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Determination of Functional γ-GTase Genes and Investigation of the Biological Activity of Proteins in *Arabidopsis thaliana* at Different Stages of Growth

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Abstract: The results in the present study emphasize that in *Arabidopsis thaliana* genome, γ-GTase encoded by a gene family comprised of four genes. Three of these genes are functionally expressed while the fourth is a pseudogene. The three functional genes express different active protein isoforms that appeared to have different functions. Two genes (GGT1 and GGT2) are structurally similar and expresses proteins with the same molecular weight and both target to the same cellular compartment (plasma membrane). However, both demonstrated different tissue localization, physiological activities and different patterns of response to environmental stress. The majority of GGTI encoded by the first gene was localized in the rosette leaves, It is also expressed throughout the whole plant.

Key words: Arabidopsis thalina, γ-GTase gene, growth, biological activity, proteins

INTRODUCTION

The γ-GTase, (5-L-glutamyl) peptide amino acid 5-glutamyl transferase, EC 2.3.2.2 has a unique role in catalyzing the hydrolysis of the γ-peptide linkage between the γ-carboxyl group of glutamate and the γ-amino group of the next amino acid in the γ-glutamyl peptides. The substrates susceptible to γ-GTase include Glutathione (GSH), the oxidized form of Glutathione (GSSG), Glutathione conjugates (GS-X) and a variety of dipeptides and tripeptides that have N-terminal γ-linked glutamyl group. The γ-glutamyl group is then transferred to a wide range of acceptors using amino acids or dipeptides as an acceptor, which results in transpeptidase reaction (Jaspers *et al*., 1985). Another significant physiological function of γ-GTase and the γ-glutamyl cycle is that the enzyme catalyzes the transpeptidation of GSH in the presence of amino acids and thus results in the formation of γ-glutamyl amino acids dipeptides. Plants form GS-X of many exogenous compounds, such as herbicides and pesticides, in a reaction catalyzed by GSTs (Marrs, 1996). These conjugates might be transported to the vacuole by ABC transporters (Martinoia *et al*., 1993; Gaillard *et al*., 1994; Li *et al*., 1995; Rea *et al*., 1998), where they undergo further modifications that start with the cleavage of the γ-glutamyl moiety by γ-GTase and continue by the removal of the glycine moiety (Lamoureux *et al*., 1991; Lamoureux and Rusness, 1993). In the present study we investigate the functional γ-GTase genes and the investigation of the biological activity of their proteins and the steady state levels of their thiol substrates throughout the plant.

In order to investigate the previous criteria, two *ggt* homozygous mutants were selected. The mutants have been identified and characterized by an insertional mutation of a transposon element in the open reading frame of *GGT1* gene (Sundaresan *et al*., 1995) and a T-DNA in the open reading frame of *GGT2* gene Syngenta *Arabidopsis* Insertion library (SAIL). The analysis of the four *GGT* genes revealed that *GGTI*, *GGT2* and *GGT3* genes are located on the fourth chromosome. GG*T1* and GG*T2* are directly located next to each other with only 858 bp apart and have approximately the same nucleotides length of 2.253 bp for *GGT1* and 2.196 bp for *GGT2* (Storozhenko *et al*., 2002). The predicted molecular weights of the encoded proteins indicated that *GGT1* and *GGT2* encode for two similar proteins with 61.12 kDa and 61.19 kDa, respectively. Meanwhile, the *GGT3* gene located far away on the same chromosome with 2.329 bp length of nucleotide sequence and encodes for a predicted protein of 69.15 kDa. Either *GGT1* or *GGT2* genes may result from gene duplication during the successive development(Martin and Slovin, 2000 and Storozhenko *et al*., 2002). The two genes may encode two similar protein isoforms for γ-GTase with a molecular weight of 61.1 kDa. These findings agree with the results of Kushnir *et al*. (1995). They have identified a cDNA for the D22 gene that encodes a plant homologue for γ-GTase. On the other hand, the different localization of *GGT3* on the fourth chromosome and its longer nucleotide sequence that encodes a high molecular weight protein may categorize this gene differently from *GGTI* and GG*T2* genes.

