Localization of Members of the γ-Glutamyl Transpeptidase Family Identifies Sites of Glutathione and Glutathione S-Conjugate Hydrolysis[^W][OA]

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γ-Glutamyl transpeptidases (GGTs) are essential for hydrolysis of the tripeptide glutathione (γ-glutamyl-cysteine-glycine) and glutathione S-conjugates since they are the only enzymes known to cleave the amide bond linking the γ-carboxylate of glutamate to cysteine. In Arabidopsis thaliana, four GGT genes have been identified based on homology with animal GGTs. They are designated GGT1 (At4g39640), GGT2 (At4g39650), GGT3 (At1g69820), and GGT4 (At4g29210). By analyzing the expression of each GGT in plants containing GGT:β-glucuronidase fusions, the temporal and spatial pattern of degradation of glutathione and its metabolites was established, revealing appreciable overlap among GGTs. GGT2 exhibited narrow temporal and spatial expression primarily in immature trichomes, developing seeds, and pollen. GGT1 and GGT3 were coexpressed in most organs/tissues. Their expression was highest at sites of rapid growth including the rosette apex, floral stem apex, and seeds and might pinpoint locations where glutathione is delivered to sink tissues to supplement high demand for cysteine. In mature tissues, they were expressed only in vascular tissue. Knockout mutants of GGT2 and GGT4 showed no phenotype. The rosettes of GGT1 knockouts showed premature senescence after flowering. Knockouts of GGT3 showed reduced number of siliques and reduced seed yield. Knockouts were used to localize and assign catalytic activity to each GGT. In the standard GGT assay with γ-glutamyl-p-nitroanilide as substrate, GGT1 accounted for 80% to 99% of the activity in all tissues except seeds where GGT2 was 50% of the activity. Protoplasting experiments indicated that both GGT1 and GGT2 are localized extracellularly but have different physical or chemical associations.

Glutathione (GSH) is the most abundant nonprotein thiol in eukaryotic organisms and is one component of their response to environmental assaults. The mitigation of stress is accomplished in part by the reaction of GSH or GSH metabolites such as phytochelatins with environmental pollutants including reactive oxygen species, heavy metals, arsenic, selenium, pesticides, herbicides, and pharmaceuticals. Additionally, GSH serves as a redox buffer (Leustek et al., 2000) and it reacts with protein cysteinyl residues. Since the glutathionylation of Cys is reversible, it has the potential to modulate enzymatic activity in response to changing environmental conditions (Klatt and Lamas, 2000; Bick et al., 2001; Dixon et al., 2005). GSH also serves as substrate or cofactor in many housekeeping reactions. For example, it is involved in control of transcription.

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for Cys or Met, and they sequester and transport most of their nonprotein Cys in the form of GSH. In animals, the GGTs and MBDS that participate in recovery of Cys from GSH are highly expressed on the outer surface of the plasma membrane of organs with secretory or absorptive functions such as the kidney and small intestines. Although GGT and MB are sufficient for GSH hydrolysis, in many instances they operate as part of the γ-glutamyl cycle that in animals results in hydrolysis of GSH outside the cell followed by transport of the component amino acids or dipeptides into the cell for reutilization including the synthesis of GSH. The cycle is illustrated in Figure 1. In addition to membrane-anchored GGTs and MBs, the cycle consists of transporters for Gly, Cys, and γ-Glu amino acids, a cytosolic γ-glutamyl cyclotransferase, 5-oxoprolinase, γ-glutamyl-Cys synthetase, and glutathione synthetase. The γ-glutamyl cyclotransferase hydrolyzes γ-Glu amino acids that result from the GGT-catalyzed transpeptidation reaction forming an amino acid and 5-oxo-l-Pro, which is converted to Glu by 5-oxoprolinase. The lethality of the mouse GGT knockout and the ability to complement the knockout with N-acetyl Cys suggest that, at least in some instances, GSH cannot be imported or that GSH cannot be hydrolyzed intracellularly, and that delivery of Cys from an extracellular source is an essential function.

In animals, GGTs and MBs are also the first two steps in the catabolism of most metabolites of GSH. For example, glutathione S-conjugates of xenobiotics and pharmaceuticals are hydrolyzed to mercapturic acids for urinary excretion (Fig. 1). Members of the GGT and MB gene families also modulate the activity of signaling molecules such as leukotrienes and prostaglan-

**Figure 1.** The γ-glutamyl cycle as it operates in animals. Extracellular GSH is hydrolyzed by GGTs and MBs to amino acids and dipeptides. The resulting amino acids and dipeptides are transported into the cell, thus making them available for a wide range of functions including intracellular synthesis of GSH and protein synthesis. Intracellularly, GSH also serves as substrate for glutathione S-transferase-catalyzed conjugation reactions. Glutathione S-transferase conjugates of many xenobiotics are exported and hydrolyzed by GGTs and MBs prior to excretion.
has not been identified. Thus, it is not yet known whether a γ-glutamyl cycle operates in plants. GGTs are the only enzymes known to hydrolyze the γ-glutamyl bond in GSH. Hydrolysis occurs with release of Glu or transfer of Glu to an acceptor amino acid generating γ-Glu amino acid. In a number of eukaryotes and microorganisms, GGTs initiate GSH and glutathione S-conjugate hydrolysis. Animal GGTs have been extensively studied, and generally the proteins are composed of two subunits (a heavy, 55–60 kD, and a light, 21–30 kD form) originating from a single precursor that is cleaved during biogenesis. Several animal GGTs have been shown to be anchored at the N terminus of the heavy chain to the external surface of the plasma membrane (Meister et al., 1981; Meister, 1989). GGTs purified from tomato (Lycopersicon esculentum), onion (Allium cepa), and radish (Raphanus sativus) have also been shown to consist of heavy and light chains (Martin and Slovin, 2000; Shaw et al., 2005; Nakano et al., 2006b). Four putative GGTs have been identified in the Arabidopsis genome (Storozhenko et al., 2002). One of these (At4g39640) was overexpressed in tobacco and was shown to be processed from a precursor protein into a 41 kD large subunit and a 29 kD small subunit.

Herein we describe the characterization of the Arabidopsis GGT family. Toward that end, we establish the organ and tissue localization of each Arabidopsis GGT using GGT:GUS reporter constructs. The catalytic activity and in vivo function of each GGT was examined by analyzing TDNA insertion mutants.

RESULTS
The GGT Genes in Arabidopsis

Each of the four putative GGT genes previously identified in Arabidopsis (Storozhenko et al., 2002) has been assigned the gene symbol GGT at The Arabidopsis Information Resource Gene Symbol Registry, consistent with the nomenclature established for the mammalian, fungal, yeast (Saccharomyces cerevisiae), and bacterial GGTs. The Arabidopsis gene assignments correspond to the following locus identifiers: At4g39640 (GGT1), At4g39650 (GGT2), At1g69820 (GGT3), and At4g29210 (GGT4).

GGT Structures Are Conserved

GGT1 and GGT2 are located adjacent to each other on chromosome 4. Their intron-exon structures are perfectly conserved and their amino acid sequences exhibit 83% identity and 90% similarity (Figs. 2 and 3). GGT3, located on chromosome 1, has regions that are highly conserved with respect to GGT1 and GGT2. However, alignment of the three sequences shows that GGT3 lacks a segment encompassing exon 1, introns 1 and 2, most of exon 2, and all of exon 3 of GGT1/GGT2 (Figs. 2 and 3). As currently annotated, the GGT3 translational initiation site (at amino acid 62 of the protein sequence in Fig. 2) occurs at a position homologous with amino acid 319/315 of GGT1/GGT2. However, the high degree of homology of the region upstream of the annotated GGT3 translation initiation site with exon 2 of GGT1/GGT2 (81% identity with respect to GGT2) and the presence of two other upstream potential translational initiation sites suggests that the GGT3 might be incorrectly annotated and that the correct annotation might be as shown in Figures 2 and 3. The central region of GGT3 shows a conservation of exon/intron structure with GGT1/GGT2, and the amino acid sequence of this region exhibits 85% and 81% identity with GGT1/GGT2, respectively. At the 3' end of the GGT3 gene, a nonsense codon truncates 67 codons from the last exon. Yet, the downstream region is highly homologous with the 3' coding sequence of GGT1/GGT2, suggesting that the nonsense codon might be a sequencing error. However, our sequencing of the GGT3 gene in the Columbia (Col) ecotype confirmed the presence of the nonsense codon resulting in termination of the protein sequence with amino acid 252. A full-length cDNA sequence will be required to resolve the uncertainties about the actual structure of the GGT3 gene.

GGT4, located on chromosome 4, is divergent from the other three GGTs in sequence and intron/exon structure. The protein sequence identity is about 50% with respect to GGT1/GGT2. Exon 3 of GGT4 is highly homologous with exons 3, 4, and 5 of GGT1/GGT2, but exon 1 is not homologous to any segment of GGT1/GGT2 (Figs. 2 and 3). Despite the sequence divergence, all four Arabidopsis GGTs have conserved regions corresponding to the catalytic domain and the site for proteolytic processing into large and small subunits of animal GGTs (noted on Fig. 2).

All Arabidopsis GGTs Are Transcribed

cDNA clones were obtained for GGT1, GGT2, and GGT4, demonstrating that they are functional genes. Although we and others (http://signal.salk.edu/csearch) have been unable to obtain a cDNA clone for GGT3, low level expression of this gene has been detected in numerous microarray experiments. Selected data from Genevestigator (Zimmermann et al., 2004; https://www.genevestigator.ethz.ch/at/index) comparing expression of GGT1, GGT2, GGT3, and GGT4 in organs, tissues, or developmental stages are shown in Figure 4, A and B. In most tissues, the expression of GGT1 and GGT4 was 5- to 10-fold higher than the expression level of GGT2 and GGT3. Only in pollen and siliques (ovary wall plus seeds) was GGT2 mRNA at a level comparable to GGT1 and GGT4.

