplasts could be due to a more abundant accumulation of TGase in these fractions, as shown by western blotting with the anti-AtPNG1 polyclonal antibody, which proved a cross-reacting band of 58 kDa (Fig.1B). No immuno-signal was detected in the nuclei compartment, nor in the microsomal and soluble fractions (data not shown).

3.2. The *AtPng1* KO line shows altered morphological differentiation

KO line was compared with that of the WT line in an attempt to verify possible morphological differences caused by its loss of function. As shown in Fig. 2A, the WT line was characterized by many basal rosette leaves with several cauline inflorescences, relatively large and whose stems were pink-pigmented (Fig. 2A, arrow). The cauline leaves were lost during plant growth and numerous siliques were produced. Compared to WT, KO plants showed a reduced number of rosette leaves, as well as numerous larger green cauline leaves (insert in Fig. 2A) with inflorescences characterized by fewer siliques. Cauline leaves in the KO line were characterized by a higher percentage of fresh weight: 92.6 ± 1 with respect to 90.1 ± 0.6 in WT and by a higher content in chlorophylls *a/b* (96.4 ug/g fw ± 4.8 in KO and 90.5 ug/g fw ± 0.9 in WT). Carotenoid content was not significantly different (9.5 ug/g fw ± 1 in KO and 10.5 ug/g fw ± 0.8 in WT).

The KO and WT lines differed in stem diameter, due to a reduced development mainly of the sclerenchyma and medullar tissues in the KO (Fig. 2C) compared to the WT line (Fig. 2B). The photosynthetic cortex of WT (Fig. 2B, red fluorescence) surrounded a thick layer of sclerenchyma cells (green fluorescence). In the WT line, the sclerenchyma developed as a thick continuous layer deepen into the medulla which incorporated several large vascular bundles. This sclerenchyma layer was less developed in the KO line (Fig 2C). In the inserts of Fig. 2B-C epidermal, cortical and sclerenchyma tissues are shown at high magnification. The multiple nonhomogeneous cell layers formed a continuous sclerenchyma ring in the WT line (insert in Fig. 2B, yellow line), while the sclerenchyma in KO line was formed by only 3 or 4 regular cell layers separated in short arches which did not include vascular bundles (insert in Fig. 2C, yellow line).

Details of the basal stem sections of WT and KO lines at the same age are shown in Fig. 3. In particular, the sclerenchyma layer (blue arrows) and the vascular bundles (yellow arrows) were considerably larger in the WT stem (Fig. 3A) compared to KO stem (Fig. 3B). In the WT stem (Fig. 3C) the cortex layer (red arrow) showed a pink color in the subepidermal region (white arrow) whose color was visible also macroscopically, as observed in Fig. 2A, whereas these pigments were absent in the green photosynthetic cortex of KO stem (Fig. 3D).

Histological observations of the stem tissues of both lines are comparatively shown in Fig. 4 and in Fig. 5. In WT, the epidermal cells were larger, with a thicker cell wall and distributed in several size classes (ranging from 6 to 30 μm) compared to the KO (Fig. 4A1 and 4A3). In the KO line, cells were smaller, missing the largest categories (maximum 18-21μm) (Fig. 4A2 and 4A3). Xylem vessels showed well-developed cell walls in the WT line (Fig. 4B1 and 4B2 respectively). In the KO line, the diameter range of the highest percentage of vessels was smaller when compared to the highest percentage diameter range of WT vessels (Fig. 4B3).

Sclerenchyma cells showed variable cell diameters in WT (Fig. 5A1 and 5A2) whereas KO was characterized by a prevalent frequency of small cells, large ones being sporadic (Fig. 5A3). Also sclerenchyma cell wall thickness was reduced in the KO line, where about 70% of cells showed a cell wall thickness of 0.5-1 μm (Fig. 5B1, 5B2 and 5B3).. In summary, epidermis, vessels and sclerenchyma all showed that in KO cells were homogeneous, smaller with the cell walls apparently thinner.

Summarizing the morphological and histological evidence, KO plants showed a reduced differentiation and altered development, presenting a pronounced juvenile phenotype, characterized by a lower number of floral stems and rosette leaves, by the longer permanence of cauline leaves on the stem, by the lower production of siliques and by cytological features.

3.3. TGase enzymatic activity persist in the plastid fraction of the *AtPng1* KO line

The WT line showed a TGase of 58 kDa, band that was not detectable in the KO line (Fig. 6A). Concomitantly, the isopeptide Nε-(γ glutamyl)-lysine, the product of *in vitro* TGase reaction, was significantly higher in the WT line compared to the KO mutant (Fig. 6B) .

