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Short communication

Immunolocalization of glutathione biosynthesis enzymes in *Arabidopsis thaliana*

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ABSTRACT

In plants, glutathione serves as a versatile redox buffer and cellular protective compound against a range of biotic and abiotic stresses. Glutathione production involves glutamate-cysteine ligase (GCL), the redox-regulated limiting enzyme of the pathway, and glutathione synthetase (GS). Because the sub-cellular and sub-organellar localization of these enzymes will have an impact on metabolism, here we examine the localization of GCL and GS in the leaves of *Arabidopsis thaliana*. Immuno-electron microscopy of leaf cells indicates localization of GCL primarily to the chloroplast with GS found in both the chloroplast and cytosol. Detailed examination of the localization of both enzymes within chloroplasts was performed using fractionation followed by immunoblot analysis and indicates that GCL and GS are found in the stroma. The localization of these enzymes to the stroma of chloroplasts has implications for the redox-regulation of GCL and plant glutathione biosynthesis.

1. Introduction

Glutathione is an important tripeptide in plants that helps protect the organism from oxidative stresses (Ogawa, 2005; Galant et al., 2011). As part of the ascorbate–glutathione cycle, glutathione acts as a major redox buffer to ameliorate the effect of reactive oxygen species and is used for the detoxification of various xenobiotic molecules (Ogawa, 2005; Galant et al., 2011). In plants, glutathione is distributed widely across tissue and cell type, but often shows differences in levels between cellular compartments and during changing growth conditions (Fernandez-Garcia et al., 2009; Zechmann and Müller, 2010; Koffler et al., 2011, 2013; Heyneke et al., 2013). The biosynthesis of glutathione occurs in two enzymatic steps (Fig. 1). The first reaction is catalyzed by glutamate cysteine ligase (GCL; also known as γ-glutamylcysteine synthetase; E.C. 6.3.2.2), which synthesizes γ-glutamylcysteine from glutamate, cysteine, and ATP (Ravilious and Jez, 2012; May and Leaver, 1994; Jez et al., 2004; Musgrave et al., 2013). Next, glutathione synthetase (GS; E.C. 6.3.2.3) catalyzes the ATP-dependent reaction that links γ-glutamylcysteine and glycine to yield glutathione (Ravilious and Jez, 2012; Musgrave et al., 2013; Jez and Cahoon, 2004; Herrera et al., 2007; Galant et al., 2009). Of the two enzymes, GCL is the primary control enzyme for glutathione synthesis and is regulated by a range of metabolic and post-translational regulatory mechanisms (Yi et al., 2010). In response to a multiple oxidative stresses, GCL activity in *Arabidopsis thaliana* (thale cress) undergoes rapid post-transcriptional activation (May et al., 1998). Subsequent work identified thiol-based redox-regulation as a critical feature for modulating GCL activity in plants (Fig. 1) (Fernandez-Garcia et al., 2009; Hothorn et al., 2006; Hicks et al., 2007; Gromes et al., 2008). The oxidized dimeric form of GCL is fully active, whereas, transition to a reduced monomeric form yields a less active enzyme. Structural and functional studies of the GCL from *A. thaliana* and *Brassica juncea* (Indian mustard) demonstrate that intramolecular disulfide bonds play key roles in the regulation of GCL in response to oxidizing and reducing conditions (Fernandez-Garcia et al., 2009; Hothorn et al., 2006; Hicks et al., 2007; Gromes et al., 2008).

The sub-cellular and sub-organellar localization of GCL will play a major role in the activity of the enzyme because the multiple compartments of eukaryotic cells have different redox environments. Early biochemical studies in tobacco suspension cells indicated that GCL activity is found primarily in the chloroplasts with GS activity detected in both the cytosol and chloroplast (Hell and Bergmann, 1988, 1990). Based on transcript analysis and reporter fusions, GCL appears targeted to the plastid with localization of GS

Abbreviations: BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; GCL, glutamate cysteine ligase; GS, glutathione synthetase; RbcL, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit; TBS-T, Tris-buffered saline with Tween-20.

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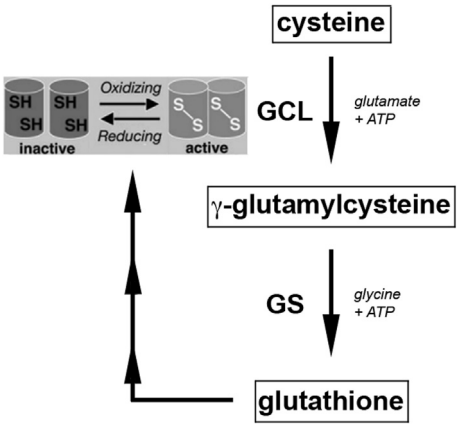

Fig. 1. Overview of glutathione biosynthesis and regulation. Synthesis of glutathione requires glutamate-cysteine ligase (GCL) and glutathione synthetase (GS). Redox environment mediated by glutathione helps to regulate the form of GCL between an active oxidized dimeric form and a less active reduced monomeric form.

to both the plastid and the cytosol (Wachter et al., 2005). Within the chloroplast, there are different compartments with distinctive redox environments. Through the capture of light energy and movement of electrons during photosynthesis, a reducing environment is found within the stroma versus an oxidizing environment in the thylakoid lumen (Rouhier et al., 2008). Therefore, an understanding of its precise localization is needed to fully understand the biochemical activity and regulation of GCL. To date, no studies have been performed to directly confirm the sub-cellular and/or sub-organellar localization of either GCL or GS in *A. thaliana*.

