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Identification and characterization of γ-glutamyltransferase knockout mutants in Arabidopsis

Rafael Prieto¹*, Yoshihiro Nakano² and Jiro Sekiya¹

¹Department of Bioscience and Biotechnology, Faculty of Bioenvironmental Science, Kyoto Gakuen University, Sogabe-cho, Kameoka, Kyoto 621-8555, Japan.
²Shikoku Research Center, National Agriculture Research Center for Western Region, NARO, Zentsuji, Kagawa 765-8508, Japan.

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Arabidopsis mutants harboring T-DNA or transposon insertions in γ-glutamyltransferase (GGT) genes AtGGT1, AtGGT2 and AtGGT3 were characterized. Gene expression analysis indicated that the identified atggt1-1, atggt1-2, atggt2-1, atggt2-2 and atggt3-1 plants were GGT-null mutants. GGT activity analysis indicated that the atggt1 mutant alleles were deficient in cell wall bound GGT, whereas the two atggt2 mutant alleles and the atggt3 mutant allele were deficient in soluble GGT. GSH contents and GSH catabolic rates in wild type and atggt1 plants suggest that AtGGT1 is involved in GSH catabolism.

Keywords: Arabidopsis, γ-glutamyltransferase, glutathione, glutathione catabolism, mutant analysis

Introduction

γ-Glutamyltransferase (GGT, EC. 2.3.2.2.) is found in plants, mammals and microorganisms. In mammals, yeast and Escherichia coli, GGTs are well characterized and have been shown to possess several physiologically important functions including glutathione (GSH) catabolism (Taniguchi and Ikeda, 1998; Suzuki et al., 1993; Mehdi et al., 2001). The function of plant GGTs has not yet been clearly defined, although they are
thought to be involved in GSH catabolism (Bergmann and Rennenberg 1993; Martin and Slovin, 2000; Shaw et al., 2005; Nakano et al., 2006a, Ohkama-Ohtsu et al., 2007a,b).

Plant GGTs are classified into soluble GGTs and bound GGTs, the latter of which are solubilized by high ionic strength buffer containing 0.5-1 M NaCl (Nakano et al., 2004). Several plants, including radish, Arabidopsis and rice, have both soluble and bound isoforms, whereas tobacco and onion have only the bound isoforms (Nakano et al., 2004). In radish, bound GGT is mainly associated with the cell wall (Nakano et al., 2004), whereas soluble GGT is mainly localized to the vacuole (Nakano et al., 2006b). Taking into account that: a) oxidized glutathione (GSSG) and GSH S-conjugate (GSX) are transported to the vacuole by ABC-type transporters (Rea, 1999), b) both GSX and GSH are thought to be transported to the extracellular space, and c) a small amount of mainly GSSG are localized to the apoplast (Vanacker et al., 1999; Ohkama-Ohtsu et al., 2007a, Okawa and Nakano unpublished), the subcellular localization of radish GGT isoforms is in agreement with a possible involvement of GGT in GSH catabolism. According with this, characterization of Arabidopsis GGT knockout mutants suggested that GGT is involved in GSH degradation in the apoplast and the vacuole (Ohkama-Ohtsu et al., 2007a,b; Martin et al., 2007; Grzam et al., 2007).

Plant cDNAs encoding heterodimeric GGTs that have molecular structures very similar to those of animals and microorganisms have been characterized in Arabidopsis (AtGGT; Storozhenko et al., 2002; GGT1-3; Ohkama-Ohtsu et al., 2007a,b; Martin et al., 2007; Grzam et al., 2007); onion (AcGGT; Shaw et al., 2005); and radish (RsGGT1-3; Nakano et al., 2006c). Heterodimeric plant GGTs are composed of one large and one small subunit that are thought to be generated by the proteolytic cleavage of a single polypeptide chain precursor. Gene database search revealed that, besides AtGGT, which is also named At4g39640, in the Arabidopsis genome there are at least two more genes, which are named At4g39650 and At4g29210, encoding heterodimeric GGTs. The Arabidopsis genome contains another potential heterodimeric GGT gene that is named At1g69820. However, analysis of the At1g69820 deduced amino acid sequence (accession no. NP177140) revealed a putative protein
that apparently lacks approximately 70% of the GGT large subunit.