To detect the enzymatic activity *in vitro*, whole plants of WT and KO were analyzed along with their life span during vegetative and reproductive phases. In the WT line, TGase activity significantly increased during plant growth, decreasing after 60-70 days when plants developed the inflorescence, and reaching the lowest value during senescence. On contrary, TGase activity in KO line was halfed compared to the WT line and did not significantly change during growth. In the KO line, TGase activity decreased after inflorescence development (90-100 days), to values not significantly different from those of the WT line (Fig. 6C).

As a critical point *in vivo* is the onset of flowering, TGase activity was determined in the two life stages, vegetative and reproductive, in the organelles of rosette leaves and of inflorescence (Fig. 7). The activity was prevalent in the chloroplast fraction and it was not significantly different in WT or KO, either when measured in the inflorescence or rosette. Soluble and microsomal fraction showed a low activity in both lines.

Together, these data showed that the KO line did not present (or its level is too low to be detected) the 58 kDa protein immuno-recognized in the WT line by the antibody against AtPNG1P; nevertheless, a low level of TGase activity was present in the plastid fraction of the KO line.

3.4. KO and WT lines differently respond to abiotic stresses

As TGase activity was shown to be at basal level in physiological conditions, to further emphasize the differences between KO and WT, plants of both lines were subjected to heat and wound stresses. Seeds of both lines were sown on sterile medium at 30 °C and plantlets were allowed to develop for 5 and 10 days. Both roots and cotyledons were affected by heat stress, as shown in Fig. 8. Compared to WT (Fig. 8A1, B1), the KO line showed a reduced primary roots development , followed by impaired development of lateral roots after 5 days (Fig. 8C1) and after 10 days (Fig. 8D1). WT cotyledons were green after 5 days (Fig. 8A2) and developed trichomes after 10 days (Fig. 8B2), whereas those of the KO line were smaller, and accumulated red pigments (Fig. 8C2); cotyledons neither grew significantly nor differentiated trichomes until day 10 (Fig. 8D2).

Leaves and inflorescence stems were studied also after wound stress. The change in TGase activity in WT leaves at the wound was rapid but transient (Fig. 9A). TGase activity increased within 15 min of about three-fold in respect to the basal activity observed at time 0 (Fig. 9B), remained constant until 30 min and decreased further on. On the contrary, in KO leaves the wound was effectiveless, being the activity constantly at low level, until 24 h.

Wound induced *AtPng1* transcription within the first 5 min and reached minimum transcription levels after 15-30 min in the WT line (Fig. 9C). Subsequently, TGase expression increased after 1 hour and it remained visible for several hours. In the KO line, wound stress in leaves did not induce any stimulation of *AtPng1* transcription (Fig. 9C).

Wound stress was induced also on the cauline inflorescence, and the progression of wound repair was studied until day 21. The non-wounded unstained stems (day 0, Fig. 10A) showed a chlorophyll rich subepidermal tissue (characterized by red autofluorescence at 450 nm), a multilayer of peripheral sclerenchyma (green under fluorescence) and a medullary parenchyma (dark under fluorescence and colorless under white light). In the WT line, at day 1 (Fig. 10B, d1) no signs of repair were visible, whereas on day 5 (Fig. 10C, d5) a lignin- and suberin-enriched tissue (data not shown), impermeabilized the wound edges (Fig. 10C, red arrows). The underlying cells of the medullary parenchyma enlarged and divided disorderly, forming a small mass of meristematic tissue (Fig. 10C, d5, green arrow). At day 9 the protecting process continued (Fig. 10D2, red arrows and details in D1 and D3). At day 14 the shape of the wound assumed a “V” form, forming an angle of about 30 degrees (Fig. 10E1). Under some dead cells, the wound edges were straight and formed by a bi- three-layer of modified cells, especially in their external cell walls (Fig. 10E2, E3). Wound edges appeared protected by protruding tissue (Fig. 10E1, red arrows). At day 21 the edges of the wound were straight (red parenthesis in Fig. 10F1 and F3), formed by cells with a modified cell walls (fluorescent towards the wound, but not modified towards medullary parenchyma, Fig. 10F2). Some parenchyma cells were still dividing, showing two brother nuclei on either side of the newly formed wall (Fig. 10F4, green arrow).

The most significant difference between the WT and KO lines was the shape of wound, as shown in Fig. 11. Whereas in WT (Fig. 11A) the wound had a “V” shape with straight sides and protruding edges, in the KO line the wound assumed a “U” shape (Fig. 11B). The layer of repair cells was thinner and the underlying parenchyma was formed by large spherical cells (details in Fig. 11C and D).