GGTs Are Coexpressed in Most Organs and Tissues

The temporal and spatial pattern of expression of each GGT was mapped by creating stable Arabidopsis transformants that harbored a construct containing 700 to 1,000 bp upstream of the translation initiation
**Figure 2.** Sequence homology of the Arabidopsis GGT gene family. The coding sequences of the four GGT genes were aligned by Clustal W with the GCG program PileUp. Regions of identity in at least three of the four sequences are shaded black. Gaps introduced into the sequences are indicated by dots. The inverted triangles indicate the position of introns in the sequences of GGT1, -2, and -3. The GGT1 and GGT2 sequence begins with exon 2 since exon 1 encodes zero or two amino acids, respectively. GGT3 lacks sequence corresponding to GGT1 and GGT2 exons 1 and 3 and most or all of exon 2, depending on the location of the initiation site(s). Additionally, the GGT3 sequence actually has a single nucleotide mutation (TGG to TAG) that results in the amino acid at position 253 (W) becoming a stop codon. The W was included simply to illustrate that the translated GGT3 sequence downstream of the W has high homology with GGT1 and GGT2. The arrow indicates a predicted signal peptide cleavage site of GGT1, -2, and -3. Triangles pointing upward indicate the position of introns in the GGT4 sequence. The predicted signal peptide cleavage site of GGT4 protein is at the position of the first intron. Based on homology with mouse and human GGT sequences, a conserved cleavage site of the precursor protein is predicted to be between amino acids 372 and 373 on the GGT1 sequence, and substrate-binding sites are predicted to be at amino acids 414 to 417 and 443 to 446.

**Table 1.** Site plus the entire GGT coding sequence with GUS fused in frame at the C terminus.

GGT1 driven GUS activity is shown in Figure 5. GUS activity was evident within 2 to 3 d after transfer of stratified seeds from 5°C to growth conditions. The highest activity was initially localized exclusively in the vascular tissues of the hypocotyl and cotyledons and at the shoot apex of 3- to 4-d-old plants (A), but by 5 d intense staining of the entire seedling was observed (data not shown). In roots, the highest GUS activity was observed in the elongation zone and the vascular bundle (data not shown). The temporal
changes in GUS expression in rosette leaves indicates that GGT1 expression is under developmental control. The highest activity was observed at the shoot apex over the entire surface of expanding leaves. Magnification of the youngest leaves revealed intense GUS activity particularly at the base of immature trichomes (B). GUS expression was not evident around mature trichomes. As leaves matured, GUS activity declined in mesophyll cells and was restricted to the major veins (C). At the point of inflorescence emergence, GUS activity was almost undetectable in the leaves of the rosette (data not shown), but was very high in the expanding cauline leaves, the floral stem, and the rosette stem (D). As seed fill began, GUS activity was much higher at the apex (F) of the floral stem than at the base (E). GUS expression in the rosette of mature flowering plants was restricted to the vascular tissue at the base of the leaf and the stem (G). High GUS activity was present in roots throughout vegetative growth (H), but dropped appreciably after the onset of reproductive development (data not shown). GUS activity was detected in sepal, petal, and stamen of flowers from bud formation (I) through maturity (J) and senescence (data not shown). In mature and senescing tissues activity was restricted to vascular tissue. GUS activity in the anther peaked after pollen maturation and persisted through senescence, but was not detected in the pollen itself. GUS activity was present in the ovary (silique) wall before fertilization through to senescence (J and K). In senescent siliques, GUS activity was restricted to the vascular tissue. The embryo (L), but not the endosperm or seed coat, of mature green seeds showed GUS activity.

The GGT3:GUS expression patterns in Figure 6 clearly show that the GGT3 gene has a functional promoter capable of driving GUS expression. It should be noted that control plants transformed with the GUS gene alone showed no activity. Furthermore, the temporal and spatial expression of GGT3 is nearly indistinguishable from GGT1 except that the level of expression is considerably lower. Whereas GGT1:GUS expression was detected after 6 to 8 h of reaction with substrate, 18 to 24 h was required for optimum detection of GGT3:GUS expression. The assessment of relative expression levels of GGT3:GUS and GGT1:GUS is based on a preliminary screen of 18 independent transgenic lines for each of the constructs and thus is not a function of insertion site. The lower level of GGT3:GUS expression observed here is supported by microarray data assembled using Genevestigator (Fig. 4) and Arabidopsis eFP Browser (http://bbc.botany.utoronto.ca/efp/) and the failure to detect GGT3 expression by reverse transcription (RT)-PCR analysis. With few exceptions, microarray analysis and GUS analysis were in agreement. One exception was pollen and anthers. GGT1:GUS activity, but not GGT3:GUS

Figure 3. Genomic structure of each GGT. Exons are shown as boxes, introns as lines, and TDNA insertion sites as triangles. Dotted lines are drawn to show the relationship between genes. Localization and orientation of TDNA or transposon insertions into each gene are illustrated on the left. Insertion line number, insertion site, and partial sequence obtained in confirmation of insertion site are shown on the right. For Exotic and Cold Spring Harbor Laboratory (CSHL) lines minus strand sequences obtained using the Ds3 primer are shown. Gels of RT-PCR products show the absence of gene-specific transcript in 20 d rosettes of both knockout lines of GGT1 and GGT4. RT-PCR analysis also shows the absence of gene-specific transcripts in green cotyledon stage siliques of both GGT2 knockout lines and elevated GGT2 transcript in the Sail_1161D03 knockup line with a TDNA insert 122 bp upstream of the GGT2 translation initiation site.
activity, was detected in anthers. Yet microarray data showed expression of both GGT1 and GGT3 in both anthers (data not shown) and pollen (Fig. 4). The failure to detect GUS expression in pollen of GGT1/GGT3 may be due to a failure to select the proper conditions for low and/or very transient expression.

GGT2:GUS was highly expressed only at three sites: immature trichomes, pollen, and the seed (Fig. 7). In the case of trichomes and pollen, expression was observed only in a narrow developmental window. GUS activity in developing trichomes was evident on the first expanding leaf (data not shown) and on subsequent rosette leaves as they expanded (A). GUS activity was absent in mature trichomes. Staining of immature trichomes was also evident on the floral stem at the onset of bolting (data not shown). On first examination, GGT2:GUS expression showed little overlap with the other GGTs. However, longer GUS reaction times (up to 36 h) revealed a very low level of GGT2:GUS expression in the vascular tissue of expanding leaf (data not shown), floral stems (B), and in root (C) similar to what would be observed for GGT1:GUS and GGT3:GUS expression with 1 to 2 h reaction times. Intense GUS staining of pollen, but not the anther itself, appeared transiently at the time of dehiscence and can be clearly seen by enlarging photograph E. Since the developmental window of expression is narrow (the time of dehiscence), expression could be detected simultaneously in only one or two flowers in a cluster (D). GGT2:GUS activity was detected in unfertilized ovules (G) and in embryos during the entire embryonic period (data not shown). Isolated torpedo and curled embryos expressing GUS activity are shown (F). The endosperm and integument with the embryo removed also exhibited intense staining (H). Unlike GGT1 and GGT3, GGT2:GUS staining was not detected in the ovary (silique) wall of full-size green siliques (I).

GGT4:GUS activity was expressed in most tissues throughout vegetative and reproductive growth. Examples are shown in Figure 8. They include the cotyledons of 4-d-old germinating seedlings (A). GUS activity was highest in roots during the vegetative stage of growth but decreased after the onset of reproductive growth. The roots of a 14-d-old plant are shown (Fig. 8B). Expression was absent from the root tip. GUS was expressed in all regions of the rosette throughout development showing relatively little tissue specificity. However, with short GUS reaction times, it was evident that the highest activity was in the expanding apical leaves and in veins of the leaves and stem (C and D, enlarged view). Unlike the other GGTs, the GGT4-GUS expression was not detected in trichomes. Like GGT1:GUS and GGT3:GUS expression, expression of GGT4:GUS was observed in the stem and rosette base at the time of bolting (E). GGT4:GUS activity was present in the floral stem and all flower parts (F).

Figure 4. Expression data for GGT genes (A) in vegetative tissues and in reproductive structures (B). Data were retrieved from the Genevestigor Data Mining site (https://www.genevestigor.etha.ch).

Analysis of Knockout Mutants Reveals Localization and Catalytic Activity of GGTs

Two independent TDNA or transposon insertion mutants were obtained for each of the four GGT genes. The results of PCR amplification and sequencing to verify insertion site are summarized in Figure 3. For each mutant line, the TDNA/transposon insertion is in an exon near the 5′ end or near the sequence encoding the putative catalytic domain of the enzyme, thus assuring complete knockout of function. Each mutant allele was isolated in the homozygous state, confirmed by segregation of antibiotic resistance, genotyping by PCR, and the absence of detectable mRNA for the GGT1, GGT2, and GGT4 alleles. GGT3 mRNA was not
detectable using the RT-PCR method. The viability of homozygous mutants of each GGT indicated that none of the GGT genes alone is essential.

Since GGTs were previously shown to consist of soluble and particulate associated isoforms in plants, the availability of the Arabidopsis mutants provided the opportunity to assess which genes give rise to the different isoforms. The 10 different algorithms for prediction of subcellular localization assembled at the SubCellular Proteomic Database (http://www.plantenergy.uwa.edu.au/application/suba/flatfile/) were used to predict the localization of each GGT (Supplemental Table S1). The results were conflicting or inconclusive, emphasizing the need for experimental assessment of protein localization (Heazlewood et al., 2005, 2007).

All of the GGT activity from onion bulbs and tomato fruit, and 95% of the activity from radish cotyledons was reported to pellet following low speed centrifugation (10,000–20,000 g) and was quantitatively released from the pellet with 1 M NaCl (Martin and Slovin, 2000; Shaw et al., 2005; Nakano et al., 2006b). These results, plus the failure to recover GGT activity from the pellet fractions of tomato fruit using detergents such as Triton X-100 (M.N. Martin, unpublished data) were interpreted to mean that GGT is bound to a pelleted fraction via an ionic association as might be expected for a protein associated with the cell wall rather than a membrane. In addition, Storozhenko et al. (2002) reported that 0.5 M NaCl was required to extract or solubilize Arabidopsis GGT1 when it was expressed in transgenic tobacco. They further showed using confocal imaging of tobacco leaf discs infiltrated with the artificial GGT substrate, ω-glutamyl-7-amido-4-methylcoumarin, that the fluorescent product resulting from hydrolysis of this compound accumulated outside the plasma membrane (Storozhenko et al., 2002). However, with an overexpression strategy there is danger that the protein was mistargeted.