Here we present a detailed analysis of the localization of GCL and GS in the leaves of *A. thaliana*. For localization of native GCL and GS in *A. thaliana*, immuno-electron microscopy was performed on leaf cells. Furthermore, to understand the native environment in which GCL and GS are found, chloroplast fractionation followed by immunoblot analysis was performed to determine their sub-organellar localization. The localization of the glutathione biosynthesis enzymes to the stroma of chloroplasts has implications for the redox-regulation of GCL.

2. Materials and methods

2.1. Antibodies

Recombinant *A. thaliana* GCL and GS proteins were expressed and purified as previously described (Jez et al., 2004; Jez and Cahoon, 2004). The purified proteins were used to raise polyclonal antibodies (α-GCL and α-GS) in rabbits (Washington University Antibody Facility) (Hicks et al., 2007). Antibodies to the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (α-RbcL) were purchased from Agrisera. The α-33 kDa rabbit antibody raised to the lumen-localized PsbO protein of photosystem II (α-PsbO) was a gift of the Pakrasi lab (Thornton et al., 2004). Secondary anti-rabbit antibodies conjugated to alkaline phosphatase (Sigma) were used for immunoblot analysis. For immuno-electron microscopy, anti-rabbit antibodies conjugated to 15 nm gold particles were used (BB International).

2.2. Immuno-electron microscopy

A. thaliana (ecotype Columbia) plants were grown for four weeks in controlled environment room under a short day 10-h-light/14-h-dark cycle at constant temperature of 22 °C, 60% relative humidity, and light intensity of 160 μE s^{-1} m^{-2}. Older leaves (~5 × 5 cm) were harvested from plants and leaf discs (~1 mm) loaded in hexadecane (Acros Organics), frozen in a Baltec HPM 010 high-pressure freezer (Technotrade), and transferred to liquid nitrogen. Substitution was performed in 0.1% (w/v) uranyl acetate (Sigma Aldrich) plus 0.25% (v/v) glutaraldehyde (Sigma Aldrich) in acetone at −80 °C for 172 h and warmed to −50 °C slowly over 10 h. After three acetone rinses, samples were infiltrated with Lowicryl HM20 (Electron Microscopy Sciences) for 48 h and polymerized at −50 °C under UV light for 24 h. Sections were mounted on formvar-coated nickel grids and blocked for 30 min with 2% (w/v) fetal bovine serum albumin (BSA) in Tris-buffered saline with 0.05% (v/v) Tween-20 (TBS-T). Sections were incubated with primary antibody (either α-GCL or α-GS) for 1 h at room temperature. Sections were rinsed with TBS-T and transferred to the anti-rabbit secondary antibodies conjugated to 15 nm gold particles for 1 h. Controls were performed in the same manner but with omission of the primary antibody and incubation with anti-rabbit secondary antibodies conjugated to 15 nm gold particles. Sections were observed using a Leo 912 transmission electron microscope (Zeiss) in the Donald Danforth Plant Science Center Microscopy Facility.

2.3. Chloroplast fractionation

A. thaliana seedlings (ecotype Columbia) were grown as described above. Leaves were collected and chopped finely with a razor blade in homogenization buffer (0.45 M sorbitol, 20 mM Tricine·KOH pH 8.4, 10 mM ethylene diamine tetraacetic acid (EDTA), 10 mM $NaHCO_3$, 0.1% (w/v) fatty acid-free BSA, and 1× protease inhibitors (Roche)). The mixture was filtered through cheesecloth and spun at 1500 × g at 4 °C. The supernatant from this spin is labeled as "cytosol", although it likely contains apoplast and broken organelles as well. Pelleted chloroplasts were suspended in resuspension buffer (0.3 M sorbitol, 20 mM Tricine·KOH pH 7.6, 5 mM $MgCl_2$, and 2.5 mM EDTA), layered over a preformed Percoll gradient, and centrifuged at 5000 × *g* (4 °C). A thin band of intact chloroplasts were collected, diluted in resuspension buffer, and centrifuged again at 3500 × *g* (4 °C). Chloroplasts were resuspended in swelling buffer (20 mM Tricine pH 7.6, 4 mM $MgCl_2$, and 1× protease inhibitors) and left on ice for 10 min to lyse the chloroplasts. The sample was centrifuged at 100,000 × *g* (4 °C) to separate the thylakoids from the stroma. Protein concentration of each fraction was measured by Bradford assay (Pierce) using bovine serum albumin as a standard and 1 μg of total protein was loaded in each lane of a 12% SDS-PAGE gel. Proteins were blotted onto a nitrocellulose membrane and probed with antibodies. Primary antibodies used were α-GCL, α-GS, α-RbcL (Agrisera Co.), and α-PsbO. Alkaline phosphatase-conjugated secondary anti-rabbit antibodies were used for detection of protein bands.

2.4. Enzyme assays

Assays for GCL and GS activity in organelle and sub-cellular fractions in the presence and absence of either 10 mM dithiothreitol (DTT^{red}) or 10 mM 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) were performed as previously described (Jez et al., 2004; Jez and Cahoon, 2004; Hicks et al., 2007).

3. Results

3.1. Sub-cellular localization of GCL and GS by immuno-electron microscopy

Previous studies suggest that GCL is localized to the chloroplasts and GS is localized both to chloroplasts and cytosol in plant cells