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MATERIALS AND METHODS

The wild type ecotype Lansberg of *Arabidopsis thaliana* L. Heynh, is used in this study, along with two homozygous lines that represent the mutation in *GGT1* gene; the two lines were termed as *ggtl. 1* mutant and *ggt1.3* mutant. The *ggt1. 1* mutant line was obtained from the National *Arabidopsis* Stock Center (NASC) Nottingham, U.K. under the stock number N100370. The insertion was identified in the *Arabidopsis GGT1* gene in BAC clone T19P19 and classified under the gene bank number 5748500.

Identification of the characteristic mutation phenotype of *ggt* mutants: Seeds of the wild type were cultivated in rows parallel to either *ggt1.1* mutant line or *ggt1.3* mutant line in one soil tray at 4°C for two days to overcome dormancy. The tray was then transformed to a growth room at a constant temperature of 22°C and continuous light intensity of 120 µ mol $m^{-2} sec^{-1}$. Plants were allowed to grow up for four weeks until clearly revealing the mutation phenotype. To avoid bias, random digits were used to select ten replicates of plants from each cultivar; the chlorotic phenotype in the leaves was visually documented. The rosette leaves diameter and the complete inflorescence length were measured to represent the leaf area and the plant height, respectively. The data were statistically analyzed using the student t-test.

Molecular analysis of *ggt1* and *ggt2* mutants: Genomic DNA was extracted from 1 or 2 rosette leaves of four-week old plants, using the DNA micro-preparation protocol described by Dellaporta *et al.* (1983). Five microgram of genomic DNA were digested in a 50 µL digestion reaction for 5-6 h using *Bam*HI and *Hind*III and the digested samples were separated by electrophoresis through a 0.7% agarose gel with ethidium bromide overnight. The λ.-DNA/HindIII fragments and the one Kb^+ DNA ladder were used to mark the digested DNA. DNA was transferred overnight onto a BioRad Zeta-probe GT blotting membrane under capillary transfer as described by Sambrook *et al.* (2001). The membranes were then exposed to BioMax MS films (Kodak) in a cassette with a BioMax MS intensifying screen at -80°C overnight and developed in Kodak M35A X-OMAT processor.

Four weeks old plants of the homozygous lines for both the *ggt1* mutant and wild type *Arabidopsis* ecotype Lansberg were reciprocally crossed to produce the heterozygous F_1 progeny. The artificially pollinated stigmas were carefully wrapped in Saran wrap to prevent self-pollination and to preserve humid conditions. After complete maturity and dryness, the seeds of the F_1 progeny were collected.

The genomic DNA of F_1 progeny and the homozygous parents (the wild type ecotype Lansberg and the *ggt1* mutants) were used to reveal the signal of the wild type gene. Two wild type *GGT1* specific primers were designed to amplify a fragment of 540 bp in the coding sequence of *GGT1* gene. These two primers were (GGT1-5' ATGTCGCTGGTTCGAACAGTGA and GGT1-3' CGCTTCTAGTCGCGTTCATCT). The amplification conditions were 94°C for 30 sec, 60°C for 1 min and 72°C for 30 sec for 35 cycles. The signal of the *GGT1* mutant gene was investigated by using primer sequence GGT1-3' CGCTTCTAGTCGCGTTCATCT along with the sequence of the *NPTII-3* (neomycin phosphotransferase II) primer CTGGCGCGAGCCCCTGATGCT. The PCR amplification conditions were 94°C for 30 sec 60°C for 1 min and 72°C for 1 min for 35 cycles. The genomic DNA extracted from four weeks old rosette plants was used as a template with the two *GGT1* specific primers. The plants were analyzed for the presence of the amplified 540 bp fragment of the wild type *GGT1* gene. The PCR amplification conditions were, 94°C for 30 sec, 60°C for 1 min and 72°C for 30 sec for 35 cycles.