Analysis of ggt1-1 and ggt1-2 revealed that greater than 95% of the total wild-type GGT activity was lost from the rosettes of these mutants. The data for ggt1-1 are shown in Table I. In the wild-type plants, 80% or more of the activity attributed to GGT1, depending on tissue age, pelleted upon low speed centrifugation. Repeated grinding and reextraction of the pellet either without (method 1) or with Triton X-100 (method 2) did not significantly increase the recovery of activity in the soluble fraction (Table I). It should be noted that solubilization of membranes with Triton X-100 was so complete that the pellet fraction was almost devoid of green color. By contrast, 1 M NaCl quantitatively released the GGT activity from the pellet. Together, these results suggest that GGT1 is bound via an ionic association. The activity released by NaCl was not pelleted by centrifugation at 100,000 g for 1 h, suggesting that it

![Figure 5](image_url)

Figure 5. Histochemical localization of GGT1 activity in plants expressing a GGT1:GUS fusion. A, GUS activity was highest at the shoot apex and in the vascular tissue of the hypocotyl and cotyledons of a 4-d-old seedling grown on one-half strength Murashige and Skoog medium. B, Enlargement of the shoot apex of an 11-d-old seedling where GUS expression was strongest showed that activity was particularly high at the base of immature trichomes. C, 11-d-old seedling showed differential, age-dependent staining of leaves. Expression was high at the rosette apex but absent in the older leaves or was restricted to the major veins. D, GUS activity was high in the floral shoot of 27-d-old plants at bolting. Cross sections of an elongated floral stem at the base (E) and apex (F), respectively, showed staining was strongest at the apex. G, A cross section through a leaf and the stem at the base of a mature rosette showed activity only in the vascular tissue. H, GUS activity was present throughout roots of mature plants. I, Floral buds and nearly all parts (J) of mature flowers showed GUS activity. K, The pistil stained prior to fertilization and the ovary (sique) wall (K) stained at all stages after fertilization. L, Removal of the seed coat showed GUS activity in the mature embryo. All GUS reactions were for 8 to 12 h.
is not associated with microsomal membranes, which pellet under this ultracentrifugation condition. Of the small amount of soluble GGT1, 50% or more pelleted after ultracentrifugation at 100,000g even in the presence of Triton X-100, suggesting that the soluble GGT is a mixed population of protein consisting of soluble and particulate associated forms, perhaps bound to small cell wall fragments. Further work will be needed to establish whether soluble GGT1 exists within cells or is generated by proteolytic activity during extraction.

GGT2 activity is below the detection limit of the assay in leaves and most other tissues of Arabidopsis. However, one insertion line (Sail_1161D03) with a T-DNA positioned 122 bp upstream of the first translation initiation site of GGT2 showed a 2- to 50-fold elevation in total GGT activity depending on the age of the plant. Fractionation of GGT activity in 16-d-old rosettes of the knockup mutant compared to a wild-type sibling showed a 4-fold increase in GGT activity in the soluble fraction and less than a 20% increase in the pelleted...
fraction, suggesting that GGT2 may not be associated with a particulate fraction and that GGT1 and GGT2 are differently localized (Table I). Approximately 50% of the soluble or Triton X-100 extracted GGT activity from the knockup pelleted upon ultracentrifugation at 100,000 g for 1 h, suggesting that this isoform also is a mixed population, as was noted for GGT1. The possibility cannot be discounted that ectopic overexpression of GGT2 might have caused spillover into either the soluble or the pellet fraction.

When protoplasts were isolated from wild type, ggt1-1, and ggt2-knockup rosette leaves only 5% to 10% of the total GGT activity was recovered with the protoplasts in each line. This suggests that GGT1 and GGT2 are neither intracellular soluble proteins nor plasma membrane-bound proteins (Table II). Rather they are localized extracellularly and are lost upon digestion of the cell wall. GGT2 activity was recovered primarily in the protoplasting buffer cleared of protoplasts by centrifugation. However, GGT1 activity was almost exclusively associated with the residual cell debris remaining from cell wall digestion even after lengthy digestion. GGT1 was quantitatively released from that fraction with 1 M NaCl.

In light of the possibility that GGT2 was mistargeted in the ggt2 knockup, localization of GGT1 and GGT2 were examined in full-size cotyledon stage green seeds. Microarray analysis showed that green siliques (ovary wall plus seeds) are one of the few tissues where both GGTs are highly expressed (Fig. 4). Furthermore, GUS expression analysis showed that GGT1, -2, and -3 are expressed in cotyledon stage green seeds. Extraction of green seeds from wild type, ggt1-1, and ggt2-1 resulted in the recovery of almost no GGT activity in the supernatant following low speed centrifugation (Table III, method 1). Addition of 1% (v/v) Triton X-100 to the extraction buffer (Table III, method 2) resulted in the recovery of approximately 50% of the total seed GGT activity in the supernatant. None of the activity in the Triton X-100 extract was knocked out in ggt1-1 seeds, but all of it was eliminated from ggt2-1 seeds. The effect of Triton X-100 on extractability suggests that GGT2 is membrane bound or soluble but associated with storage bodies. Repeated reextraction of the pellet fraction highlighted the challenge of quantitatively

![Figure 8. Histochemical localization of GUS activity in plants expressing a GGT4:GUS fusion protein. A, Activity was present in cotyledons of germinating seedlings. Roots (B) and rosette (C) of 16-d-old seedling grown on one-half-strength Murashige and Skoog + Suc showed GUS activity. The inset shows strongest staining on expanding leaves at the shoot apex. E, Upper root and stem of a 34-d-old plant stained at the time of bolting. F, All flower parts and the floral stem showed GUS activity.](image)

Table I. GGT1 and GGT2 are differentially extracted from Arabidopsis rosettes

| Extraction No. | Additions to Buffer | Total GGT Activity Recovered in Low Speed Supernatants | Method 1 | Method 2 |
|---------------|---------------------|-------------------------------------------------------|----------|----------|
|               |                     | Wild-Type Segregant ggt1-1                            | Wild-Type Segregant ggt2 Knockup |
| 1             | None                | 36 ± 3                                                | 2 ± 1    | 42 ± 3   | 158 ± 6 |
| 2             | None                | 3 ± 2                                                 | 1 ± 1    | 2 ± 2    | 8 ± 4   |
| 3             | None                | 0                                                     | 0        | 0        | 0       |
| 4             | 1 M NaCl            | 126 ± 5                                               | 0        | 119 ± 4  | 144 ± 5 |
| 5             | 1 M NaCl            | 40 ± 3                                                | 0        | 38 ± 2   | 46 ± 2  |
|               |                     |                                                       |          |          |         |
| 1             | Triton X-100        | 35 ± 3                                                | 3 ± 1    | 50 ± 4   | 140 ± 7 |
| 2             | Triton X-100        | 2 ± 1                                                 | 1 ± 1    | 2 ± 1    | 7 ± 4   |
| 3             | Triton X-100        | 0                                                     | 0        | 0        | 0       |
| 4             | 1 M NaCl            | 124 ± 3                                               | 0        | 114 ± 4  | 142 ± 2 |
| 5             | 1 M NaCl            | 32 ± 2                                                | 0        | 40 ± 2   | 44 ± 3  |

*GGT activity was measured as described using γ-GPNA as substrate. Statistical treatment of data is described in “Material and Methods.”

Rosettes of 16-d-old plants were ground with a pestle in a microtube tube and extracted three times by one of two methods: method 1 with buffer A alone or method 2 with buffer A containing 1% (v/v) Triton X-100 using a volume of buffer equal to 2 times the original tissue weight for each extraction. Pellets from the same tissues were subsequently extracted a fourth and fifth time with buffer B containing 1 M NaCl and using a volume equal to 4 times the original tissue weight. Tissue was incubated for 30 min at 5°C after each grinding and extraction prior to centrifugation for 5 min at 13,000g.
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Table II. GGT1 and GGT2 activities were not recovered with leaf mesophyll protoplasts

| Fraction                     | Wild-Type Segregant | ggt1-1 | Wild-Type Segregant | ggt2 Knockup |
|------------------------------|---------------------|--------|---------------------|--------------|
| Untreated leaf<sup>a</sup>    | 420 ± 12            | 25 ± 2 | 395 ± 23            | 743 ± 34     |
| Intact protoplasts<sup>b</sup> | 16 ± 4              | 2.0 ± 2| 15 ± 5              | 27 ± 3       |
| Broken protoplasts           | 17 ± 3              | 2.0 ± 1| 15 ± 3              | 30 ± 4       |
| Protoplast buffer, 60g       | 29 ± 3              | 15 ± 3 | 33 ± 4              | 305 ± 24     |
| Protoplast buffer, 13,000g   | 30 ± 2              | 16 ± 1 | 35 ± 5              | 299 ± 15     |
| Undigested debris            | 15 ± 4              | 1.0 ± 2| 6 ± 2               | 30 ± 4       |
| Undigested debris, NaCl extract | 328 ± 10          | 0      | 332 ± 22            | 343 ± 15     |

<sup>a</sup>The youngest leaves were harvested immediately prior to protoplasting and protein extracted for GGT activity measurement as described in “Materials and Methods.” 
<sup>b</sup>Leaf tissue was harvested and incubated for 5 h with protoplasting buffer as described in “Material and Methods.” Protoplasts and buffer were separated by centrifugation at 60g. Protoplasting buffer, cleared of protoplasts, was assayed for GGT activity before and after centrifugation at 13,000g. Undigested cell wall debris was extracted twice with buffer A and then twice with buffer B containing 1 M NaCl.

