FEBS Letters 581 (2007) 3131–3138

γ-Glutamyl transpeptidase GGT4 initiates vacuolar degradation of glutathione *S*-conjugates in *Arabidopsis*

Anke Grzam[a], Melinda N. Martin[b], Rüdiger Hell[a], Andreas J. Meyer[a,*]

[a] *Heidelberg Institute of Plant Sciences, University of Heidelberg, Im Neuenheimer Feld 360, D-69120 Heidelberg, Germany*
[b] *Biotechnology Center for Agriculture and the Environment, Rutgers University, New Brunswick, NJ, USA*

Received 24 April 2007; accepted 23 May 2007

Available online 4 June 2007

Edited by Ulf-Ingo Flügge

Abstract **The xenobiotic monochlorobimane is conjugated to glutathione in the cytosol of *Arabidopsis thaliana*, transported to the vacuole, and hydrolyzed to cysteine *S*-bimane [Grzam, A., Tennstedt, P., Clemens, S., Hell, R. and Meyer, A.J. (2006) Vacuolar sequestration of glutathione *S*-conjugates outcompetes a possible degradation of the glutathione moiety by phytochelatin synthase. FEBS Lett. 580, 6384–6390]. The work here identifies γ-glutamyl transpeptidase 4 (At4g29210, GGT4) as the first step of vacuolar degradation of glutathione conjugates. Hydrolysis of glutathione *S*-bimane is blocked in *ggt4* null mutants of *A. thaliana*. Accumulation of glutathione *S*-bimane in mutants and in wild-type plants treated with the high affinity GGT inhibitor acivicin shows that GGT4 is required to initiate the two step hydrolysis sequence. GGT4:green fluorescent protein fusions were used to demonstrate that GGT4 is localized in the lumen of the vacuole.**

Keywords: γ-Glutamyl transpeptidase; Glutathione-conjugate degradation; Monochlorobimane; Vacuolar sequestration

1. Introduction

Plants take up many toxic xenobiotics from their growth environment with relative indiscrimination but are able to detoxify or sequester these compounds by a number of pathways [1]. Detoxification of many electrophilic xenobiotics begins by reaction with reduced glutathione (GSH). Xenobiotics are covalently bound to the sulfhydryl group of GSH in a reaction catalyzed by glutathione *S*-transferases (GSTs) [2]. Following the conjugation in the cytosol, the glutathione *S*-conjugates undergo further metabolism which results in transient accumulation of degradation products [3,4]. A vacuolar carboxypeptidase activity that cleaved the Gly residue as the initial reaction with the concomitant accumulation of γ-GluCys-conjugates was shown in barley vacuoles [5]. In contrast to barley, *Arabidopsis thaliana* plants challenged with xenobiotics accumulate mainly Cys *S*-conjugates and only minute amounts of intermediate degradation products [6]. Accumulation of Cys *S*-conjugates implies that the two initial steps in glutathione *S*-conjugate metabolism involve cleavage of Glu and Gly from the GSH-moiety of the conjugates. The absence of intermediates showed that the first degradation step limits the overall reaction but provided no information as to whether the Glu or the Gly residue are cleaved first [6]. Recent reports implicate two different enzymes, phytochelatin synthase (PCS) and γ-glutamyl transpeptidase (GGT), as catalysts for the initial step of glutathione *S*-conjugate degradation in *Arabidopsis* [7,8]. Cytosolic PCS is able to initiate glutathione *S*-conjugate metabolism by removal of the Gly residue [7,9]. It needs to be considered that PCS is almost inactive unless plants are exposed to heavy metal contamination. Recent reports showed that in the absence of heavy metals vacuolar sequestration of glutathione *S*-conjugates occurs much more rapidly than hydrolysis of the glutathione moiety by PCS in the cytosol [6,10].

Significant activity towards glutathione *S*-conjugates was also shown for a vacuolar GGT [8]. Given that glutathione *S*-conjugates are rapidly sequestered to the vacuole, degradation of at least a significant proportion within the vacuole seems more plausible than degradation in the cytosol as proposed by Blum et al. [7]. GGTs are the only enzymes known to hydrolyze the unique amide bond linking the γ-carboxylic acid of Glu to Cys in GSH. Several GGT isoforms have been purified from plant species including tomato, onion, and radish [11–13]. Like most GGTs from animal tissues, the GGTs from these plants exhibited broad substrate specificity and were able to hydrolyze GSH and several glutathione *S*-conjugates [14,15]. The bulk of the GGT activity in these plants was localized in a pellet fraction and extractable only with high molarity NaCl, suggesting ionic association possibly with the cell wall. However, Nakano and colleagues showed that a less abundant soluble GGT from radish is localized to the vacuole and is able to degrade glutathione *S*-bimane (GSB) [13,16]. In the yeast *Saccharomyces cerevisiae*, GGT is a membrane bound vacuolar protein [17].