The spatial and temporal distribution profile of *GGT* genes in *Arabidoysis*: Seeds of wild type ecotype Lansberg and the *ggt1* mutants were grown in soil under illumination of white fluorescent at 120 µmol $m^{-2} sec^{-1}$ for 24 h and a constant temperature of 22°C. Plant tissues of roots, stem, rosette leaves, cauline leaves, flower buds, flowers, immature siliques and mature siliques were collected from five-weeks old plants; total RNA was isolated from these organs as described by Kirk and Kirk (1985), separated in agarose gel and blotted. The blots were prehybridized at 65° in 15 mL of the hybridization buffer (7% SDS, 1% casein, 1 mM EDTA, 0.25 M of Na_2HPO_4.7H,O, pH 7.4) for 2 h (Church and Gilbert, 1984).

Quantitation of cysteine, γ-glutamyl cysteine and glutathione: The levels of cysteine, γ-glutamyl cysteine and glutathione were estimated as described in Steffens *et al.* (1986), Fahey and Newton (1987) and Xiang and Oliver (1998). The fluorescent monobromoimane derivatives were separated and quantified by HPLC. The column was a Waters Radical Compression 10 µm C 18 reversed phase column (serial number Wat. 052885) connected to Hewlett Packard series 1100 HPLC system with HP 1046A fluorescence detector and Hp 3395 intergrator. The samples were centrifuged briefly and 20 µL were injected and differentially eluted with absolute methanol and 0.25% acetic acid, pH 3.3.

Construction of the pCB308 *GGT2* promotor and transformation of *E. coli* and *Agrobacterium*: The *GGT2* promotor fragment was digested with BamHI and XbaI

and was ligated to the pCB308 binary vector that was digested with the same restriction enzymes in a 10 μL ligation reaction. The ligation was performed in 0.5 mL sterile microfuge and the insert to the vector ratio was 1:3, respectively. The microfuge tube was incubated at 14°C in a water bath overnight. One to two μL of DNA were transformed to either *E. coli* (DH5α strain) or *Agrobacterium tumefaciens* strain bought from INVITROGEN using the electroporation method described in the manufacturer protocol. The transformed cells were collected at 8000 g and resuspended in 100 μL of supernatant. The suspension was plated out on L.B. agar plates with kanamycin and uniformly spread using sterile glass beads. The transformed bacterial cells were allowed to grow by incubating the plates at 37°C overnight.

Floral dip transformation of *Arabidopsis* plants: The wild type plants ecotype Columbia was transformed by the *Agrobacterium* strain carrying the GGT2: GUS construction in the pCB308 binary vector using the floral dipping method as illustrated in Clough and Bent (1998).

RESULTS AND DISCUSSION

Significant reduction in the mutants' rosette leaves diameter was evident, it was reduced to 9.19±1.23 and 7.62±1.86 mm in the mutant lines *ggt1.1* and *ggt1.3*, respectively compared to 10.52 mm in the wild type. An increase in the inflorescence height of the mutant lines was also clearly expressed. In four-weeks old mutant plants the inflorescence length was 10.44 cm compared to 4.6 cm in the wild type plants (Table 1).

The appearance of the chlorotic phenotype of *ggtl* knockout mutants that was accompanied by reduction in the leaves size may be explained by assuming that the γ-GTase encoded by *GGTI* may have a role in the GSH metabolism in the leaves (source tissues) that mediate the synthesis of γ-glutamyl amino acid and facilitate their availability and transport inside the cells, where they could be accumulated or exported through phloem to the sink tissue. Also, GGTI has a role to mediate the transport of the individual components of the reduced glutathione through the ABC transporters located on the plasma membrane to the apoplast space (Martin and Slovin, 2000 and Storozhenko *et al*., 2002). This reduced GSH will be degraded and returned back to the cell for further processing to its individual amino acids or for de novo synthesis.

In the second model, the γ-GTase encoded by *GGT1* gene may play a role in protecting plants against the Reactive Oxygen Species (ROS) produced naturally during electron transport chains in photosynthesis or under the stressful conditions including the nutrient deficiency. The inability to scaving the ROS could result in destroying the photosynthesis apparatus with oxidative damage of the cells (Marrs, 1996). Therefore, *ggt1* mutants that are missing *GGT1* gene were unable to face this challenge under soil conditions and therefore, showed the symptoms of chlorosis and reduction in size. This may point out to the Ascorbate/GSH cycle as evidence to the continuous oxidation of glutathione that may be accompanied by an increase in the reduced GSH pool.