Table III. GGT1 and GGT2 activities fractionate differentially from green seeds

| Extraction No.<sup>a</sup> | Additions to Buffer | Total GGT Activity Recovered | Wild-Type Segregant | ggt1-1 | Wild-Type Segregant | ggt2 Knockup |
|-----------------------------|---------------------|------------------------------|---------------------|--------|---------------------|--------------|
| Method 1                    |                     |                              |                     |        |                     |              |
| 1                           | None                | 14 ± 4                       | 11 ± 3              | 12 ± 3 | 0                   |              |
| 2                           | None                | 34 ± 2                       | 35 ± 2              | 25 ± 3 | 0                   |              |
| 3                           | None                | 21 ± 3                       | 19 ± 2              | 22 ± 2 | 0                   |              |
| 4                           | None                | 12 ± 2                       | 13 ± 2              | 12 ± 3 | 0                   |              |
| 5                           | 1 M NaCl            | 114 ± 5                      | 14 ± 3              | 129 ± 5| 111 ± 9             |              |
| 6                           | 1 M NaCl            | 50 ± 2                       | 12 ± 3              | 66 ± 4 | 63 ± 4              |              |
| Method 2                    |                     |                              |                     |        |                     |              |
| 1                           | Triton X-100        | 456 ± 29                     | 460 ± 15            | 373 ± 13| 0                  |              |
| 2                           | Triton X-100        | 150 ± 6                      | 162 ± 10            | 108 ± 4| 0                  |              |
| 3                           | Triton X-100        | 67 ± 4                       | 68 ± 4              | 52 ± 3 | 0                  |              |
| 4                           | Triton X-100        | 22 ± 2                       | 19 ± 2              | 29 ± 3 | 0                  |              |
| 5                           | 1 M NaCl            | 327 ± 4                      | 24 ± 2              | 349 ± 5| 363 ± 8             |              |
| 6                           | 1 M NaCl            | 105 ± 1                      | 16 ± 2              | 111 ± 6| 103 ± 4             |              |

<sup>a</sup>Full-size green seeds at the cotyledon stage were extracted four times by one of two methods: method 1 with buffer A alone or method 2 with buffer A containing 1% (v/v) Triton X-100 and using a volume of buffer equal to 8 times the original tissue weight for each extraction. The pellets were subsequently extracted two additional times with buffer B containing 1 M NaCl and using a volume equal to 12 times the original tissue weight. Tissue was incubated 30 min at 5°C with each buffer prior to centrifugation. 
<sup>b</sup>GGT activity measurement using γ-GPNA as substrate and data analysis are described in “Material and Methods.”
in seeds and ovary walls was a larger percent of the GGT activity in the Triton X-100-solubilized fraction (Table V). While GGT2 was responsible for 100% of the soluble/Triton-solubilized activity in seeds, GGT1 and GGT4 appeared to account for nearly equal percentages of the Triton X-100 extracted activity in ovary walls and flower buds. For both GGT1 and GGT4 the soluble/Triton-solubilized activities were at the detection limit of the assay. In all tissues, GGT1 was responsible for all activity in the pellet fraction.

Genevestigator analysis and GGT:GUS expression profiling showed that GGT1, GGT3, and GGT4 are expressed in nearly all tissues. In fact, both our RT-PCR results (data not shown) and Genevestigator analysis showed that the expression of GGT4 is comparable to or higher than GGT1 in many tissues. Yet, nearly all activity was eliminated with knockout of GGT1 and GGT2 leading to the conclusion that GGT3 and GGT4 are catalytically inactive or minimally active in the standard assay with γ-GPNA as substrate.

To further evaluate substrate specificity for each GGT, the activities of mutant lines and the corresponding wild-type segregants were measured using a range of γ-glutamyl donor substrates. GGT activity in green seed extracts using GSH as the γ-glutamyl donor and 1-amino cyclopropane-1-carboxylic acid (ACC) as the γ-glutamyl acceptor is shown in Figure 9. As when using γ-GPNA as γ-glutamyl donor (Table III), all of the activity in the Triton X-100-solubilized protein extract was eliminated in the ggt2-1 mutant and all of the NaCl-solubilized activity was eliminated in the ggt1-1 mutant. Identical results were obtained using S-(p-nitrobenzyl) glutathione or S-decyl glutathione as γ-glutamyl donors (data not shown) showing that both GGT1 and GGT2 hydrolyze GSH and several conjugates of GSH. With the GGT3 and GGT4 knockout lines and corresponding wild-type segregants, no GGT4 or GGT3 activity was observed in either fraction. The conclusion from these experiments is that GGT1 and GGT2 can use a number of γ-glutamyl donors (data not shown) showing that both GGT1 and GGT2 hydrolyze GSH and several conjugates of GSH. With the GGT3 and GGT4 knockout lines and corresponding wild-type segregants, no GGT4 or GGT3 activity was observed in either fraction.

Growth of GGT Knockouts Is Not Impaired on GSH as Sole Sulfur Source

To assess the phenotype of the GGT mutants, their germination and growth was compared with wild-type

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**Table IV. GGT1 is responsible for most of the γ-GPNA-dependent activity in several tissues**

| Tissue | Insertion Line | GGT Activity | 1 μM NaCl Extracted |
|--------|---------------|--------------|---------------------|
|        | Wild-Type Segregant | Mutant | Wild-Type Segregant | Mutant |
| Root   | ggt1-1 | 9 ± 3 | 9 ± 3 | 41 ± 1 | 0 |
|        | ggt1-2 | 10 ± 2 | 9 ± 1 | 37 ± 3 | 0 |
|        | ggt2-1 | 10 ± 1 | 10 ± 1 | 33 ± 1 | 33 ± 3 |
|        | ggt2-2 | 11 ± 2 | 10 ± 1 | 37 ± 4 | 42 ± 2 |
|        | ggt3-1 | 15 ± 3 | 16 ± 4 | 55 ± 7 | 60 ± 5 |
|        | ggt3-2 | 18 ± 4 | 16 ± 3 | 60 ± 4 | 57 ± 3 |
|        | ggt4-1 | 19 ± 4 | 12 ± 3 | 75 ± 4 | 77 ± 5 |
|        | ggt4-2 | 16 ± 3 | 11 ± 4 | 63 ± 2 | 60 ± 3 |
| Rosette| ggt1-1 | 38 ± 4 | 4 ± 1 | 180 ± 10 | 0 |
|        | ggt1-2 | 35 ± 3 | 5 ± 3 | 165 ± 8 | 0 |
|        | ggt2-1 | 37 ± 5 | 36 ± 3 | 175 ± 18 | 170 ± 9 |
|        | ggt2-2 | 33 ± 4 | 35 ± 6 | 170 ± 5 | 176 ± 6 |
|        | ggt3-1 | 40 ± 3 | 38 ± 5 | 210 ± 15 | 208 ± 11 |
|        | ggt3-2 | 37 ± 4 | 36 ± 3 | 220 ± 12 | 218 ± 15 |
|        | ggt4-1 | 35 ± 4 | 32 ± 5 | 230 ± 13 | 234 ± 14 |
|        | ggt4-2 | 37 ± 5 | 34 ± 3 | 205 ± 15 | 199 ± 18 |
| Stem   | ggt1-1 | 6 ± 2 | 4 ± 1 | 101 ± 3 | 18 ± 2 |
|        | ggt1-2 | 7 ± 3 | 3 ± 2 | 98 ± 7 | 12 ± 5 |
|        | ggt2-1 | 6 ± 2 | 5 ± 2 | 95 ± 4 | 94 ± 5 |
|        | ggt2-2 | 7 ± 3 | 7 ± 2 | 105 ± 8 | 99 ± 6 |
|        | ggt3-1 | 4 ± 1 | 6 ± 2 | 73 ± 4 | 68 ± 2 |
|        | ggt3-2 | 5 ± 2 | 6 ± 3 | 68 ± 5 | 65 ± 3 |
|        | ggt4-1 | 5 ± 2 | 2 ± 2 | 58 ± 1 | 70 ± 2 |
|        | ggt4-2 | 6 ± 3 | 3 ± 2 | 63 ± 3 | 65 ± 5 |

*Rosettes were harvested from 16- to 18-d-old soil-grown plants. Entire primary stems with all leaves, flowers, and siliques removed were harvested from plants 6 to 8 d after bolting. Roots were harvested from 14-d-old plants grown on half-strength Murashige and Skoog media supplemented with 1% Suc. Tissues were extracted three times with buffer A containing 1% Triton X-100 using volumes equal to 2 times the original tissue weight for each extraction. Pellets were subsequently extracted a fourth and fifth time with buffer B containing 1 M NaCl and using a volume equal to 4 times the original tissue weight. Tissue was incubated 30 min at 5°C with each buffer prior to centrifugation. Mutants of GGT1 and GGT2 are in the Col background while the GGT3 and GGT4 mutants are in the Landsberg background. GGT activity was measured using γ-GPNA as substrate and data was analyzed as described in "Materials and Methods."
segregants on one-half-strength Murashige and Skoog media containing GSH or sulfate as a sole sulfur source or on medium without sulfur. None of the mutants were impaired in germination rate, percent germination, or growth for up to 14 d on either sulfur-containing medium. Sulfate and GSH supported the growth of wild type and mutants equally well, indicating that the mutants are not impaired in the ability to use GSH as a sole sulfur source. It should be noted that nonenzymatic hydrolysis of GSH was not detected by HPLC, which, if it had occurred, would have bypassed the need for GGT activity. Seedlings of wild-type and mutant lines began showing the symptoms of sulfur starvation only after 5 d of growth on sulfur-free medium, indicating that sulfur reserves in the seeds were adequate for germination growth up to 5 d. The fact that GGT mutants performed like wild type on sulfur-free medium indicated that they are not impaired in either the storage of sulfur or the mobilization of stored sulfur.