As TGase was identified in a highly active form in the cell walls (Fig. 1A), its presence in repairing cells was investigated by fluorescence microscopy with AtPNG1P antibody. At time 0 after wound induction, TGase was hardly immunodetected (Fig. 12A), while at day 21 wound edges clearly showed TGase labeling (Fig. 12B, white arrows). In the KO mutant, TGase was not immunodetected at time 0 nor after 21 days (Fig. 12C and D).

4. Discussion

The comparison between KO mutant and the WT line clarify TGase role during *A. thaliana* development. The data here reported allow concluding that AtPNG1P and its TGase activity are involved in plant cell development and in stress response. Macroscopically, the KO line showed delayed and reduced development, reduced dimension of the inflorescence, bigger cauline leaves and a decreased production of smaller siliques. Anatomically, in the KO, vascular bundles were less numerous and smaller and the sclerenchyma layer in the stem was less developed. Also, the cortical layer was less developed and did not present the pink color typical of WT stem. Epidermis, vessel, and sclerenchyma cells were significantly smaller in the KO line and the thickness of the sclerenchyma cell wall was significantly reduced. All those aspects were related to a less differentiated secondary cell walls, and to a reduced number of cells, generally of smaller dimension in KO than WT.

The macro- and microscopical differences between the two lines could be related to the absence of a 58 kDa TGase in the KO line, which, on the contrary, was clearly present in chloroplasts and in cell walls of WT. Nevertheless, a low residual TGase activity was detectable also in the KO line, suggesting the possible existence of other active forms, mainly in the chloroplasts. The hypothesis of the existence of other TGase forms is supported by data in literature (Della Mea et al. 2004b). The 58 kDa TGase form is probably the most common one, also in other plants, as shown in the plastid, microsome and cell wall fractions of the *Nicotiana* flower corolla (Della Mea et al. 2007). Other TGase isoforms have been identified in different plant organelles, for example, a 52 kDa isoform was found in the soluble fraction, while 39 and 58 kDa isoforms were studied in the chloroplast of higher plants (Dondini et al. 2003). In the apical meristem of *Helianthus tuberosus*, three isoforms of 58, 75 and 85 kDa have been identified, while a 70 kDa TGase was found in apple pollen (Beninati et al. 2013; Di Sandro et al. 2010; Del Duca et al. 2009; Serafini-Fracassini and Del Duca 2008). These forms could derive by post-translational modification of a single protein of 86 kDa immunodetected in *Arabidopsis* enriched microsomal fraction, identified as the first plant TGase by Della Mea et al. (2004).

The residual activity in KO could thus be tentatively attributed, at least in part, to TGase isoforms presents in the chloroplasts. In fact, plastidial TGase activity was not significantly different in WT or KO, either when measured in the inflorescence or in the basal leaf, probably due to a chloroplast-encoded isoform.

Here it was found that KO residual TGase activity poorly correlated to the development events; in WT, on the contrary, the activity increased with stem growth and decreased during the flowering stages, namely approaching senescence.

The effects of the KO mutation, could be explaind at least in part, by highlighting the involvement of PAs, substrates of TGase, in the deposition of secondary wall, in cell differentiation and cell death in early metaxylem (Tisi et al. 2011; Aloisi et al. 2017). In addition, the amount of PAs is known to be severely modified after different stresses (Minocha et al. 2014; Tiburcio et al. 2014;

Liu et al. 2015; Aloisi et al. 2016a), and induced stress conditions enhanced the differences between WT and KO. Nevertheless, TGase might also be directly affected by stress, as shown, for example, by salt and light stresses in *Dunaliella salina* (Dondini et al. 2001). Finally, wound stress has been reported to affect the neo-synthesis and activity of two TGase forms, one of which of 58-kDa, in dormant tissues of *Helianthus tuberosus* when activated *in vitro* (Del Duca et al. 2000).

In the present work, plantlets of the TGaseKO line showed a more juvenile aspect, both under standard and heat stress conditions. These evidences could be explained, at least in part, by the involvement of PAs, in particular spermine, in *A. thaliana* stress tolerance (Sagor et al. 2013). Finally higher levels of free and bound PAs were reported in heat tolerant rice (Pang et al. 2007). In stressed KO plantlets, as suggested by their more juvenile aspect, a lower catalysis of conjugated PAs can be hypothesized, due to the lack of 58 kDa TGase. By transcriptomic assays, among thousand genes involved in tomato heat-stressed seedlings, as unique report, at least to our knowledge, overexpression of TGase was found to enhance, within few hours, heat tolerance by maintaining membrane integrity and holding higher net photosynthetic rate. Numerous genes were closely associated with the protein processing in the endoplasmic reticulum, carbon fixation, and photosynthetic metabolism (Jahan et al. 2021).