Alignment of the proteins encoded by *GGT* genes in *Arabidopsis* revealed high homology of two of these genes *GGT1* and *GGT* with a percentage identity of 82%. Table 2 indicating that the two enzymes are structurally similar. This strongly agrees with Martin and Slovin (2000), who purified two isoforms of GGT in tomato. The two GGT isoforms exhibit identity in the native molecular mass with a gylcosylated hydrophyllic domain. Both enzymes were found to hydrolyze dipeptides and tripeptides with N-terminal γ-linked glutamate and transfer the γ-glutamyl moiety to a broad range of acceptors.

Table 1: The phenotypic criteria (rosette leaf diameter in mm and the inflorescence height in cm) of *Arabidopsis thaliana* wild Type (wt.) and mutant lines ggt1.1 and ggt1.3. p>0.05 indicates insignificant difference ≤ 0.05 indicates significant difference

Arabidopsis line	Wt	*ggt1.1*	*ggt1.3*
Rosette leaves diameter	10.52±0.99	9.19±1.23	7.62±1.86
Inflorescence height	4.6±1.53	5.99±1.91	10.44±2.07
t-test	*wt* vs. *ggt 1.1*	*wt* vs. *ggt 1.3*	*ggt 1.1* vs. *ggt 1.3*
Rosette leaves diameter	0.016	0.001	0.041
Inflorescence height	0.164	0.000	0.001

Table 2: Characterization and comparison of the deduced proteins encoded by the four *GGT* genes in *Arabidopsm thaliana*. The table illustrates the predicted length and molecular mass for each encoded protein, the percentage identity of the pairwise alignment of the GGT proteins and the cellular localization of each protein as created using the Clustal W prediction results

Gene	AGI code	Predicted protein length	Calculated molecular weight	Proposed localization	GGT1	GGT2
GGT1	At4g 39640	574	61.12	Plasma membrane	-	-
GGT2	At4g 39650	637	69.15	Plasma membrane	80	
GGT3	At4g 29210	572	61.19	Vacuolar membrane	48	49

The putative signal sequence targets either *GGT1* or *GGT2* to the plasma membrane. These data are in agreement with the results reported by Storozhenko *et al.* (2002) and confirmed the localization of *GGT1* enzyme on the plasma membrane with an extracellular active site in the apoplastic space. Taken together the data reported by Martin and Slovin (2000) and Storozhenko *et al.* (2002) indicate that *GGT2* enzyme is located also on the plasma membrane with its active site facing outside the cell. However, *GGT1* and *GGT2* may be functionally different one might be specialized for GSH transport, while the other is involved in the γ-glutamyl amino acid synthesis.

On the other hand, the deduced protein sequence of *GGT3* gene was partially different. It showed only 46-48% identity with GGT1 and GGT2, respectively. The GGT3 has long N-terminal (47 amino acids). The Signal P prediction targets this protein to the vascular membrane or the endoplasmic reticulum. Storozhenko *et al.* (2002) also reported this third copy of *GGT* on the fourth chromosome with percentage identity of 50-59% with the first GGT isoform. The putative protein has a mw of 69.2 kDa with long N-terminal predicted to contain a trans-membrane domain.

In crosses of *ggt1* mutants to the wild type plants, the F_1 progeny exhibited the wild type phenotype, which indicate that ggt1 mutant phenotype is recessive to the wild type. Also it showed 100% kanamycin resistance that indicates a heterozygous progeny. The PCR screening for the kanamycin resistant F_1 progeny was performed using either the wild type *GGT1* gene specific primers or the mutant *GGT1* primers. The PCR screening revealed two lines that possessed either the wild type gene or the mutated gene. The data therefore may be regarded to demonstrate a heterozygous mutant genotype. The signal was strongly detected in the parent's *GGT1* mutant homozygous line and in lesser intensity in the mentioned two lines of F1 progeny, as shown in Fig. 1.