In *Arabidopsis* four GGT genes have been identified. According to gene symbols registered with the *Arabidopsis* information resource (TAIR) these genes are named GGT1 (At4g39640), GGT2 (At4g39650), GGT3 (At1g69820), and GGT4 (At4g29210) [18,19]. GGT1, GGT2, and GGT3 exhibit between 80% and 90% identity among each other and have

*Corresponding author. Fax: +49 6221 54 5859.
E-mail address: ameyer@hip.uni-hd.de (A.J. Meyer).

Abbreviations: CHX, cycloheximide; CLSM, confocal laser scanning microscopy; Cys-B, cysteine *S*-bimane; CysGly-B, cysteinylglycine-bimane; GGT, γ-glutamyl-transpeptidase; GSB, glutathione *S*-bimane; GSH, reduced glutathione; GST, glutathione *S*-transferase; LMWT, low molecular weight thiol; MBB, monobromobimane; MCB, monochlorobimane; PCS, phytochelatin synthetase

0014-5793/$32.00
doi:10.1016/j.febslet.2007.05.071

highly conserved intron/exon structures. GGT4 is divergent from the other GGTs with homology of about 50% [18–20]. Heterologous expression of *At*GGT1 in tobacco and analysis of GGT1 knockouts indicated that GGT activity is localized outside the plasma membrane and likely associated ionically with the cell wall [18–20]. The other GGTs have not been characterized in detail and GGTs were not identified in recent surveys of the vacuolar proteome [21,22]. However, prediction of subcellular targeting of the four GGTs using the combination of algorithms assembled at the SubCellular Proteomic Database (SUBA) site (http://www.plantenergy.uwa.edu.au.application/suba/flatfile/) identified GGT4 as possibly targeted to the vacuole [23].

Fluorescent in situ labelling of GSH with monochlorobimane (MCB) offers the unsurpassed opportunity to trace the GSH-dependent detoxification pathway in living cells [10]. The initially non-fluorescent and membrane-permeable MCB is conjugated to GSH in a reaction catalyzed by GSTs leading to formation of fluorescent glutathione *S*-bimane (GSB). After vacuolar sequestration, the fluorescence is not affected by the first two hydrolysis reactions that lead to formation of cysteine *S*-bimane (Cys-B). The persistent fluorescence also allows extraction of MCB labelled thiols from tissues for direct analysis [6].

In situ labelling of metabolites with MCB in combination with reverse genetics were used to show that GGT4 is the vacuolar enzyme responsible for cleaving the γ-glutamyl residue from GSB as the initial glutathione *S*-conjugate degradation step following exposure of *Arabidopsis* to toxic levels of xenobiotics.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. (accessions Landsberg *erecta*, L*er* and Columbia, Col-0) and 2 mutant lines, lacking GGT4 (At4g29210) were used. The transposon insertion mutants designated *ggt4-1* and *ggt4-2* both in background L*er* were obtained from the EU Exon Trapping Consortium (Exotic-GT-5-57895) and Cold Spring Harbor Laboratory (CSHL-GT11203), respectively [24].

For experiments with leaf material, plants were sown on soil and grown for 8–10 weeks at 21 °C under short day conditions (9 h light; 15 h dark) in a controlled growth chamber. For in situ experiments with root material, plants were grown hydroponically. Seeds were sterilized and sown on growth medium containing 0.8% agar in 0.5 ml Eppendorf tubes with bottoms removed. These seed holders were placed on containers filled with 0.5× Hoagland medium and plants were grown for 10 weeks.

2.2. Analysis of mutant lines

For verification of the insertion site, genomic DNA was extracted from 20 d old plants grown at 24 °C with 16 h light; 8 h dark. PCR amplification was performed using an Extract N-Amp Kit (Sigma Chemical Co., St. Louis, MO) and primers specific for the transposon LB and a flanking GGT4 sequence. Primers used were: GGT4 specific FP, aatcggtggtggttctttcttgatt; GGT4 specific RP, tgttgagaaatcatccatctcgttg; and Exotic-GT-TPS-3′DS, acccgaccggatcgtatcggt. Amplified products were sequenced to verify the insertion site.