The functional characterization of GGT genes is important to understand the physiological role of the GGT isoforms. We have named the At4g39640 cDNA sequence as \textit{AtGGT1} (accession no. Z49240), the At4g39650 cDNA sequence as \textit{AtGGT2} (accession no. AK220806) and the At4g29210 cDNA sequence as \textit{AtGGT3} (accession no. NM119065). In the present study, we describe the identification and characterization of \textit{Arabidopsis} knockout mutants having affected GGT isoforms that may differ in their functions and cellular localizations.

**Materials and Methods**

**Plant Materials**

The \textit{atggt1-1}, \textit{atggt2-1} and \textit{atggt2-2} T-DNA insertion mutants (SALK_080363, SALK_069311 and SALK_147881, ecotype Columbia, Col, Alonso et al., 2003) were identified by a homology search of the Salk Institute Genomic Analysis Laboratory database (http://signal.salk.edu/cgi-bin/tdnaexpress), and were provided by the \textit{Arabidopsis} Biological Resource Center (Ohio State University, Columbus). The \textit{atggt1-2} transposon insertion mutant (CSHL_GT1235, ecotype Larnsberg erecta, Ler) was provided by the Cold Spring Harbor Laboratory (http://genetrap.cshl.edu). The \textit{atggt3-1} transposon insertion mutant (N161036, ecotype Larnsberg erecta, Ler) was identified from the Exotic database (http://www.jic.bbsrc.ac.uk/science/cdb/exotic) and was provided by the Nottingham \textit{Arabidopsis} Stock Centre (University of Nottingham, Loughborough).

Bulk seeds were grown on gardening soil supplemented with 1/1000 Hyponex (Hyponex Japan) once a month. Alternatively, previously sterilized seeds were planted on 1/2 Murashige and Skoog agar medium supplemented with 1.5% sucrose (MSS). After 3 days at 4°C, plants were grown at 25°C under continuous light.

**Genotype Analysis**

\textit{Arabidopsis} genomic DNA was prepared from 12 to 15 T4 segregants according to Liu et al. (1995). The genotypes of the T-DNA insertion lines were verified by genomic PCR, using the T-DNA left border primer LBb1 (5’-GCAGAGAGTCTGAACAATCGCT-3’) and \textit{AtGGT} specific primers. The genotype of the \textit{atggt1-1} mutant was confirmed using LBb1, At1-5.1 (5’-GCAGAGAGTCTGAACAATCGCT-3’) and At1.2-3.1 (5’-TTCCACGGAACGCTTACTGA-3’) primers, and the two \textit{atggt2}
alleles were verified using LBb1, At2-5g.1 (5’-ACATTGACCAACAATA-GATGGA-3’) and At1-2.3-1 primers. On the other hand, the genotype of the transposon insertion line atggt1-2 was confirmed by genomic PCR, using the transposon primer DS3-2 (5’-CGATTCGCTATTTATCCCGTTC-3’), At1-5.1 and At1.2-3.2 (5’-TCAATATCCTGAA-GGAACC-3’) primers. The genotype of the atggt3 mutant was verified using DS3.2 and the AtGGT3 specific primers At3-5.1 (5’-CTAGCTCAGAGTCTTGACT-3’) and At4g29210 3’-2 (5’-CTCCACCTGATAGCTCCT-3’). The PCR products were analyzed as described in a previous paper (Nakano et al., 2006c).

RNA isolation and RT-PCR analysis

Total RNA was prepared from 4 week-old soil grown plants using RNeasy Plant mini kit (QIAGEN, USA) following manufacturer’s instructions. The total RNA was treated with RNase free DNase (Takara, Japan) and reverse transcription was performed using oligo dT primer and ReverTraAce (TOYOBO, Japan) as described by the manufacturer. The synthesized first strand cDNA was treated with RNase H (Takara).

For RT-PCR, the primer set Act2 (5’-GTTGGTGAAGCACAA-3’ and 5’-CAAGACTTCTGGGCATCT-3’) was used to amplify a 425 bp fragment of Actin2 cDNA as the internal standard of gene expression. An 855 bp AtGGT1 cDNA fragment, a 1725 bp AtGGT2 cDNA fragment, and a 456 bp AtGGT3 cDNA fragment were amplified using primer sets AtGGT1 (At1-5.3: 5’-GGGATTCCATCAGGTGTTTC-3’ and At1.2-3.2), AtGGT2 (At2-5.1: 5’-ATGTCGCTCGTTCGTACTGC-3’ and At1.2-3.2) and AtGGT3 (At3-5.2: 5’-CCTCAACCCTGAAATTACCGT-3’ and At4g29210 3’-2), respectively. The expression of AtGGT genes in atggt mutant genotypes was analyzed by RT-PCR, using the following primers: At1-5.1 and At1.2-3.1 primers to amplify a 451 bp AtGGT1 cDNA fragment, At1-5.2: 5’-ATGTCGCTGGTTCGAACAGT-3’ and At1.2-3.2 primers to amplify a 1719 bp AtGGT1 cDNA fragment, At2-5.1 and At1.2-3.2 primers to amplify a 1725 bp AtGGT2 cDNA fragment, and At4g29210 5’-UTR (5’-GAGATAGAGATTGAGATTGAA-G-3’) and At4g29210 3’-2 primers to amplify a 1772 bp AtGGT3 cDNA fragment.