On the other hand, no genetic or molecular analysis was accomplished for *ggt2* mutants as the phenotypic screening did not show any significant differences for the mutant compared to the wild type. Wilson *et al.* (1990) envisioned that the function of many genes that have multiple roles acting at early as well as at late stages in development, may be obscured in phenotypic screens. The functionally redundant gene because of the presence of a second locus that can substitute the same function, may not result in any phenotype (Goebl and Petes, 1986; Oliver *et al.*, 1992). This may be similar to the GGT genes, if we take in mind that *GGT1* and *GGT2* genes are structurally very similar. The blots was hybridized with the GUS marker gene for the *GGT1* mutants and with BAR marker gene for the *ggt2* mutants. Both autoradiograms showed different single copy of the insertions, as shown in Fig. 2.

The spatial localization of different *GGTs* in *Arabidopsis thaliana* was investigated using northern analysis screening for different plant organs. The clear difference between the mRNA expression patterns of *GGT1* and *GGT2* genes indicated different organ localization and therefore, selects each gene to a different role through the GSH metabolic pathway. In all the investigated organs, (roots, stems, cauline leaves, rosette leaves, flower buds, flowers, young and mature siliques), it was observed that the *GGT1* mRNA expression pattern

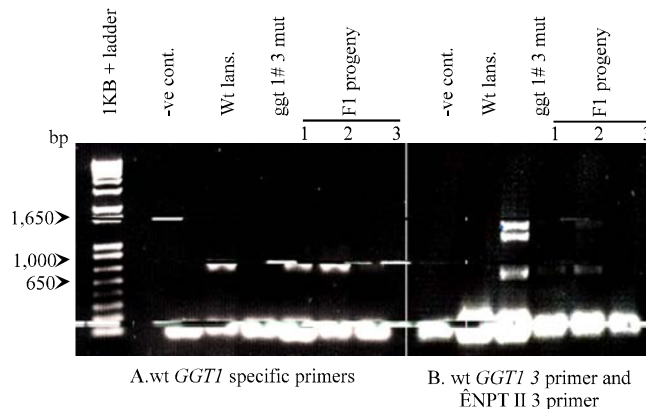

Fig. 1: PCR screening of the F_1 progeny of the artificial crossing of the homozygous wild type ecotype Lansberg and the *ggt1* mutant lines. The genomic DNA extracted from the homozygous parents revealed the control signal by using A. the specific wild type *GGT1* primers and B the mutant *GGT1* gene primers. The genomic DNA extracted from 3 individual siblings of kanamycin resistant F_1 progeny represented as 1, 2 and 3, was screened with each primers set. Ten microliter of the PCR products were run over 0.8% agarose gel

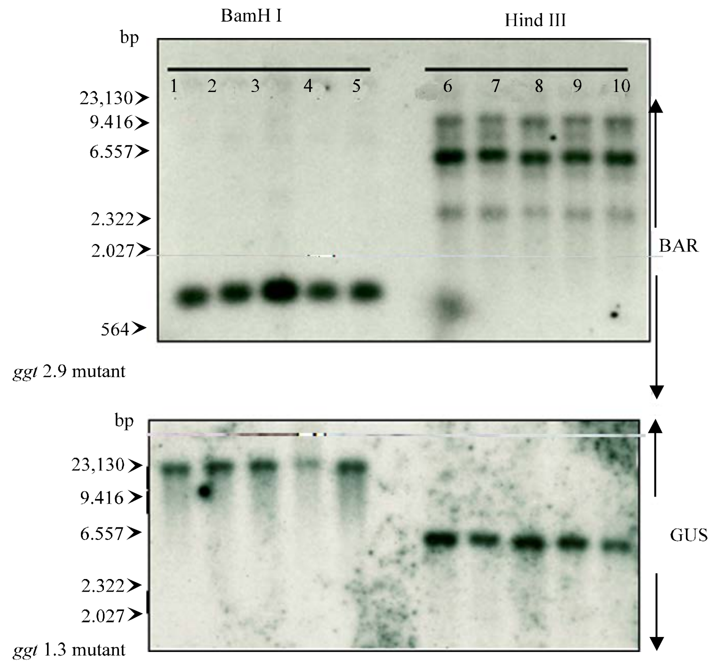

Fig. 2: Southern blots for *GGT1* and *GGT2* genes. Ten replicates of either *ggt1.3* mutant or the *ggt* 2.9 mutant lines were investigated. For each five replicates, 5 μg of the genomic DNA was fractionated by BamHI or HindIII and run over 0.7% agarose gel. Blotting was applied overnight on Zeta membrane and hybridization was performed using the cDNA radioactive probes of *GUS* for *GGTI* gene and of *BAR* for *GGT2* gene

