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Histochemical quantification of glutathione contents in guard cells of *Arabidopsis thaliana*

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ABSTRACT: Glutathione (GSH), one of the most abundant low molecular-weight thiol compounds, maintains redox homoeostasis in plants. To measure GSH content in guard cells of *Arabidopsis thaliana*, we have developed a technique to quantify the GSH content in guard cells using the staining dye monochlorobimane (MCB). The fluorescent intensity of glutathione S-bimane (GSB) gradually increased in guard cells with increasing incubation time and reached steady state 2 h after epidermal tissues were treated with MCB. The guard cells contained larger amount of GSH content than other cells of the leaves except trichome cell. MCB staining showed that guard cells of *ch1-1*, a GSH deficient mutant, accumulated significantly lower GSH content than that of wild type guard cells. When the guard cells were treated with 1-chloro-2,4-dinitrobenzene to reduce GSH content in the guard cells, the GSB fluorescence in the guard cells was abolished. In addition, GSH monoethyl ester restored GSH level in the guard cells of *ch1-1* mutant plants. These results suggest that GSH content in guard cells can be quantified using MCB dye.

KEYWORDS: *ch1-1*, monochlorobimane, glutathione S-bimane, GSH monoethyl ester, oxidative stress

INTRODUCTION

Glutathione, a tripeptide, has many functions, including sulphur metabolism, regulation of growth and development, cell defence, redox signalling, and regulation of gene expression. It has been reported that these functions depend on the concentration and/or redox state of GSH pools[1,2]. GSH levels vary during plant development and in response to a wide array of stimuli such as atmospheric pollutants, biotic and abiotic stress, and light[3,4]. The GSH level in the aerial parts of *ch1-1* mutant, which lacks the light-harvesting protein of photosystem II (PSII), was lower than that in the wild types[5]. Quantifying of GSH content of guard cells is important to understand the physiology of plant stress.

Although GSH contents have been examined in roots[4,6], seedlings[7], suspension cells[8–10], and trichome cells[11], there are very few studies in guard cells, particularly the guard cells of the plant model Arabidopsis. Because of its small size, traditional methods are difficult to apply to Arabidopsis guard cells. In this study, we provide a new method to quantify GSH content in guard cells of *Arabidopsis thaliana* using a GSH specific dye, monochlorobimane (MCB).

MATERIALS AND METHODS

Arabidopsis wild type, ecotype Columbia, and *ch1-1* (Columbia accession; At1g44446) mutant plants were grown in a plastic square pot filled with 70% (v/v) vermiculite (Asahi-Kogyo, Okayama, Japan) and 30% (v/v) peat soil in a growth chamber under a 16-h light and 8-h dark cycle condition. Temperature and relative humidity were controlled at 22 ± 2 °C and 60 ± 10%, respectively.

The viability of guard cells and epidermal cells was examined using fluorescein diacetate (FDA) staining[12]. The excised leaves were treated with 5 µg/ml FDA solution and incubated for 2 h at room temperature. Epidermal peels were prepared using medical adhesive (Hollister Inc., USA) to attach a leaf lower epidermis side down onto a slide glass. The cuticle and mesophyll layers were carefully removed with a razor blade and the fluorescence was observed under a Fluorescent Microscope BX60F5 (Olympus Optical Co. LTD.) with U-MV BV2 Olympus lens attached with PC based VIEWFINDER LITE Version 1.0.135 (Pixera Corporation) software.

GSH content in the guard cells was determined using MCB dye that reacts with GSH to form a cell-impermeable fluorescent glutathione S-bimane (GSB)[4,7,11,13]. Excised leaves that were treated with 1-chloro-2,4-dinitrobenzene (CDNB) or GSH monoethyl ester (GSHmee) were incubated in a staining solution containing 100 µM of MCB for 2 h at room temperature. Then, epidermal peels were prepared using medical adhesive (Hollister Inc., USA) to attach a leaf abaxial side down onto a slide glass. The cuticle and mesophyll layers were carefully removed with a razor blade, so that the lower leaf epidermal layer containing intact stomatal complexes remained on the slide glass. Besides the above technique, we have modified the technique to prepare guard cells. Firstly, leaf abaxial side attached onto a slide glass and the cuticle and mesophyll layers were removed before MCB incubation and, secondly, epidermal peels were incubated in MCB solution after blending with a commercial blender. GSB fluorescence intensity in guard cells was observed under a fluorescence microscope. The fluorescence image was captured, ROI (region of interest) around each guard cell was drawn and the pixels/intensity of the fluorescence in the guard cell was measured using ADOBE PHOTOSHOP CS3 software (Adobe Systems Inc., San Jose, CA).

RESULTS

We used FDA staining to investigate whether removal of upper epidermal tissue with a razor blade affects cell viability. When the epidermal tissues were stained with FDA, not only guard cells but also epidermal cells displayed green fluorescence (Fig. 1a), suggesting that these cells were alive.