Enzyme Assay

Soluble and bound GGTs were extracted from the samples as previously
described by Nakano et al. (2004). Briefly, the samples were homogenized with 10 vol. of L buffer (20 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM PMSF) supplemented with 5% (w/w) insoluble polyvinylpirrolidone (PVP) and centrifuged at 10,000 x g for 10 min. The supernatant (S-1 fraction), which represents soluble plus microsomal fractions, was recovered and further centrifuged at 100,000 x g for 1 h. After that, the soluble protein fraction (S-2) was recovered from the supernatant. The pellet (P fraction), which represents the microsomal fraction, was resuspended in L buffer. The cell debris pellet derived from the initial 10,000 x g centrifugation was washed twice with L buffer and resuspended in either H buffer (20 mM Tris-HCl, pH 8.0, 1 M NaCl) or T buffer (20 mM Tris-HCl, pH 8.0, 1% Triton X-100). After centrifugation at 10,000 x g for 10 min, the protein fractions extracted with H buffer (CW fraction) and T buffer (PS fraction) were recovered from their corresponding supernatants.

**Preparation of Arabidopsis protoplasts**

*Arabidopsis* protoplasts were prepared and the enzyme activity was measured as described previously (Nakano et al., 2004).

**Measurement of thiol compounds**

Thiol compounds were extracted and analyzed from rosette leaves of 3-week-old *Arabidopsis* plants grown on MSS, and from 15±2 mm siliques and dry seeds from soil grown plants as described elsewhere (Nakano et al., 2006b).

**Results and Discussion**

**Identification of atggt mutants**

In order to undertake the functional characterization of *Arabidopsis* GGT isoforms, we obtained one T-DNA insertion mutant and one transposon insertion mutant alleles of *AtGGT1*, two T-DNA insertion mutant alleles of *AtGGT2*, and one transposon insertion mutant allele of *AtGGT3* (Fig. 1A). The genotype of the putative GGT insertion mutants was confirmed by genomic PCR, and homozygous lines were recovered at the expected 1/4 ratio for single gene insertion. DNA sequencing of the corresponding T-DNA-GGT and transposon-GGT chimeric PCR products indicated that 1) *atggt1-1* had T-DNA inserted in the third *AtGGT1* exon, 2) *atggt1-2* had the transposon inserted in the fifth *AtGGT1* exon, 3) both *atggt2-1* and *atggt2-2* mutant alleles had T-DNA inserted in the second
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AtGGT2 exon, and 4) the transposon insertion in the atgg1-3 mutant allele was localized to the third AtGGT3 exon (Fig. 1A).

On the other hand, RT-PCR gene expression analysis of atggt mutants indicated that atggt1-1, atggt1-2, atggt2-1, atggt2-2, and atggt3-1 showed no de-
detectable *AtGGT1*, *AtGGT2*, and *AtGGT3* mRNA accumulation, respectively, and therefore, they appeared to be null mutant alleles (Fig. 1B). With the exception of *atggt1*, which, as described by Ohkama-Ohtsu et al. (2007a) showed increased chlorosis compared with the wild type (data not shown), the rest of *atggt* mutants grew normally on soil and MSS medium.

**AtGGT gene expression analysis**

*AtGGT* gene expression in different *Arabidopsis* organs was analyzed by the semi-quantitative RT-PCR method. Both *AtGGT1* and *AtGGT3* were expressed in all the tested organs (Fig. 1C). In contrast, the *AtGGT2* gene was mainly expressed in siliques, at a significant level in flowers, but not in roots, stems or rosette leaves (Fig. 1C). These results are similar to the ones described by Ohkama-Ohtsu et al. (2007a,b). Besides, the *AtGGT* expression profiles indicate similarities to those of different radish *RsGGT* genes (Nakano et al., 2006c), and suggest that GGT isoforms may play various roles at the germination, vegetative growth and reproductive stages. On the other hand, *GGT::GUS* expression analysis indicated that *GGT1* expression is limited to the vascular tissue, whereas *GGT2* expression is limited to embryo, endosperm, outer integument, and a small portion of the funiculus in developing siliques (Ohkama-Ohtsu et al., 2007a).