Table 3: Specific activity (units/mg protein) of γ-glutamyl transpeptidase in the indicated organs of the transgenic *Arabidopsis* plants disrupted in *GGT1* gene and the wild type plants at successive developmental stages. Means with * are significantly different from the control wild type plants. $p>0.05$ indicates insignificant difference. $p\leq 0.05$ indicates significant difference

Plant organ	Arabidopsis line	Specific activity as units/mg protein	t-test	
3-weeks old rosette leaves	wt (Lansberg)	1.51±0.18	wt vs. *ggt* 1.1	0.003
	ggt 1.1	0.25±0.08*	wt vs. *ggt* 1.3	0.003
	ggt 1.3	0.33±0.23	*ggt* 1.1 vs. *ggt* 1.3	0.613
4-weeks old rosette leaves	wt (Lansberg)	1.71±0.32	wt vs. *ggt* 1.1	0.016
	ggt 1.1	0.38±0.06	wt vs. *ggt* 1.3	0.017
	ggt 1.3	0.34±0.01	*ggt* 1.1 vs. *ggt* 1.3	0.359
5-weeks old rosette leaves	wt (Lansberg)	3.19±0.58	wt vs. *ggt* 1.1	0.008
	ggt 1.1	0.34±0.19	wt vs. *ggt* 1.3	0.013
	ggt 1.3	0.32±0.06	*ggt* 1.1 vs. *ggt* 1.3	0.829
Immature siliques	wt (columbia)	0.84±0.18	wt vs. *ggt* 1.1	0.222
	ggt 1.1	0.66±0.04	wt vs. *ggt* 1.3	0.417
	ggt 1.3	0.72±0.10	*ggt* 1.1 vs. *ggt* 1.3	0.357
Immature siliques	wt (Lansberg)	0.79±0.13	wt vs. *ggt* 1.1	0.204
	ggt 1.1	0.65±0.09	wt vs. *ggt* 1.3	0.017
	ggt 1.3	0.56±0.05	*ggt* 1.1 vs. *ggt* 1.3	0.236
Roots	wt (Lansberg)	0.56±0.04	wt vs. *ggt* 1.1	0.130
	ggt 1.1	0.47±0.0	wt vs. *ggt* 1.3	0.244
	ggt 1.3	0.48±0.08	*ggt* 1.1 vs. *ggt* 1.3	0.893

was mainly expressed in both the cauline and rosette leaves and at lower level in the roots: It was also recognized with a detectable level in the flower buds, flowers and immature siliques. In agreement with these results, Storozhenko *et al*. (2002) revealed that the transgenic tobacco plants, that had been transformed with the *Arabidopsis thialiana GGT1* gene demonstrated high transgene expression level of mRNA and showed a high catalytic properties of *GGT1* in the leaves extract.

The study of the γ-GTase expression pattern throughout the development of the siliques revealed no significant differences between the *ggtl* mutants and the wild type plants. Moreover, the assay of γ-GTase activity of the roots in the liquid culture systems showed only 15% reduction in the mutant activity compared to the wild type controls. In *GGT1* mutant lines, γ-GTase activity was dramatically decreased in the three successive developmental stages of rosette leaves, as shown in

Table 3. This different pattern of expression in the mutant may refer to the activity of the 35S promoter being located on the DsE transposable element (Sundaresan *et al*., 1995). The 35S promoter can transcribe the whole sequence of the transposon and run over through the downstream region of *GGTI* gene. On the other hand, the GGT3 messages revealed a different pattern. The GGT3 mRNAs were distributed widely with high levels in all the surveyed organs. The results may suggest GGT3 to perform a common important role in GSH metabolism throughout the plant.