Both \textit{ggt1} and \textit{ggt3} Show Altered Phenotypes

Phenotypic assessment of the GGT mutants revealed that both \textit{ggt1}-1 and \textit{ggt1}-2 showed premature leaf senescence. Both alleles appeared similar to wild type until the flowering stage of development. When the plants began to form seeds, the rosette leaves of the mutants began to yellow and rapidly senesce (Fig. 10). All progeny of \textit{ggt1}-1 \times \textit{ggt1}-2 crosses showed the same phenotype, indicating that the two mutations are allelic. The other GGT mutants did not show premature leaf senescence, indicating that GGT1 has a unique function that is not complemented by another GGT gene. Measurement of GSH, Cys, or Cys-Gly content in all tissues, including isolated ovary walls and seeds at several developmental stages, did not reveal any major changes in the GGT1 mutants. The metabolite analysis did not, therefore, support the idea that premature leaf senescence is related to a major perturbation of GSH metabolism. Despite the premature death of rosette leaves, flowering ceased only a few days earlier than wild-type plants, and the total seed yield was not significantly reduced by the absence of GGT1 activity. Both \textit{ggt3} alleles showed a postflowering phenotype. The mutants did not show growth aberration prior to flowering and they flowered at the same time as wild type. However, the mutants showed a reduction in the height of the inflorescence (Fig. 10). Flowering ceased 5 to 7 d sooner than wild type and resulted in a 30% reduction in seed yield. Failure of the other GGT mutants to show this phenotype, indicates that

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**Table V. GGT activity measurements in wild-type and mutants show that GGT1 and GGT2 are responsible for all γ-GPNAdependent activity in reproductive organs**

| Tissue | Insertion Line | GGT Activity\(^b\) Soluble and Triton Extracted | GGT Activity\(^b\) 1 M NaCl Extracted |
|--------|----------------|-----------------------------------------------|----------------------------------------|
|        |                | Wild-Type Seg | Mutant | Wild-Type Seg | Mutant |
| Flower buds | \textit{ggt1}-1 | 15 ± 2 | 8 ± 3 | 157 ± 4 | 24 ± 3 |
|           | \textit{ggt1}-2 | 15 ± 3 | 7 ± 2 | 165 ± 3 | 20 ± 2 |
|           | \textit{ggt2}-1 | 17 ± 3 | 17 ± 2 | 200 ± 10 | 226 ± 5 |
|           | \textit{ggt2}-2 | 16 ± 2 | 17 ± 3 | 175 ± 5 | 177 ± 4 |
|           | \textit{ggt3}-1 | 16 ± 3 | 18 ± 4 | 168 ± 4 | 165 ± 5 |
|           | \textit{ggt3}-2 | 15 ± 4 | 16 ± 3 | 166 ± 12 | 157 ± 4 |
|           | \textit{ggt4}-1 | 16 ± 4 | 6 ± 3 | 152 ± 6 | 144 ± 7 |
|           | \textit{ggt4}-2 | 15 ± 3 | 5 ± 2 | 167 ± 5 | 156 ± 6 |
| Green seeds | \textit{ggt1}-1 | 704 ± 59 | 742 ± 45 | 482 ± 17 | 45 ± 5 |
|           | \textit{ggt1}-2 | 650 ± 49 | 625 ± 57 | 515 ± 37 | 38 ± 4 |
|           | \textit{ggt2}-1 | 545 ± 52 | 0 | 518 ± 11 | 529 ± 20 |
|           | \textit{ggt2}-2 | 625 ± 45 | 0 | 498 ± 25 | 513 ± 35 |
|           | \textit{ggt3}-1 | 545 ± 55 | 554 ± 47 | 727 ± 22 | 715 ± 38 |
|           | \textit{ggt3}-2 | 525 ± 66 | 535 ± 57 | 695 ± 19 | 702 ± 15 |
|           | \textit{ggt4}-1 | 313 ± 34 | 379 ± 31 | 629 ± 29 | 621 ± 33 |
|           | \textit{ggt4}-2 | 490 ± 57 | 476 ± 48 | 688 ± 21 | 695 ± 37 |
| Ovary wall | \textit{ggt1}-1 | 21 ± 4 | 16 ± 3 | 28 ± 2 | 3 ± 2 |
|           | \textit{ggt1}-2 | 19 ± 3 | 15 ± 2 | 30 ± 4 | 2 ± 1 |
|           | \textit{ggt2}-1 | 23 ± 5 | 17 ± 3 | 26 ± 2 | 28 ± 2 |
|           | \textit{ggt2}-2 | 21 ± 3 | 15 ± 4 | 25 ± 1 | 26 ± 3 |
|           | \textit{ggt3}-1 | 29 ± 4 | 32 ± 3 | 54 ± 2 | 53 ± 5 |
|           | \textit{ggt3}-2 | 32 ± 3 | 31 ± 4 | 56 ± 3 | 54 ± 3 |
|           | \textit{ggt4}-1 | 35 ± 4 | 20 ± 5 | 62 ± 3 | 52 ± 4 |
|           | \textit{ggt4}-2 | 32 ± 5 | 20 ± 4 | 61 ± 4 | 60 ± 3 |

\(^a\)Seeds and ovary walls from siliques with full-size green cotyledon stage embryos were separated for assay. Flower buds and ovary walls were extracted three times with buffer \(A\) containing 1% (v/v) Triton X-100 using volumes equal to 2 times the original tissue weight for each extraction. Pellets were subsequently extracted a fourth and fifth time with buffer \(B\) containing 1 M NaCl and using a volume equal to 4 times the original tissue weight. Tissue was incubated 30 min at 5°C with each buffer prior to centrifugation. Seeds were extracted using the volumes indicated in Table III. \(^b\)GGT activity was measured using γ-GPNAd as substrate and data was analyzed as described in “Materials and Methods.”
GGT3, like GGT1, has a unique function that is not complemented by another GGT gene. The progeny from crosses of ggt3-1 × ggt3-2 showed the phenotype, indicating that the mutations are allelic. Thiols were measured in several tissues and, as with the GGT1 mutants, no alteration in the levels of thiols could be detected.

Both GGT2 and GGT4 mutants were indistinguishable from wild-type plants at all stages of growth with regards to phenotype. Similarly, neither GGT2 nor GGT4 mutants showed changes in the levels of GSH, its precursors, Cys and γ-Glu-Cys, or the potential products of GSH hydrolysis Cys-Gly and γ-Glu-Cys in any tissues. All parts of the plant were tested including isolated seeds and ovary walls at several developmental stages. The GGT4 mutants are altered in the ability to metabolize S-conjugates of GSH, a finding that is reported in depth in a separate article (Grzam et al., 2007).

**DISCUSSION**

GGT activity was reported in both plants and in animals about 50 years ago. Yet, most of what is known about GGTs comes from studies with animals where they are involved in Cys delivery, metabolism of glutathione S-conjugates of xenobiotic for excretion, and metabolism of glutathione S-conjugates of bioactive compounds such as eicosanoid hormones to modulate activity. The results described herein indicate that although GGTs in plants and animals have separately evolved, they may have retained many properties and functions in common, thus making the animal GGTs and the γ-glutamyl cycle within which they function a useful framework for examining GSH and glutathione S-conjugates catabolism in plants.

In many cases, the animal GGTs are localized on the extracellular surface of the cell membrane where, in conjunction with dipeptidases (MBDs), they hydrolyze GSH to its component amino acids (Fig. 1). These amino acids are transported into the cell where they are used for a range of processes including the synthesis of GSH. It is for this reason that GGTs are sometimes described in the context of a γ-glutamyl cycle, which includes the extracellular enzymes for catabolism, transporters, and the intracellular enzymes for resynthesis of GSH (Fig. 1). The essentiality of the hydrolysis step was clearly indicated by the conditional lethality of a mutation in the single mouse GGT gene, which demonstrated that the delivery of Cys is completely dependent on the activity of GGT (Lieberman et al., 1996). Knockout of the GGT gene resulted in massive excretion of GSH and sulfur starvation, suggesting that the intact GSH molecule is not imported. The severe phenotype resulting from inborn errors (mutations) in five of the γ-glutamyl cycle genes in humans suggest a need for the complete cycle (Ristoff and Larsson, 2007).

There is considerable indirect evidence that GSH is also used by plants to store and transport reduced sulfur (Cys) and that it may be hydrolyzed by GGTs, as in animals, and imported as the component amino acids. GSH is present in xylem and phloem (Rennenberg et al., 1979; Rauzer et al., 1991; Kuzuhara et al., 2000), and indirect evidence suggests that it is transported from mature vegetative tissues to young vegetative tissues and from vegetative tissue to the seeds during development (Sunarpi and Anderson, 1997a, 1997b). Until recently, it was presumed that GSH was imported intact into cells, but this idea has been called into question by the recent finding that an Arabidopsis γ-glutamyl-Cys synthetase (gsh1) mutant is embryo lethal. In addition to demonstrating that GSH is essential, the phenotype

Figure 9. All GSH-dependent GGT activity was abolished in green seeds by knocking out GGT1 and GGT2. The radioisotope images show the separation of [14C]ACC and the product of the GGT reaction, [14C]-γ-GluACC, on Si:C18 TLC plates following assay of GGT activity in wild type, ggt1-1, and ggt2-1 mutant seeds using GSH as the γ-glutamyl donor and [14C]ACC and the γ-glutamyl acceptor amino acid. The reactions were performed using the Triton X-100 extracted proteins from green seeds or using the NaCl extracted residual proteins from green seeds. Extractions were performed as described in Table II. Assays were performed as described in “Materials and Methods.”

Figure 10. Both alleles of the GGT1 and GGT3 knockouts have altered phenotypes. Rosette leaves of ggt1-1 and ggt1-2 plants are dead 50 d after planting while leaves of the wild-type plant are only beginning to senesce. Plants homozygous for the ggt3-1 and ggt3-2 alleles are 30% to 50% shorter than the wild-type plants at maturity and have up to 30% to 40% fewer siliques at maturity.
indicates that the mutant embryo is unable to obtain GSH from its heterozygous parent that is fully capable of GSH synthesis (Cairns et al., 2006). If embryos are unable to import GSH, then they must synthesize it from component amino acids that can come from in situ synthesis or import of constituent amino acids resulting from extracellular hydrolysis of GSH by GGTs. We have shown that at least two GGTs are appropriately localized at the organ and cellular level to serve this function and exhibit catalytic properties consistent with such a function.