**Distribution of GGT activity in Arabidopsis**

By low-speed centrifugation (10,000 x g for 10 min) of rosette leaf homogenates, we found that *Arabidopsis* GGT activity was localized to both the supernatant (S-1 fraction), which

| Table 1 Fractionation of GGT activities in *Arabidopsis* rosette leaf homogenates |
|-----------------------------------|-------------------|
| Fraction                          | GGT activity (nkat/g fresh wt) |
| 10,000 x g supernatant (S-1)      | 0.256±0.042        |
| 100,000 x g supernatant (S-2)     | 0.235±0.026        |
| 100,000 x g precipitate (P)       | nd                 |
| II buffer extract of 10,000 x g precipitate (CW) | 1.44±0.174 |
| T buffer extract of 10,000 x g precipitate (PS) | nd              |

Values are means±SD (n=3). nd: not detected.
contained soluble and microsomal protein fractions, and the corresponding cell debris pellet, which contained the crude cell wall fraction and other cellular components (Nakano et al., 2004; Table 1). Here, we proceeded further with the analysis of Arabidopsis GGT distribution by conducting high-speed centrifugation (100,000 x g for 1 h) of the S-1 protein fraction in order to obtain soluble protein fraction (S-2) and microsomal fraction (P). The results indicate that the GGT activity of the S-1 fraction was essentially recovered in the S-2 fraction, but not in the P fraction (Table 1). These results are in agreement with the distribution of GGT activity in radish cotyledons (Nakano et al., 2004) and indicate the existence of soluble GGT, but not microsomal bound GGT, in rosette leaves. As a consequence, the GGT activity that localized to the Arabidopsis S-1 fraction will be subsequently referred to as soluble GGT activity.

As in the case of radish bound GGT activity, which is mainly associated with the cell wall (Nakano et al., 2004), the Arabidopsis GGT activity that localized to the cell debris pellet could be solubilized with high ionic strength buffer containing 0.5 M NaCl (CW fraction), but not with 1% Triton X-100 (PS fraction) (Table 1). Moreover, as reported in radish protoplast (Nakano et al., 2004), cell wall degradation of Arabidopsis cells also led to the disappearance of NaCl extractable GGT activity but not that of the soluble one (Table 2). These results indi-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Extraction buffer</th>
<th>GGT activity (nkat/mg chlorophyll)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosette leaf</td>
<td>L</td>
<td>0.138±0.021</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>2.59±0.17</td>
</tr>
<tr>
<td>Protoplast</td>
<td>L</td>
<td>0.118±0.021</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>0.122±0.028</td>
</tr>
</tbody>
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Enzyme was extracted with L buffer or H buffer, and 5% insoluble PVP from the plant materials. The homogenate was centrifuged at 10,000 x g for 10 min at 4°C and GGT activity in each supernatant was measured. Values are means±SD (n=3).
cate that this type of *Arabidopsis* GGT activity is associated with the cell wall. The GGT activity that localized to the CW fraction will be subsequently referred to as bound GGT activity.

**Fractionation analysis of GGT activity in atggt mutants**

The *atggt1* mutant alleles presented soluble GGT activity similar to those of wild type plants in siliques (Fig. 2A). In contrast, bound GGT activity in *atggt1* mutants was not significant compared with those of wild type plants (Figs. 2B). Similar results were found when comparing soluble and bound GGT activities in leaves of wild type and *atggt1* plants (data not shown). These results indicate that *AtGGT1* encodes the main *Arabidopsis* bound GGT. Based on the heterologous expression of *Arabidopsis* *AtGGT1* cDNA in tobacco plants, it was proposed that *AtGGT1* encodes a heterodimeric GGT that is localized to the

![Fig. 2 GGT activity in wild type and atggt mutants. GGT activity in soluble (A) and bound (B) protein fractions was extracted from siliques as described in the manuscript. Values are means±SD (n=3).](image)

plasma membrane outside tobacco transgenic cells (Storozhenko et al., 2002). However, our results clearly indicate that AtGGT1 is not localized to the Arabidopsis plasma membrane; rather, it is likely localized to the cell wall, similar to radish bound GGT (Nakano et al., 2004). These results agree with those of Martin et al. (2007), who suggested that AtGGT1 is associated with a particulate fraction via an ionic interaction, and partially agree with those published by Ohkama-Ohtsu et al. (2007a) who proposed that AtGGT1 is a protein that may be bound to both the cell wall and the plasma membrane.

