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ORIGINAL ARTICLE

Glutathione is not involved in light-, Dark-, Ca- and H_2O_2-induced stomatal movement in *Arabidopsis*

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Glutathione (GSH), is a thiol-containing tripeptide, maintains redox homeostasis in plants under normal and stressful conditions. In this study, we investigated whether GSH involved in light-, dark-, Ca- and H_2O_2-induced stomatal movement in *Arabidopsis.* Application of GSH and a GSH decreasing chemical (CDNB; 1-chloro-2,4-dinitrobenzene) did not affect stomatal aperture in guard cells of *Arabidopsis.* Dark induced stomatal closure and light induced stomatal opening but pre-treatment of GSH and CDNB did not alter dark- and light-induced stomatal aperture. Treatment of guard cells with Ca and H_2O_2 did not affect GSH contents in guard cells but induced stomatal closure in both wild type and *chorinal-1 (ch1-1)* mutant plants. In addition, pre-treatment of GSH and CDNB did not affect Ca- and H_2O_2-induced stomatal closure in both plants. Taken together these results suggest that GSH might not directly affect light-, dark-, Ca- and H_2O_2-induced stomatal movement in guard cells of *Arabidopsis*.

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Glutathione has many functions including sulfur metabolism, regulation of growth and development, cell defence, redox signalling, and regulation of gene expression (May *et al.*, 1998; Noctor and Foyer, 1998). Different factors, atmospheric pollutants, biotic and abiotic stress, and light, affect glutathione contents in plant (Alscher, 1989; Sánchez-Fernández *et al.*, 1997). Therefore, control of GSH content in plants can be expected to have important consequences through modification of metabolic functions in plant cells. Glutathione contents in aerial parts of *ch1-1* mutant plants are lower than wild type plants (Ogawa *et al.*, 2004, Jahan *et al.*, 2008, Jahan *et al.*, 2011).

Increment of $[Ca^{2+}]_{cyt}$ in guard cells is closely related to stomatal closure (McAinsh *et al.*, 1995). In previous, several authors stated that ABA and H_2O_2 elicits I_{Ca} currents and $[Ca^{2+}]_{cyt}$ oscillation in guard cells (Allen *et al.*, 1999; Pei *et al.*, 2000;

Köhler *et al.*, 2003, Okuma *et al.,* 2011). The glutathione peroxidise-3 (ATGPX3) protein involved in scavenging H_2O_2. Therefore, the *Arabidopsis* mutant *atgpx3* plants are less sensitive to ABA than wild type (Miao *et al.*, 2006). Glutathione levels enhanced ABA sensitivity to guard cells (Jahan *et al.*, 2008; Okuma *et al.,* 2011). Increment of GSH levels in guard cells decreased Allylisothiocyanate sensitivity to stomatal closure (Khokon *et al.,* 2011). However, the effect of GSH contents on light-, dark-, Ca- and H_2O_2-induced stomatal movement in guard cells remained unclear.

To date, very few reports have been published on GSH function in guard cells of *Arabidopsis.* Recently Jahan *et al.* (2011) stated that GSH can be quantified in guard cells. In order to understand the function of GSH contents on light-, dark-, Ca- and H_2O_2-induced stomatal movement in *Arabidopsis*, we presented results in this report that GSH contents might not directly involve in light-, dark-, Ca- and H_2O_2-induced stomatal movement in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions

We used *Arabidopsis* wild type, ecotype Columbia (Col-0), and *ch1-1* mutant plants in this study. Plants were grown in a plastic pot (5.5 d × 5.5 h) according to (Jahan *et al.*, 2008; Jahan *et al.*, 2011).

Measurement of stomatal aperture

Stomatal assay was prepared and aperture was measured as described previously (Jahan *et al.*, 2008; Jahan *et al.*, 2011). Rosette leaves from 5 to 6 week-old plants were incubated on solution containing 10 mM KCl, 50 µM $CaCl_2$, and 10 mM Mes-Tris (pH 6.15) under light for 2 h to facilitate stomatal opening and/or followed by 2 h light incubation with GSH or CDNB or Ca or H_2O_2. Then stomatal aperture was measured under Leica DM5000B fluorescence microscope connected with digital imaging color camera and image analysis software. There were twenty stomatal apertures were measured for each replicate.

Measurement of GSH content in guard cells

Glutathione contents in guard cells were quantified using monochlorobimane (MCB) according to Jahan *et al.* (2011). Epidermal peels were incubated in a 100 µM MCB staining solution for 2 h at room temperature. Leaf was attached onto a microscope slide glass with adhesive then cuticle and upper mesophyll layer were carefully removed. Fluorescence intensity of GSB in guard cells was observed under a fluorescent microscope (Leica DM5000B fluorescence microscope**,** Germany). The fluorescence of guard cell image was captured and pixels/intensity of the fluorescence was measured using Adobe Photoshop CS3 software (Adobe Systems Inc. San Jose, CA).