These results in Table 3 indicate an extensive activity of γ-GTase encoded by *GGTI* gene in the rosette leaves with the greater functionally active existence in the later developmental stages. Taken together this active distribution pattern in the leaves along with the chlorotic phenotype reported in the same organ of *ggtl* mutants may suggest a role of the GSH catabolism catalyzed by GGT1 in accumulating and transporting the γ-glutamyl amino acid residue (particularly γ-glutamyl sulfur amino acid) in leaf tissues. Later, the accumulated γ-glutamyl amino acids could be exported through phloem elements to the different sink tissues such as the developing siliques where it can be stored.

The above findings are totally in agreement with Lieberman *et al.* (1996). In that, the catabolism of GSH by *GGT* is a major source of circulating cysteine. In addition, other studies have suggested that *GGT* is involved in amino acid re-absorption and cysteine transport (Hanigan and Ricketts, 1993; Hanigan, 1995; Meister and Larsson, 1995). The biochemical analysis of the thiol levels in *ggtl* mutants revealed that in all the investigated organs, including the rosette leaves, cauline leaves, stems, flowers, young and mature siliques, there was no significant differences in the GSH levels in the *ggtl* mutants as compared to the wild type plants (Table 4). However, in the flower buds a significant decrease was recognized in the *ggtl* mutants compared to their controls. These data indicate that the disruption of *GGTI* gene has no obvious effect on the steady state levels of thiols. This includes the leaves, which are known to be the major source tissue for GSH synthesis. In the present study, the leaves of *Arabidopsis* plants had been reported to contain 80-90% of the γ-GTase activity encoded by *GGTI* gene. Moreover, the northern analysis has confirmed major existence of *GGT1* messages in the leaves.

These findings demonstrate no significant accumulation in GSH pool of the leaves, despite of, the dominant representative role of the GGT1 protein in the leaf tissues. This may suggest a compensatory role of GGT3 in these tissues. In fact, the northern analysis revealed a fairly major distribution of GGT1 messages throughout the leaves. Therefore, the accumulation of GSH in a specific tissue or cellular compartment that may result from the disruption *of GGTI* gene and the absence of GGTI protein could be manipulated. An intensive regulatory increase in *GGT3* gene expression and/or an increase in the activity rate of GGT3 protein could occur.

Table 4: Biochemical analysis of different thiols (cysteine, γ-glutamyl cysteine and glutathione) in the *ggt1* knockout mutants as compared to the wild type. The indicated organs were collected from 4-weeks old plants. Means with * are significantly different from the control wild type plants. $p>0.05$ indicates insignificant difference. $p\leq0.05$ indicates significant difference

Arabidopsis line / Plant organ	Cysteine concentration			t-test	
	wt.	*ggt 1.1*	*ggt 1.3*	wt. vs *ggt 1.1*	wt. vs *ggt 1.3*
Rosette leaves	0.023±0.00	0.028±0.00	0.026±0.00	0.367	0.242
Caulline leaves	0.023±0.00	0.038±0.00	0.030*±0.00	0.103	0.033
Flowers	0.136±0.00	0.146±0.01	0.133±0.00	0.378	0.464
Flower buds	0.103±0.00	0.093±0.00	0.097±0.02	0.109	0.675
Young silliques	0.120±0.00	0.138±0.01	0.117±0.01	0.096	0.715
Mature silliques	0.092±0.00	0.077±0.00	0.081±0.00	0.084	0.189
Stem	0.045±0.01	0.046±0.00	0.021±0.00	0.900	0.038
t-test γ-glutamyl cysteine concentration					
Rosette leaves	0.005±0.00	0.008±0.00	0.004±0.00	0.15	0.518
Caulline leaves	0.018±0.02	0.025±0.01	0.038±0.01	0.697	0.355
Flowers	0.49±0.01	0.047±0.01	0.040±0.00	0.788	0.387
Flower buds	0.104±0.00	0.084±0.00	0.071±0.02	0.889	0.551
Young silliques	0.105±0.00	0.139±0.00	0.084±0.01	0.064	0.608
Mature silliques	0.121±0.03	0.152±0.02*	0.115±0.00	0.031	0.193
Stem	0.031±0.01	0.032±0.00	0.016±0.00	0.923	0.119
t-test GSH concentration					
Rosette leaves	0.458±0.00	0.559±0.05	0.462±0.04	0.215	0.932
Caulline leaves	0.729±0.13	0.846±0.04	0.775±0.03	0.425	0.712
Flowers	1.890±0.15	1.908±0.03	1.849±0.01	0.899	0.77
Flower buds	2.581±0.14	2.277±0.03	1.778±0.11*	0.198	0.026
Young silliques	1.878±0.04	2.203±0.16	1.886±0.02	0.198	0.824
Mature silliques	1.418±0.16	1.369±0.13	1.312±0.03	0.765	0.509
Stem	0.928±0.03	0.925±0.00	0.650±0.11	0.882	0.15