The possibility that TGase can participate in healing processes is rather unknown for plants but already demonstrated for some animals (Greenberg et al. 1991; Telci and Griffin 2006; Qin et al. 2013). Present data confirmed that wound stress affected TGase, thereby causing clear differences in reactivity between WT and KO lines. In WT, the transcription of TGase occurred very rapidly within 5 minutes and allowed the enzyme activity to reach a three-fold peak already within 15 minutes, while in the KO line the enzyme transcription was not detectable.

The evaluation of wound stress in the stem allowed to observe the recovery from wounding for several days and the formation of a scarring tissue that covered the entire wound. TGase accumulated in the stem of the WT line, in the 2-3 cell layers underneath some superficial dead cells. This distribution pattern suggests that TGase could be involved in the injury response, probably by exerting its glue function and strengthening cell walls, as previously observed during senescence in *Nicotiana* petals (Della Mea et al. 2007). The lack of TGase activity in the cell walls of the KO line, might be related to the weaker anatomical structure observed, characterized by large intercellular spaces in the parenchyma, and large, spherical parenchyma cells, which is probably due to reduced stiffness of the KO cell wall, failing to counteract the internal turgor pressure (Carter et al. 2017). The different cell features of the wounded stem tissues cause differences in the shape of the wound, exhibiting a “V” shape in WT and a “U” shape in KO line (Waffenschmidt et al. 1999; Iranzo et al.

2002). The cytological comparison between WT and *AtPng1* KO plants suggested that many of the differences between KO and WT lines were related to a different distribution of TGase in the cell walls of the scarring layer, probably leading to a less differentiated secondary cell walls in the former.

Present data allow to assume the important role of cell wall TGase in cell differentiation, organization, and plant development. In fact, the *AtPng1* KO was characterized by lower development and by a reduced reactivity to abiotic stresses when compared to WT. In conclusion, our data highlight the involvement of TGase, and consequently probably its reaction products, in the normal growth and differentiation of *A. thaliana,* as shown by its phenotypic and physiological profiling analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The research involves neither human nor animals.

Credit authorship contribution statement

I. Aloisi and **L. Parrotta**: Investigation, Methodology, Formal analysis, Writing - original draft.

C. Faleri and **M. Della Mea**: Investigation, Methodology, Formal analysis.

G. Cai, S. Del Duca and **D. Serafini-Fracassini**: Investigation, Writing - review and editing.

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Figure legends

Fig. 1. TGase in WT. TGase activity in subcellular enriched fractions, *i.e.* Cell Wall (CW), Nuclei, microsomal (MICRO), soluble (SOL) and chloroplast (CHL). Means were compared by Dunnett's Multiple Comparison Test. Bars with the same letter are not significantly different ($p \geq 0.01$) (**A**). Immunorecognition in Western blot analysis by the *A. thaliana* TGase antibody (AtPNG1P) of the cell walls (CW), and chloroplast (CHL) fractions. Non-immunoreacted fractions (*i.e.* Microsomal, Nuclei, Soluble) are not shown (**B**).

Fig. 2. Comparison of WT and KO lines. Morphological comparison of 3 months-old plants. Arrow highlights pink color pigmentation in WT stems (**A**). Particular of the caulinar leaves is reported in the squared detail. Stem transverse section observed by fluorescence microscopy in WT line (**B**) and *AtPng1* KO line (**C**). Green fluorescence: lignified sclerenchyma and xylem elements. Red fluorescence: chlorenchyma. The yellow lines in the magnifications reported in squares label the thickness of the sclerenchyma layers. Bars in figure B and C 250 µm; bars in inserts 50 µm.

Fig. 3. Histological comparison of WT and KO lines. Basal stem transverse section in WT line, stained with acid fluoroglucine (**A**) and unstained KO (**B**). Blue arrows indicate the thickness of the schlerenchyma layer; yellow arrows indicate the radial diameter of vascular collateral bundles. Detail of the unstained cortex of WT (**C**) and stained KO (**D**) inflorescence. Red arrows mark the cortical parenchyma in both lines; in WT the external layers showed a natural pink color (C, white arrow). Bars: 50 µm.

Fig. 4. Comparison of cell dimension of WT and KO line epidermis and vessels. Transverse section of the epidermis of the basal stem in WT (**A1**) and KO (**A2**) lines. Comparison of epidermis cell diameter, recorded by dimension classes, is reported in **A3**. Transverse section of the vascular bundles of the basal stem in WT (**B1**) and KO (**B2**) lines. Comparison of vessel diameter, recorded by dimension classes, is reported in **B3**. Bars: 50 µm.