Establishing the location of expression of all GGTs in Arabidopsis very likely establishes the location of all GSH or glutathione S-conjugate hydrolysis. If GSH serves as a transported form of Cys, one would expect GGTs to be highly expressed in sink tissues. Such tissues might include locations of rapid growth, rapid accumulation of storage compounds, or tissues with a transport function. Indeed, the expression patterns of GGT1 and GGT3 are completely overlapping and are consistent with this prediction. Both are highly expressed in rapidly growing seedlings, and growth points that include the rosette apex, the floral stem apex, and reproductive tissues (immature flowers, seeds, and ovary wall). In mature tissues, expression is restricted to vascular tissues. In developing seeds, all four GGTs are highly expressed and indeed reproductive structures are likely sink tissues for GSH. However, the function served by high expression of GGT1, GGT2, and GGT3 at locations in or around immature trichomes is unclear. The trichomes of Arabidopsis are reported to contain a high concentration of GSH and are hypothesized to serve in detoxification of xenobiotics (Gutierrez-Alcala et al., 2000).

Appropriate subcellular location is crucial if a GGT is to hydrolyze extracellular GSH and glutathione S-conjugates following translocation from source sites. In animals, most GGTs are anchored on the external surface of the cell. The N-terminal consensus amino acid sequences of both GGT1 and GGT2 exhibit homology with the human and mouse membrane anchoring domain (Storozhenko et al., 2002). Furthermore, at least one Arabidopsis GGT (GGT1), when expressed in tobacco, appears to be localized outside the plasma membrane (Storozhenko et al., 2002). Analysis of GGT1 and GGT2 mutants shed further light on the localization of GGT1 and GGT2 and suggested that both may be located extracellularly but with apparent difference in the nature of their association. In Arabidopsis protein extracts, GGT1 was found to be associated with a particulate fraction via an ionic interaction (it could be released only by treatment with high molarity NaCl). GGT2 was found to be soluble (in leaf tissue) or associated with a particulate fraction and could be released by treatment with a nonionic detergent (green seeds). Most of the GGT1 and GGT2 activities in leaves are not recovered with protoplasts, suggesting an apoplastic location. Recovery of GGT2 in the digestion buffer suggests that it is a soluble protein. Recovery of GGT1 with undigested debris and release with 1 M NaCl again suggests an ionic association perhaps with cell wall fragments that are recalcitrant to degradation. For both GGTs, it remains to be established whether location is native or an artifact of isolation. Both GGT1 and GGT2 are predicted to be targeted to the secretory system. Although the two GGTs show extremely high homology, their localization is clearly different. The release of GGT2 from green seeds by detergent suggests that it is associated with a membrane fraction or simply sequestered in a storage body. What role these two GGTs might play remains to be determined, but a clue might be that both GGT1 and GGT2 expression and activity are high in developing embryos.

The analysis of the GGT mutants provided a means to link individual GGT genes with specific isoforms. Using the standard GGT assay developed to measure animal GGT activity (with γ-GPNA as substrate), the GGT1 gene was shown to be the source of the majority of the GGT activity in nearly every Arabidopsis tissue with the exception of developing embryos, where GGT2 accounts for approximately 50% of the total activity. These results were obtained by comparing GGT activity in wild-type and mutant lines. The same results were obtained using a range of other γ-glutamyl donor substrates including GSH and several glutathione S-conjugates. These results were corroborated by the expression pattern of GGT:GUS fusion constructs that showed the GGT2 promoter to be highly active primarily in developing embryos and the GGT1 promoter to be active in both reproductive and vegetative tissues.

Assignment of activity to GGT3 was not possible. Although there are several possible explanations, a potentially relevant point is that GGT3 expression is very low in comparison to the others. Thus, GGT3 may be a minor form whose loss would not have been detected in the GGT3 mutant above plant to plant deviation. The structural divergence of GGT3 has resulted in loss of most of the N terminus and thus most of the heavy chain. This may have resulted in alteration of catalytic properties.

Low level of expression is not an explanation for very low GGT4 activity in the standard assay. Data available from public microarray sets show GGT4 mRNA is expressed at a higher level than the other GGTs in many tissues. In a separate article, we present evidence that structurally divergent GGT4 has also diverged functionally so as to serve a specialized function. It is localized in the vacuole where it hydrolyzes glutathione S-conjugates that are in some instances rapidly targeted to the vacuole in plants (Grzam et al., 2007). This is unlike in animals where glutathione S-conjugates are transported to the exterior of cells. GGTs in animals are also involved in modulating the activity of glutathione S-conjugates of several classes of eicosanoid hormones (Shi et al., 2001). In animals most glutathione S-conjugates are hydrolyzed by GGT, but others are hydrolyzed by an evolutionarily divergent member of the GGT family, GGL, that is able to use only the glutathione S-conjugate of leukotrienes as substrate (Carter et al., 1997).
Animal GGTs have high affinity for a wide range of γ-glutamyl donor substrates that include GSH and many glutathione S-conjugates consistent with their wide range of in vivo roles. Like the animal enzymes, GGT1 and GGT2 also show broad specificity for the γ-glutamyl donors. Both GGT1 and GGT2 also have $K_m$ values between 50 and 100 μM for GSH and several glutathione S-conjugates (M.N. Martin, unpublished data). GGTs from tomato, onion, and radish were also reported to have high affinity and broad specificity for γ-glutamyl donor (Martin and Slovin, 2000; Shaw et al., 2005; Nakano et al., 2006b). Although high affinity GGTs have been identified, it should be noted that high affinity GSH-specific transporters able to import the intact GSH molecule have not been identified in plants. For example, bean (Phaseolus vulgaris) protoplasts were shown to take up GSH, GSSG, and glutathione S-conjugates, but the affinity of the transporter(s) was nearly 50-fold lower for GSH ($K_m$ 0.4 mM) than GSSG ($K_m$ 7 μM). Furthermore, GSH uptake was competitively inhibited by GSSG and glutathione S-conjugates (Jamai et al., 1996). Complementation of the yeast mutant, hgt1, which is unable to take up GSH, was used to characterize oligopeptide transporters from rice (Oryza sativa; Zhang et al., 2004), Brassica juncea (Bogs et al., 2003), and Arabidopsis (Cagnac et al., 2004) that were able to transport GSH. In each case, the $K_m$ values for GSH were greater than 0.4 mM, and these transporters, like the bean transporter, exhibited much higher affinity for GSSG and glutathione S-conjugates. Failure to identify a high affinity GSH importer and the presence of high affinity GGTs might underscore the importance of GSH hydrolysis and import of its component amino acids.

Analysis of the GGT mutants did not provide proof that GGTs function in the utilization of GSH as a sulfur source. None of the mutants were defective in germination or in early growth on GSH as sole sulfur source. The early leaf senescence phenotype of ggt1 mutants or the reduced height of ggt3 mutants could not be correlated with altered level of GSH in any tissue. There are several possible explanations. The simplest is that the GGT genes are functionally redundant, a hypothesis that points the way for future experimentation to examine how mutations in multiple GGTs affect the ability to utilize GSH. This explanation is supported by the finding that Arabidopsis seeds are unable to germinate on medium supplemented with as little as 1 μM of the high affinity GGT-specific inhibitor acivicin (M.N. Martin, unpublished data). It is, however, perplexing that the ggt1 mutant does not show significantly compromised usage of GSH as sole sulfur source or increased GSH content when it has only 1% of the total GGT activity of wild type. This suggests that under the conditions used to grow the mutants GGT activity is in enormous excess of what is required. It is noteworthy that GGT1:GUS plants grown with GSH as sole sulfur source showed no increase in level of expression as compared to plants grown with sulfate as sulfur source (M.N. Martin, unpublished data). Another possible explanation is that changes in GSH concentration in the mutants might be restricted to a small compartment, such as the apoplast, where differences would not be detected with the analytical method employed here. A third possibility is that GGTs do not play a role in GSH utilization or they have a minor role. Unlike animals, plants are capable of de novo Cys synthesis, meaning that GGT mutants would manifest a phenotype only if Cys synthesis was blocked. However, the latter possibility seems unlikely given that all the GGT knockouts were fully capable of early growth on medium with GSH as the only sulfur source, a condition where Cys synthesis would not have been possible. Since GSH transport and hydrolysis to recover Cys may be a supplementary function rather than a required function in plants, the severity of the phenotype observed in animals is unlikely in plants. Expression analysis using Genevestigator shows that genes responsible for sulfate transport, sulfur assimilation and reduction, and Cys synthesis are expressed in all organs and tissues of Arabidopsis with the exception of pollen. It is noteworthy that the phenotypes of both GGT1 and GGT3 mutants manifest themselves during reproductive growth.

### MATERIALS AND METHODS

#### Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) lines were sown on potting mix (Promix BX, Premier Horticulture Inc.) and fertilized with one-quarter-strength Jack's Classic with micronutrients (10-50-20) J.R. Peters. Following stratification for 3 d at 5°C, plants were grown in Environmental Growth chambers (Chagrin) equipped with fluorescent and incandescent lighting at 120 μmol photons m$^{-2}$s$^{-1}$ with 16 h light at 24°C and 8 h dark at 20°C. All tissues were harvested 9 to 13 h into the light cycle.

#### DNA Constructions and Plant Transformation

Gateway recombination technology (Invitrogen) was used to create plant vectors with GUS fused in frame at the C terminus of each GGT. The GGT sequences containing 700 to 1,000 bp upstream of the annotated translation initiation codon and the entire structural gene were amplified by PCR from Arabidopsis Col 7 genomic DNA. The following primers designed with Gateway attB1 and attB2 sites were used: GGT1, 5′-GGGGACCACTTTGTACA-AAAAAACGAGCTGCAATAAATAATGCGGGGAGATGT-3′ and 5′-GGGGACCACTTTGTACCA-CTAAAGGTTTACCTCTAGAGGAAACCTCC-3′; GGT2, 5′-GGGGACCACTTTGTACCA-CTAAAGGTTTACCTCTAGAGGAAACCTCC-5′ and 5′-GGGGACCACTTTGTACCA-CTAAAGGTTTACCTCTAGAGGAAACCTCC-3′. All tissues were harvested.