Statistical analysis

Student's t-test was used to assess significance of differences between mean values.

Accession numbers

Arabidopsis Genome Initiative number for the genes discussed in this article is as follows: *CH1-1*, At1g44446.

RESULTS

Increment or depletion of GSH in guard cell did not affect stomatal aperture

Whether GSH content directly affect stomatal aperture, we measured stomatal aperture after guard cells were treated with GSH which increases GSH and CDNB which decreases GSH. When guard cells were treated with different concentration of

GSH, stomatal apertures were similar to GSH untreated guard cell (Fig. 1, open bars). Whether depletion of GSH affects stomatal aperture, we measured CDNB-induced stomatal aperture. Figure 1 (closed bars) showed that CDNB-induced stomatal aperture was similar to CDNB-untreated guard cells. These results suggest that increment or depletion of GSH in guard cells might not affect stomatal aperture in guard cells of *Arabidopsis* plants.

Effects of GSH content on dark- and light-induced stomatal aperture

Dark induced stomatal closure and light induced stomatal opening (Shimazaki *et al.*, 2007). Whether GSH content affects dark- and light-induced stomatal aperture in guard cells, we tested dark- and light-induced GSH content and stomatal aperture in guard cells of *Arabidopsis*. We found that the treatment of dark decreased but light increased GSH content in guard cells of *Arabidopsis* plants (Fig 2a). We measured dark- and light-induced stomatal aperture after guard cells were treated with CDNB and GSH. Figure 2b showed that dark-induced stomatal closure and light-induced stomatal opening were similar to that of guard cells were pre-treated with CDNB and GSH. These results indicate that GSH might not affect light- and dark-induced stomatal movement in guard cells of *Arabidopsis.*

Effects of GSH content in Ca^{+2}- and H_2O_2-induced stomatal aperture

In previous, several authors stated that many signalling compounds enhanced Ca^{+2} concentration and H_2O_2 production in guard cell. Whether GSH content mediates Ca^{+2}- and H_2O_2-induced stomatal closure, we measure Ca and H_2O_2 induced GSH content and stomatal closure in guard cells. The treatment of Ca and H_2O_2 did not affect GSH content in guard cells of wild types and *ch1-1* mutant plants (Fig 3a). But both Ca and H_2O_2 treatments significantly enhanced stomatal closure in both plants (Fig. 3b) which were consistent with previous results (Munemasa *et al.*, 2006). In addition, pre-treatment of GSH and CDNB did not affect Ca- and H_2O_2-induced stomatal closure (Fig. 3c). These results indicated that GSH content in guard cells did not increase Ca^{+2} and H_2O_2 sensitivity to guard cells of *Arabidopsis.* Taken together, our results suggest that GSH might not affect Ca- and H_2O_2-induced stomatal movement in guard cells of *Arabidopsis.*

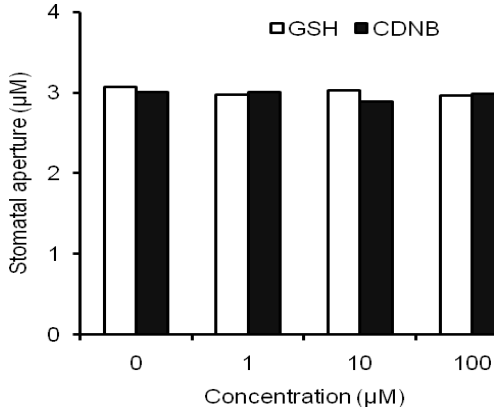

Figure 1. Glutathione and CDNB induced stomatal aperture. Excised leaves of wild type plants were treated with different concentration of GSH. Averages from three independent experiments (60 total stomata per bar) are shown.

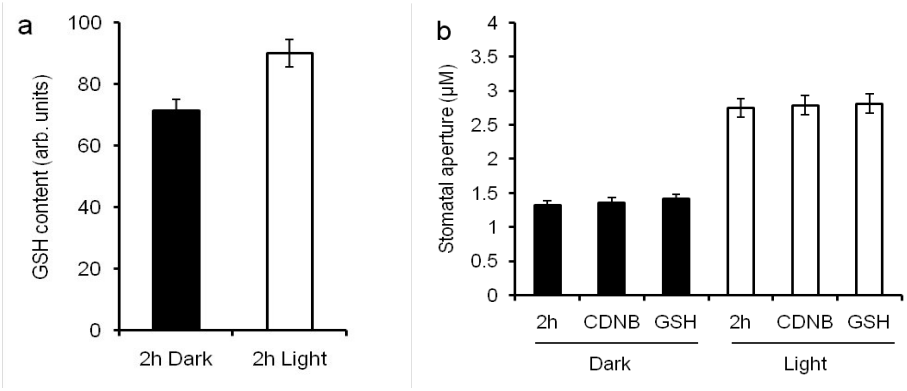

Figure 2. GSH contents in guard cells and dark- and light-induced stomatal aperture.

a, Glutathione contents in guard cells of wild type plants incubated for 2h dark (closed bar) and light (open bar). Glutathione contents were measured using MCB fluorescence dye. Error bars represent standard error (n=5).

b, Glutathione and CDNB did not affect stomatal aperture in dark- (closed bars) and light- (open bars) induced stomatal aperture in guard cells of wild type plants. Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent standard error.