Homozygous plants of each line were selected on kanamycin and were also confirmed by PCR. Knockout of gene function was confirmed by RT-PCR. RNA was extracted from wild-type and mutants using TRIzol reagent (Invitrogen, Carlsbad, CA), first strand cDNA synthesized using Superscript II reverse transcriptase (Invitrogen), and products were amplified by PCR using Platinum Taq DNA Polymerase (Invitrogen) and the gene specific primers indicated above. Actin 2 (ACT 2) was used as an internal control.

2.3. DNA constructions and plant transformation

For transient expression of GGT4:GFP fusions, a short N-terminal fragments of 85 amino acids of AtGGT4 cDNA (RAFL15-32-I09; RIKEN Genomic Sciences Centre, Japan) were amplified and cloned with *Bam*HI and *Sal*I in front of eGFP in the vector pFF19 [25]. Transformation of onion epidermal cells and *Arabidopsis* leaves was done by particle bombardment. Gold particles were prepared according to the manufacturers protocol (Bio-Rad, München, Germany) and the particle suspension was spotted to macro carriers with 3–6 μg DNA per shot. Tissues were subjected to particle bombardment at 650 psi (onion) or 900 psi (*Arabidopsis*) (Biolistic PDS-1000-He; Bio-Rad). The bombarded tissue was kept in Petri dishes on wet filter paper for 24–72 h at room temperature in the dark.

2.4. Leaf infiltration for GSH labelling

For analysis of GSB and its degradation products, infiltration, incubation, harvesting and reverse-phase HPLC analysis of leaf samples was performed as described [6]. Where indicated, buffers for leaf infiltration and subsequent incubation were supplemented with 500 μM acivicin and 1.4 mM cycloheximide (CHX) as inhibitors for degradation and protein biosynthesis as indicated. For analysis of gene expression 100 mg leaf material was used. This material was infiltrated with either buffer (control) or with buffer containing 300–500 μM MCB or monobromobimane (MBB). All other conditions for infiltration, incubation and harvesting were the same as described above.

2.5. Root incubation for GSH labelling

Roots of 10 weeks old hydroponically grown plants were cut off and washed with infiltration buffer. After careful removal of adhering water 150–250 mg fresh root material was cut in pieces of 4–8 mm length and placed in fresh infiltration buffer. The root material was incubated in MCB solution for 15 min at RT in the dark. After washing twice with fresh incubation buffer, a sample of 50–100 mg was taken immediately after washing (time point 0), and two further samples were taken after extended incubation for 5 h and 24 h at RT in the dark. Extraction and analysis of thiol bimane conjugates was performed as described for leaves. Due to different labelling efficiencies and to avoid possible errors with determination of the fresh weight of small root samples the amounts of GSB and its degradation products are presented as % of all recovered bimane-labelled low molecular weight thiols (LMWT).

2.6. Confocal microscopy

Imaging of bimane conjugates formed in situ after labelling of GSH with 100 μM MCB was done as described earlier [6,10]. GFP fluorescence was observed with either a 25× multi-immersion lens (Plan-Neofluar, NA 0.8, Zeiss, Jena, Germany) or a 63× water-immersion lens (C-Apochromat, NA 1.2; Zeiss). Images were assembled in Photoshop (Adobe Systems).

3. Results

3.1. Identification and molecular characterization of ggt4 insertional mutants

To investigate the role of GGT4 in the metabolism of glutathione *S*-conjugates, two independent transposon insertion lines for this gene were identified. The GGT4 gene consists of a 3300 bp open reading frame, and the gene model, supported by cDNAs, shows 5 exons (Fig. 1A). Each knockout line contained a single locus transposon insertion in exon 3. The sequence of DNA spanning the insertion site in each mutant was determined to confirm the position of insertion and is shown in Fig. 1A. The lines were designated *ggt4-1* and *ggt4-2*.

Neither *ggt4-1* nor *ggt4-2* differed from wild-type plants at any stage during development when grown under several different growth conditions. RT-PCR analysis with mRNA from 20-day-old leaves established that both insertion lines are indeed null mutants with respect to GGT4 expression