PCR products were cloned into Gateway entry vectors pDONR221 or pDONR223 and 8 h dark at 20°C. All tissues were harvested. These were sequenced to ensure that no mutations were present. The entry clones were then used to transfer the GGT sequence to the plant Gateway destination vector pMD163 using Invitrogen protocols. Promoterless Gateway-compatible TDNA destination vector pMDC163 was obtained from Mark Curtis (Curtis and Grossniklaus, 2003). The recombinant plant binary vector was used to transform Agrobacterium tumefaciens GV3101 (MP90).

Arabidopsis ecotype Col 7 (Arabidopsis Biological Resource Center no. CSS731), obtained from the Arabidopsis Biological Resource Center, was grown on potting mix for 35 to 40 d and transformed by the dipping method...
Histochemical Analysis of GUS

Arabidopsis tissue was harvested into ice-cold 90% (v/v) acetone, incubated for 15 to 20 min on ice to permeabilize tissue, and then washed with GUS assay buffer. The GUS reaction contained 50 mM potassium phosphate, pH 7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% (v/v) Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Gold Biotechnology). The tissue was vacuum infiltrated for 5 to 10 min and incubated for 12 to 36 h at 37°C. Reactions were terminated by transfer to 70% (v/v) ethanol for removal of chlorophyll and preservation of the samples.

For visualization of the GUS reaction, tissues were examined using an Olympus SZ-CTV dissecting microscope interfaced with a Nikon DMS1200 digital camera and ACT-1 image capture software. Images were processed using Adobe Photoshop.

Analysis of Mutant Lines

Information regarding construction of T-DNA or transposon mutant lines and the sources of seeds is referenced in Supplemental Table S2. For verification of insertion site, genomic DNA was extracted from 20-d-old plants and the region around the insertion site was PCR amplified using an Extract N-Amp kit (Sigma Chemical) and the primer pairs indicated in Supplemental Table S2. Amplified products were sequenced.

Homozygous plants of SAIL lines were selected on Basta. Homozygous plants of all other lines were selected on kanamycin. Homozygous lines were confirmed by PCR using the primer pairs indicated in Supplemental Table S2. Knockout or knockup of gene function was confirmed by semiquantitative RT-PCR. RNA was extracted using TRIzol reagent and first-strand cDNA synthesized using Superscript II reverse transcriptase (Invitrogen).

Protein Extraction

Plant tissue was extracted using modifications of previously described methods (Martin and Slovin, 2003). Tissue was frozen in liquid nitrogen, ground to a powder using a mortar and pestle assembly (Bel-Art Products), and extracted with buffer A containing 100 mM Tris-Cl, pH 8.0, 1 mM EDTA, and a plant protease inhibitor cocktail (no. P9599, Sigma Chemical) using a ratio of 2 to 12 μL of buffer A per 1 mg of tissue depending on the tissue source. Where indicated, 1% (v/v) Triton X-100 was added to buffer A. Seeds required 8 to 12 μL of buffer for quantitative solubilization/extraction of GGT activity. The crude extract was incubated 30 min on ice to assure quantitative recovery of proteins and was centrifuged for 15 min at 13,000g at 5°C. The supernatant was recovered for assay of soluble GGT activity. When there was a need to quantitatively recover soluble GGTs, the pellet was reextracted one to three times with buffer A followed by centrifugation and recovery of supernatant as above. The pellet was then reextracted with buffer B containing the components of buffer A plus 1 mM NaCl, using a ratio of at least 4 μL (12 μL for seeds) of buffer per milligram of original tissue weight. The extract was centrifuged as above and the supernatant was recovered.

Microsomal Membrane Preparation

Plant material was frozen in liquid nitrogen, ground to a powder with a mortar and pestle, and further ground with extraction buffer consisting of 100 mM Tris-Cl, pH 8.0, 300 mM Suc, and 10 mM EDTA. The extract was centrifuged 15 min at 13,000g, the supernatant was recovered and centrifuged at 100,000g, 5°C for 1 h to pellet microsomal membranes (Dammann et al., 2003).

Spectrophotometric Measurement of GGT Activity

The assays using γ-GPN as γ-glutamyl donor were performed at 30°C in 96-well microtiter plates. Each well contained in a volume of 100 μL, 100 μM Tris-Cl, pH 8.0, 10 μL of plant extract, 5 μM γ-GPN, and 20 mM glycylglycine. Assays were initiated with γ-GPN, and the rate of formation of p-nitroaniline was measured spectrophotometrically at 405 nm using a Synergy HT plate reader with Kineticale Software (Bio-TEK). One unit of enzyme activity was defined as the amount catalyzing the formation of 1 pmol p-nitroanilide per minute. Total activity measurements are the average of three independent tissue samples with triplicate assays performed on each tissue sample. Activities below 5 to 10 pmol min⁻¹ mg fresh weight⁻¹ are near the limit of sensitivity of the assay when measuring crude protein extracts. Values of zero were assigned only when absorbance reading after 24 or 48 h did not differ from the 0 time point.

Radiometric Measurement of GGT Activity

The assays were performed as described (Martin and Slovin, 2000) and contained in a volume of 50 μL, 100 mM Tris-Cl pH 8.0, 0.4 mM GSH or other γ-glutamyl donor as indicated, 5 mM ACC, 3.75 kBq [2,3-14C]ACC per assay as a γ-glutamyl acceptor, and plant extract. [2,3-14C]ACC with the specific activity of 1.87 GBq/mmol was synthesised by the Commissariat a L’Energie Atomique, France. The reaction was initiated with plant extract, incubated for 30 to 90 min at 30°C, and terminated by the addition of 50 μL of absolute ethanol. Terminated reactions were centrifuged for 5 min at 13,000g to pellet protein and other debris. Five microliters of each reaction were spotted onto a lane of a 10 × 20 cm Baker SiC₄₆ reversed-phase octadeyl TLC plate (JT Baker). [2,3-14C]ACC and the product, [14C]-γ-glutamylACC (γ-GluACC), were resolved using a solvent system of 1-propanol/water (82:19, v/v). Radiolabeled products and substrate were detected and quantified using a Storm 860 Image Acquisition system and an Image Quant Analysis system (Amersham Biosciences). One unit of enzyme activity was defined as the amount catalyzing the formation of 1 pmol of γ-GluACC per min.

Measurement of Thiols

Plant samples were harvested, fresh weight was recorded, and the samples immediately frozen and stored at −80°C until analyzed. Frozen tissue was extracted with 0.1 m HCl plus 1 mM EDTA at a ratio of 10 μL of 1 mM γ-glutamyl donor as indicated, 5% ethylene glycol with 1 mg AccI, 3.75 kBq [2,3-14C]ACC per assay as a γ-glutamyl acceptor, 0.1 μL of 10% (w/v) acetic acid was added to reduce the pH to about 3.0, a pH at which the monobromobimane adducts are more stable.

The monobromobimane adducts were resolved by liquid chromatography using a Waters Corporation Alliance LC system equipped with Millenium software, a model 2690 Separation Module, and a model 474 fluorescence detector. Chromatography was performed at 32°C with a Nova-Pak C₁₇₈₆ Sentry Guard column (3.9 mm × 20 mm) followed in series with a 3.9 mm × 150 mm Waters AccQ-Tag column. The monobromobimane adducts were measured with the fluorescence detector set to an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Eluent A, containing sodium acetate and triethylamine at pH 5.05, was purchased as a concentrate from Waters. The composition of eluent B was acetonitrile:water (30:70, v/v). The elution method was 0 to 9 min, 6% B; 9 to 16 min, linear gradient to 8.5% B; 16 to 22 min, linear gradient to 25% B; and 22 to 30 min, linear gradient to 100% B at a flow rate of 1.5 mL min⁻¹. Standard curves were established using t-Cys, GSH, γ-GluCys, and Cys-Gly (all from Sigma-Aldrich).

Growth of Plants with a Defined Sulfur Source

Where indicated, plants were grown on modified Murashige and Skoog basal salts with minimal organic nutrients (catalog no. M9999, Sigma-Aldrich), 1% (w/v) Noble agar, and 1% (w/v) Suc. In the modified Murashige and Skoog medium all other trace divalent metal sulfates were replaced with equimolar amounts of divalent metal chlorides or nitrates. Magnesium sulfate was deleted and replaced with an equimolar amount of magnesium nitrate for sulfur-free media and for media containing GSH as sulfur source. Media with GSH as sulfur source was amended with 1 mm GSH. Growth conditions are as described above.
Protoplast Isolation

For mesophyll protoplast isolation, roselet leaves were harvested from 30-d-old Arabidopsis grown in a chamber as described above. Leaves (1 g) were cut with a razor blade into small slices, plasmolyzed for 30 min in 5 mL of 10 mM MES pH 5.7, 500 mM mannitol, and transferred to 5 mL protoplasting solution containing 2% (w/v) cellulase from Trichoderma viride (Sigma C7974), 0.5% (w/v) Macerozyme R-10 (Serva), 400 mM mannitol, 10 mM KCl, 10 mM CaCl2, and 5 mM MES-KOH, pH 5.7. The sliced leaves were vacuum infiltrated for 1 min at 15 mm Hg and allowed to incubate in the dark at 24°C for 5 h with occasional agitation. Protoplasts were separated from undigested material by filtration through a 200 μm nylon mesh. Protoplasts were pelleted by centrifugation for 5 min at 60g and washed three times in 400 mM mannitol, 5 mM MES-KOH, pH 5.7, and 5 mM KCl and 5 mM CaCl2. Protoplasts were visualized under a microscope to establish that most were intact. GGT activity was measured in roselet leaves used for protoplasting, in the protoplasting solution before and after tissue incubation, and in the isolated protoplasts before and after lysis.

Unless otherwise noted, biochemicals were purchased from Sigma Chemical or Gold Biotechnology.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Predicted localization of Arabidopsis GGTs.

Supplemental Table S2. Primers used to characterize TDNA and transposon insertion lines.