In human, where *GGT* encodes by multigenes family, a mutation in one of the *GGT* genes may not completely inactivate GGT activity in all organs (Courtay *et al*., 1994; Lieberman *et al*., 1995).

In addition, one of the key clues, that can control the GSH biosynthesis and also can effectively regulate its degradation pathway, is the feedback inhibition mechanism that controls the activity of γ-glutamyl cysteine synthetase. γ-Glutamyl cysteine synthetase activity was blocked as a result to the increase in the GSH pool (Noctor *et al*., 1998). Therefore, any minor accumulation in the GSH pool as a result to the disruption of *GGT1* gene and the absence of GGT1 activity can direct the biosynthetic pathway to stop the de novo synthesis of GSH and maintain its homeostasis in the leaf tissues. Similarly, Storozhenko *et al*. (2002), who had over expressed an *Arabidopsis GGT* in the transgenic tobacco plants and revealed a higher γ-GTase activity in the transgenic plants than its controls, found that the steady state level of GSH in both the leaves of the transgenic plants and the control plants are the same. They have been attributed that to a compensatory increase in the rate of GSH synthesis to overcome the high GSH degradation rates caused by AtGGT overproduction. However, the data in this study refer to a little decline of the GSH level in the flower buds of the *ggt1* mutants as compared to the wild type plants. This indicates a fairly small existence of GGT1 protein in this organ. In fact, the mRNA expression pattern localized the GGT1 messages with a detectable level in the flower buds. Moreover, a little expression of *GGT2* gene was investigated in this organ as well.

The decrease in the GSH level in the *ggt*1 flower buds may indicate a role of GGTl protein in the activation of the de novo synthesis of GSH inside these cells. The flower buds are considered a major sink tissue that receives the GSH being synthesized in the leaves (source tissue) and exported through phloem elements. The exported GSH can undergo further degradation in the apoplastic space by the action of GGT1 protein, which allows γ-glutamyl amino acids and cysteine glycine dipeptides to be imported into the cell via ABC transporters on the plasma membrane (Storozhenko *et al*., 2002). The individual amino acids (glutamate, cysteine and glycine) that result from further processing of these dipeptides, can be involved in the GSH de novo synthesis. The block of GSH degradation via disruption of *GGT1* may cause a deficiency in the supply with the GSH individual amino acids component, therefore, the continuous processing of the cycle for further GSH transport and de novo synthesis will be blocked.

The data presented here are totally consistent with the previous results that screened γ-GTase activity and the RNA expression in *ggt2* mutants and the wild type plants. The expression pattern of *GGT2* was reported to be active in the developed siliques. The γ-GTase assay demonstrated 55-69% of the activity encoded by *GGT2* in either the young or the mature siliques. In addition, no existence of GGT2 messages was detected on the northern blot for the rosette leaves. In consequence, no activity of GGT2 protein in the rosette leaves has been reported. These data may suggest an important role for the GSH metabolism being catalyzed by *GGT2* gene in the developing siliques. The data may further indicate that the GSH degradation that occurs in the cell apoplast of these tissues could be blocked as a result of *GGT2* gene disruption. Consequently, an accumulation of GSH in the extracellular space could occur. Furthermore, this could prevent the continuous import of the GSH degraded components (γ-glutamyl amino acids and cysteine-glycine dipeptides) from the apoplastic space to the cytosol where they can undergo further metabolic processing to be integrated in the de novo GSH synthesis.

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