Soluble GGT activity in siliques of atggt2-1 and atggt2-2 was ca. 25% of those of wild type plants (Fig. 2A). In contrast, bound GGT activity was identical in wild type and atggt2 mutants (Fig. 2B). Results indicate that AtGGT2 is a soluble GGT that is mainly expressed in siliques. Analysis of an atggt2 knockout mutant suggested that AtGGT2 is a soluble protein which is extracellularly localized when expressed in leaves (Martin et al., 2007). On the other hand, analysis of atggt2 knockout mutants suggested that, when expressed in siliques, AtGGT2 is membrane bound or soluble but associated with storage bodies (Martin et al., 2007). In contrast, Ohkama-Ohtsu et al. (2007a) suggested that, in a similar way to AtGGT1, AtGGT2 is an apoplastic enzyme that is only partially extracted under low-salt conditions.

As illustrated in Fig. 2A, atggt3-1 and wild type plants had similar levels of bound GGT activity in siliques. In contrast, GGT soluble activity in the atggt3-1 knockout plant was approximately 85% of that in wild type. Ohkama-Ohtsu et al. (2007b) and Grzam et al. (2007) have proposed that AtGGT3 is a soluble protein that catalyzes the first step in the metabolism of GSH conjugates in the vacuole.

**GSH degradation analysis**

Ohkama-Ohtsu et al. (2007a) have suggested that AtGGT1 is important in preventing oxidative stress by metabolizing extracellular GSSG in the apoplast. However, they could not find significant differences between wild type and atggt1 plants in terms of the GSH levels in leaves, roots, stems, flowers, flower buds and siliques. In a similar way, our determination of GSH contents revealed that there were no significant differences in the steady-state levels of GSH in rosette leaves, siliques and mature seeds between atggt1 and wild type plants (data
not shown). However, these results may be explained by the low GSH degradation rate in \textit{atggt1} mutants that might be compensated by a decrease in the GSH biosynthetic rate. In order to verify such a possibility, we monitored the time course of GSH degradation in the presence of L-buthionine-[S, R]-sulfoximine (BSO), a widely used GSH biosynthesis inhibitor that targets \(\gamma\)-L-glutamyl-L-cystein synthetase. Rosette leaves of non-treated wild type and \textit{atggt1-1} plants presented similar GSH contents (Fig. 3A). In contrast,

Fig. 3  GSH (A) and cysteine (B) contents in \textit{Arabidopsis} leaves. Rosette leaves of MSS grown wild type (circles) and \textit{atggt1-1} mutant (squares) were excised and transferred onto either MSS (closed symbols) or MSS supplemented with 1 mM BSO (open symbols) and incubated for 4-8 h at 25°C under continuous light. Values are means±SD (n=3).
after 8 h of BSO treatment, the GSH contents in wild type and atgg1-1 leaves gradually decreased to 44% and 67% of the contents of the untreated counterparts. We could not detect a significant amount of L-cysteinylglycine, the first product of the GGT-mediated GSH catabolic pathway, in any of the samples (data not shown). However, we determined that the cysteine contents were essentially the same in non-treated rosette leaves of wild type and atgg1-1 plants, whereas after 8 h of BSO treatment, rosette leaves of wild type plants contained approximately twice the amount of cysteine compared with those of the atgg1-1 mutant (Fig. 3B). Taken together, these results indicate that AtGGT1 is involved in Arabidopsis apoplastic GSH catabolism.

Our findings indicate differences in the cellular localization of Arabidopsis heterodimeric GGT isoforms and the involvement of at least one of them, AtGGT1, in GSH catabolism. The complex biological significance of the different Arabidopsis GGT isoforms should be determined in further studies.

Acknowledgments

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References


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17. Suzuki, H., Hashimoto, W., Kumagai, H. (1993) *Escherichia coli* K-12 can utilize an exogenous \( \gamma \)-glutamyl peptide as an amino acid source, for which \( \gamma \)-glutamyltranspeptidase is essential. *J. Bacteriol.* 175, 6038-6040.


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