Note Added in Proof

Subsequent to submission of this manuscript, two articles have been published describing characterization of Arabidopsis GGT1 and GGT2 (Ohkama-Ohtsu N, Radwan S, Peterson A, Zhao P, Badr AF, Xiang C, Oliver DJ [2007a] Characterization of the extracellular γ-glutamyl transpeptidases, GGT1 and GGT2, in Arabidopsis. Plant J 49: 865–877) and GGT4 (Ohkama-Ohtsu N, Zhao P, Xiang C, Oliver DJ [2007b] Glutathione conjugates in the vacuole are degraded by γ-glutamyl transpeptidase GGT3 in Arabidopsis. Plant J 49: 878–888). A third article describing characterization of Arabidopsis GGT4 is in press (Grzam et al., 2007).

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LITERATURE CITED

Bick JA, Setterdahl AT, Knaff DB, Chen Y, Pitcher LH, Zilinskas BA, Leustek T (2001) Regulation of the plant-type 5'-adenylyl sulfate reductase by oxidative stress. Biochemistry 40: 9040–9048

Bogs J, Bourbouloux A, Cagnac O, Wachter A, Rausch T, Delrot S (2003) Functional characterization and expression analysis of a glutathione transporter, BgGT1, from Brassica juncea: evidence for regulation by heavy metal exposure. Plant Cell Environ 26: 1703–1711

Cagnac O, Bourbouloux A, Chakrabarty D, Zhang MY, Delrot S (2004) AtOPT6 transports glutathione derivatives and is induced by primisulfuron. Plant Physiol 135: 1378–1387

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) AtOPT6 transports glutathione derivatives and is induced by primisulfuron. Plant Physiol 135: 1378–1387

Cairns NG, Pasternak M, Wachter A, Cobbett CS, Meyer AJ (2006) Maturation of Arabidopsis seeds is dependent on glutathione biosynthesis within the embryo. Plant Physiol 141: 446–455

Carter BZ, Wiseman AL, Orkiszewski R, Ballard KD, Ou CN, Lieberman MW (1997) Metabolism of leukothriene C4 in γ-glutamyl transpeptidase-deficient mice. J Biol Chem 272: 12305–12310

Chakravarthi S, Bulleid NJ (2004) Glutathione is required for regulation of the γ-glutamyl transpeptidase Gene Family

Characterization of the γ-glutamyl transpeptidase gene family formation of native disulfide bonds within proteins entering the secretory pathway. J Biol Chem 279: 38972–38979

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743

Curtis MD, Groseniklaus U (2003) A Gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol 133: 462–469

Dammann C, Ichida A, Hong B, Romanovsky SM, Hrabak EM, Harmon AC, Pickard BG, Harper JF (2003) Subcellular targeting of nine calcium-dependent protein kinase isoforms from Arabidopsis. Plant Physiol 132: 1840–1848

Dixon DP, Skipsey M, Grundy NM, Edwards R (2005) Stress-induced protein γ-glutathionylation in Arabidopsis. Plant Physiol 138: 2233–2244

Foyer CH, Theodoulou FL, Delrot S (2001) The functions of inter- and intracellular glutathione transport systems in plants. Trends Plant Sci 6: 486–492

Grant CM, Valev FH, Dawes I (1996) Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast Saccharomyces cerevisiae. Curr Genet 29: 511–515

Grzam A, Martin MN, Hell R, Meyer AJ (2007) γ-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione S-conjugates in Arabidopsis. FEBS Lett 581: 3133–3138

Grzam A, Tennstedt P, Clemens S, Hell R, Meyer AJ (2006) Vacuolar sequestration of glutathione S-conjugates outcompetes a possible degradation of the glutathione moiety by phytochelatin synthase. FEBS Lett 580: 6384–6390

Gutierrez-Alcala G, Gortor C, Meyer AJ, Fricker M, Vega JM, Romero LC (2000) Glutathione biosynthesis in Arabidopsis trichome cells. Proc Natl Acad Sci USA 97: 11108–11113

Habib GM, Barrios R, Shi ZZ, Lieberman MW (1996) Four distinct membrane-bound dipeptidase RNAs are differentially expressed and show discordant regulation with γ-glutamyl transpeptidase. J Biol Chem 271: 16273–16280

Habib GM, Shi ZZ, Cuevas AA, Lieberman MW (2003) Identification of two additional members of the membrane-bound dipeptidase family. FASEB J 17: 1313–1315

Heazlewood J, Verbom R, Toni-Filippini J, Small I, Millar A (2007) SUBA: the Arabidopsis subcellular database. Nucleic Acids Res 35: D213–218

Heazlewood JL, Toni-Filippini J, Verbom RE, Millar AH (2005) Combining experimental and predicted datasets for determination of the subcellular location of proteins in Arabidopsis. Plant Physiol 139: 598–609

Irishimvitch V, Shapira M (2000) Glutathione reductox potential modulated by reactive oxygen species regulates translation of Rubisco large subunit in the chloroplast. J Biol Chem 275: 16289–16295

Jama A, Tommasini R, Martinova E, Delrot S (1996) Characterization of glutathione uptake in broad bean leaf protoplasts. Plant Physiol 111: 1145–1152

Kim BJ, Choi CH, Lee CH, Jeong SY, Kim JS, Kim BY, Yim HS, Kang SO (2005) Glutathione is required for growth and presupere cell differentiation in Dicotyledons. Dev Biol 284: 387–398

Klatt P, Lamas S (2000) Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Eur J Biochem 267: 4928–4944

Kuzuhara Y, Isobe A, Awaizhara M, Fujiiwara T, Hayashi H (2000) Glutathione levels in phloem sap of rice plants under sulfur deficient conditions. Soil Sci Plant Nutr 46: 265–270

Lamoureux GL, Rusness DG (1981) Catabolism of glutathione conjugates of pesticides in higher plants. In JD Rosen, PS Mager, JE Casada, eds, Sulfur in Pesticide Action and Metabolism. Am Chem Soc, Washington, DC, pp 133–164

Lamoureux GL, Rusness DG (1993) Glutathione in the metabolism and detoxification of xenobiotics in plants. In LJ de Kok, J Stulen, H Rennenberg, C Brunold, WE Rauser, eds, Sulfur Nutrition and Assimilation in Higher Plants. SPB Academic, The Hague, The Netherlands, pp 221–237

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol 51: 141–165

Lieberman MW, Barrios R, Carter BZ, Habib GM, Lebovitz RM, Rajappan S, Sepulveda AR, Shi ZZ, Wan DF (1995) γ-glutamyl transpeptidase: What does the organization and expression of a multi-promoter gene tell us about its functions? Am J Pathol 147: 1175–1185

Lieberman MW, Wiseman AL, Shi ZZ, Carter BZ, Barrios R, Ou CN, Chevez-Barrios P, Wang Y, Habib GM, Goodman JC, et al (1996)
Growth retardation and cysteine deficiency in -glutamyl transpeptidase-deficient mice. Proc Natl Acad Sci USA 93: 7923–7926

Lockwood TD (2000) Redox control of protein degradation. Antioxid Redox Signal 2: 851–878

Marrs KA (1996) The functions and regulation of glutathione S-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 127–158

Marrs KA, Alfenito MR, Lloyd AM, Walbot V (1995) A glutathione-S-transferase involved in vacuolar transfer encoded by the maize gene bronze-2. Nature 375: 397–400

Martin MN, Slovin JP (2000) Purified -glutamyl transpeptidases from tomato exhibit high affinity for glutathione and glutathione S-conjugates. Plant Physiol 122: 1417–1426

Meister A (1989) -Glutamyl transpeptidase. In D Dolphin, R Poulson, O Avramovic, eds, Glutathione: Chemical Biochemical, and Medical Aspects. Wiley, New York, pp 367–474

Meister A, Tate SS, Griffith OW (1981) -Glutamyl transpeptidase. Methods Enzymol 77: 237–253

Mueller LA, Goodman CD, Silадy RA, Walbot V (2000) AN9, a petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. Plant Physiol 123: 1561–1570

Nakano Y, Okawa S, Prieto R, Sekiya J (1989) Long-distance transport of sulfur in soybean. Plant and Soil 179: 57–62

Ristoff E, Larsson A (2007) Inborn errors in the metabolism of glutathione. Orphanet J Rare Dis 2: 1–16

Shaw ML, Pither-Joyce MD, McCallum JA (2005) Purification and cloning of a -glutamyl transpeptidase from onion (Allium cepa). Phytochemistry 66: 515–522

Shi ZZ, Han B, Habib GM, Matzuk MM, Lieberman MW (2001) Disruption of -glutamyl leukotrienase results in disruption of leukotriene D4 synthesis in vivo and attenuation of the acute inflammatory response. Mol Cell Biol 21: 5389–5395

Shi ZZ, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, Lukin DJ, Danney CM, Matzuk MM, Lieberman MW (2000) Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc Natl Acad Sci USA 97: 5101–5106

Sipos K, Lange H, Fekete Z, Ullmann F, Lill R, Kispat G (2002) Maturation of cytosolic iron-sulfur proteins requires glutathione. J Biol Chem 277: 26944–26949

Steinkamp R, Rennenberg H (1985) Degradation of glutathione in plant cells: evidence against the participation of a -glutamyltranspeptidase. Zeitschrift Fur Naturforschung C-. J Biosci 40c: 29–33

Steinkamp R, Schwelthofen B, Rennenberg H (1987) -Glutamylcysteine transferase in tobacco suspension cultures: catalytic properties and subcellular localization. Physiol Plant 68: 499–503

Storozhenko S, Belles-Boix E, Babychuk E, Herouart D, Davey MW, Slooten L, Van Montagu M, Inze D, Kushnir S (2002) -Glutamyl transpeptidase in transgenic tobacco plants: cellular localization, processing, and biochemical properties. Plant Physiol 128: 1109–1119

Sunarpi, Anderson JW (1997a) Allocation of S in generative growth of soybean. Plant Physiol 114: 687–693

Sunarpi, Anderson JW (1997b) Effect of nitrogen nutrition on the export of sulphur from leaves in soybean. Plant and Soil 188: 177–187

Zhang MY, Bourbouloux A, Cagnac O, Srikanth CV, Rentsch D, Bachhawat AK, Delrot S (2004) A novel family of transporters mediating the transport of glutathione derivatives in plants. Plant Physiol 134: 482–491

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR: Arabidopsis microarray database and analysis toolbox. Plant Physiol 136: 2621–2632