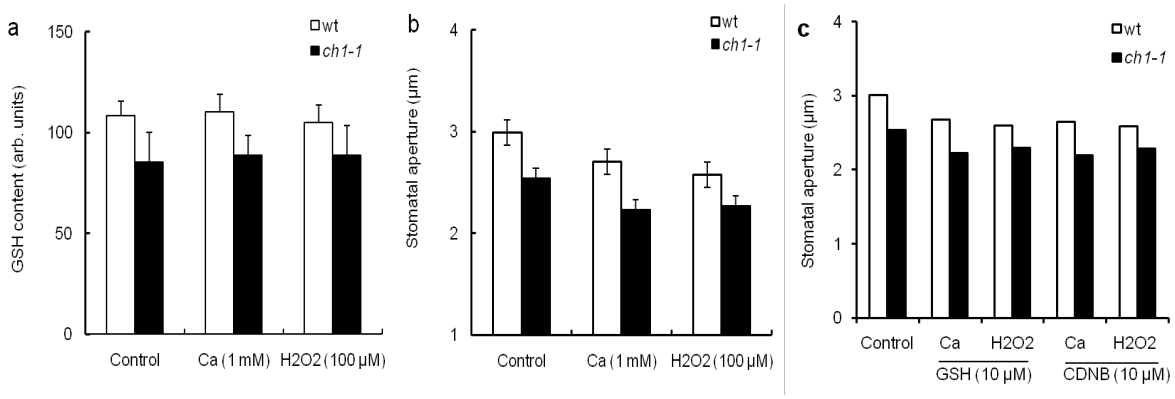

Figure 3. Effects of GSH and CDNB on Ca- and H_2O_2 -induced stomatal closure.

a, Excised leaves treated with Ca and H_2O_2 to induce GSH contents in the wild types (open bars) and *ch1-1* mutant plants (closed bars). Glutathione contents were measured using MCB dye. Error bars represent standard error (n = 5). **b**, Excised leaves were treated with Ca and H_2O_2 to induce stomatal closure in the wild types (open bars) and *ch1-1* mutant plants (closed bars). Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent standard error. **c**, Leaves were treated with Ca and H_2O_2 in GSH and CDNB pre-treated guard cells of wild types (open bars) and *ch1-1* mutant plants (closed bars). Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent standard error.

DISCUSSION

Glutathione has many functions in plant e.g. sulfur metabolism, growth, development, cell defence, redox signalling, and regulation of gene expression (Noctor and Foyer, 1998; Jahan *et al.*, 2008). Glutathione peroxidases (GPXs) are enzymes that involve in scavenging oxyradicals in animal cells (Arthur, 2000). In *Arabidopsis*, GPX3 play an important role as a H_2O_2 scavenger therefore *atgpx3* plants is more sensitive to ABA-induced stomatal closure than wild-type plants (Miao *et al.*, 2006). Deficient of intercellular GSH contents in guard cells increased ABA sensitivity to guard cell

(Jahan *et al.*, 2008). This result stated that reduced GSH level increased ABA sensitivity to guard cells. Our study was conducted to find the clue whether GSH directly affect light-, dark-, Ca- and H_2O_2-induced stomatal movement in *Arabidopsis* which finally leads ABA sensitivity to guard cells. Several factors in ABA signalling are regulated by redox conditions in guard cells (Meinhard *et al.*, 2002). In this study, we showed that treatment of guard cells with GSH and CDNB did not affect stomatal aperture (Fig. 1). In addition, GSH and CDNB treatment did not affect dark/light-induced stomatal aperture (Fig. 2). We chemically (CDNB) and genetically (*ch1-1* mutant*)* confirmed that GSH content did not increase Ca^{+2} and H_2O_2 sensitivity to guard cells (Fig. 3). Our result confirms that reduction or increment of GSH in guard cells might not activate Ca^{+2} and H_2O_2 sensitivity to guard cells of *Arabidopsis*.

In conclusion, plant responses stress condition through different physiological processes. Plant accumulates ABA in guard cells to reduce water loss through stomatal closing during drought condition. It is also proved that GSH helps in many plant defence functions. We stated here that GSH might not directly affect light-, dark-, Ca- and H_2O_2-induced stomatal movement in *Arabidopsis*.

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