Fig. 5. Comparison of cell dimension and cell wall thickness of WT and KO line schlerenchyma. Transverse section of the basal stem in WT (**A1**) and KO (**A2**) lines. Comparison of schlerenchyma cell diameter, recorded by dimension classes, is reported in **A3**. Transverse section of the basal stem in WT (**B1**) and KO (**B2**) lines. Comparison of thickness of schlerenchyma cell wall, recorded by

dimension classes, is reported in **B3**. Bars in figure A1 and A2 50 µm; bars in figure B1 and B2 10 µm.

Fig. 6. TGase presence and activity in WT and KO lines. Immunodetection by Western blotting of TGase in WT and KO. Total lysate was probed (**A**). TGase products detected in ELISA assay by the 81D4 antibody in WT and KO lines. Casein was used as internal control. C+ = TGase-treated casein, C- = untreated casein (**B**). Total TGase activity in WT and KO lines during *in vivo* plant growth (from 45 to 126 days) (**C**). Means of samples were compared by Dunnett's Multiple Comparison Test. *** = $p \leq 0.001$

Fig. 7. Comparison of the subcellular activity of TGase of the WT and KO lines. The activity was measured in soluble (SOL), microsomal (MICRO) and chloroplasts (CHL) fractions of leaves and inflorescence. Means were compared by Dunnett's Multiple Comparison Test. Bars with the same letter are not significantly different ($p \geq 0.01$).

Fig. 8. Comparison of WT and KO lines germinating seeds under heat stress. Seeds were exposed to 30°C for 5 and 10 days. Root development of WT (**A1**) and KO (**C1**) and cotyledon development of WT (**A2**) and KO (**C2**) at 5 days. Root development of WT (**B1**) and KO (**D1**) and cotyledon development of WT (**B2**) and KO (**D2**) at 10 days.

Fig. 9. TGase expression and activity after leaf wounding in WT and KO lines. Leaves from the basal rosette were wounded by longitudinal cutting along the mid-rib (**A**). Time-course TGase activity in leaves until 24 hours after wounding. Means of samples were compared by Dunnett's Multiple Comparison Test. *** = $p \leq 0.001$ (**B**). Time-course expression of AtPng1 revealed by PCR after wounding (**C**). Actin was used as housekeeping gene.

Fig. 10. Time-course histological observations after stem wounding in WT line. Transversal sections of the basal inflorescence observed by white light or fluorescence microscopy before longitudinal radial wounding; part of the collateral vascular bundles and its phloem (Phl) are in evidence (**A**). At day 1 the radial wound (W) enlarged laterally (**B**). At day 5, a new irregular protection cell layer surrounded the wound edges (red arrows) covering the wounded epidermis, photosynthetic cortex, phloem and connected with sclerenchyma (green fluorescence in squares). The medullary parenchyma formed new cells, some of which very large (green arrow) (**C**). At day 9, the

wound surface was completely covered by neo-formed protective tissue (blue fluorescence, red arrows) (**D2**). Phl was completely covered (details in **D1** and **D3**). At day 14, the wound enlarged in a “V” shape. The thickness of the new protective layer increased covering the wound margin (**E1**, red arrows), and magnification of the wound bottom in **E2**) and their cells show lignified cell walls (**E3**). At day 21, the protective tissue (red arrows, **F1**) and a lignified cell layers still increased (**F2**), as highlighted by autofluorescence (red parenthesis, **F3**). Dividing cells were visible below the protecting layer (green arrow, **F4**). Bars in figure A, B, C, D1, D3, E2, E3, F2 and F3 50 µm; bars in figure E1 and F4 20 µm. Bar in figure D2: 250 µm. Bar in figure F1: 200 µm.

Fig. 11. Comparative stem anatomy after wounding in WT and KO lines. Sections of the inflorescence stained by acid floroglucine and observed after 21 days of WT line (**A**) and of KO line (**B**) and relative magnifications (**C-D**). Bars: 50 µm.

Fig. 12. Comparative immunofluorescence of TGase distribution after wounding of stem in WT and KO line. (A-B) WT line. **(A)** At time 0, anti-TGase (AtPNG1p and secondary Alexa-Fluor 488 antibodies) labelling was weakly diffused. **(B)** At day 21, TGase labelling was located along the wound edge (white arrows). **(C-D)** KO mutant line. **(C)** Anti-TGase labelling at day 0 revealed a pattern similar to WT. **(D)** At day 21, the wound edge exhibits weak or no labelling. The red dotted lines indicate the wound position. Bars: 50 µm.

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