We used MCB staining to examine GSH content in guard cells of Arabidopsis. The fluorescence intensity in guard cells was much higher than that in epidermal cells of leaves (Fig. 1k), indicating that guard cells had larger amounts of GSH than epidermal cells. On the other hand, GSB fluorescence intensity in trichome cell was higher than that in the guard cells (Figs. 1b and 1k), suggesting a higher GSH content in trichome cells. Fig. 1 (d to j) shows time course of MCB staining in the guard cells and MCB specificity to GSH. The fluorescence intensities in guard cells reached a plateau at 2 h and did not significantly change until at least 6 h (Fig. 1j). GSB fluorescence was abolished in guard cells treated with CDNB (Fig. 1j; white and black triangles), a well-known substrate for GSTs to exhaust GSH, indicating that GSB fluorescence is dependent on GSH content. Note that fluorescence in MCB-untreated guard cells as well as other cells of epidermal tissues was undetectable

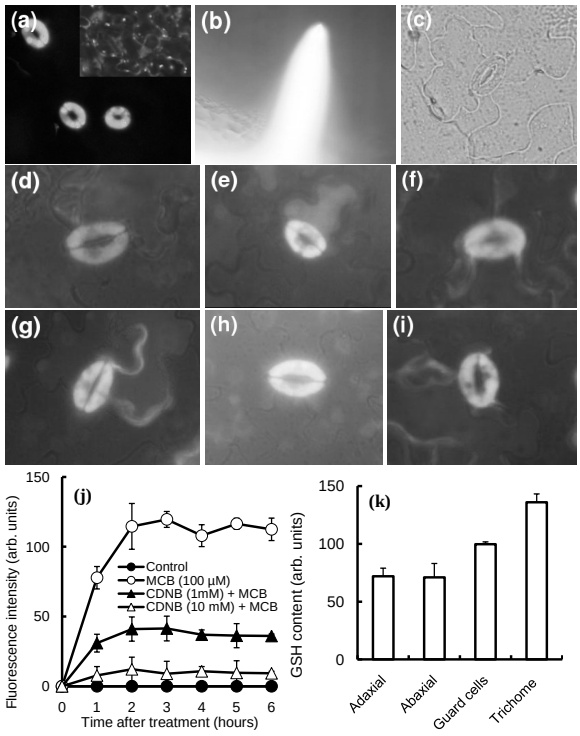

Fig. 1 FDA and MCB staining of different cells of Arabidopsis leaves. (a) Guard cells and epidermal cells emitted green fluorescence after excised leaves were treated with FDA solution. (b) Trichome cell emitted highest GSB fluorescence intensity after excised leaves were treated with MCB solution. (c) MCB-untreated guard cells did not emit detectable fluorescence. (d)–(i) Fluorescence images of guard cells were taken after excised leaves were treated with 100 µM of MCB for 1 to 6 h. (j) Time courses of MCB incubation showed GSB fluorescence intensity in guard cells without CDNB pre-treatment (white circles) and with CDNB pre-treatment (black and white triangles). (k) GSB fluorescence intensity in different cells of leaves after treated with MCB solution. Error bars represent standard deviation ($n = 5$).

(Fig. 1c). These results also suggest that MCB dye is specific to GSH. On the other hand, pretreatment of guard cells for 2 h with L-Buthionine-sulphoximine (BSO) did not abolish GSB fluorescence in guard cells of Arabidopsis (data not shown). Treatment with BSO for 24 h reduced GSH content by more than 95% in Arabidopsis roots[4]. Thus 2-h pretreatment with BSO is not enough to decrease GSH content in Arabidopsis guard cells.

We examined GSH content in guard cells of *ch1-1* mutant plants. The mutants lack the light-harvesting protein of the photosystem II (PSII) and GSH content

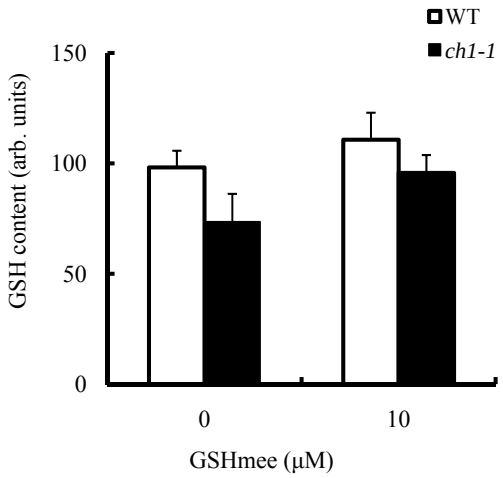

Fig. 2 GSH content was determined in guard cells of wild type and *ch1-1* mutant plants. GSHmee elevated GSH content in guard cells of wild types (white bars) and *ch1-1* mutant (black bars) plants. Error bars represent standard deviation ($n = 5$).

in aerial parts of the mutants is lower than that of wild type plants[5]. The GSB fluorescence in the guard cells of *ch1-1* mutants was lower than that in the wild type guard cells ($P < 0.05$) (Fig. 2), indicating that guard cells of *ch1-1* mutant plants accumulated significantly lower GSH content than wild types did. GSHmee can be hydrolysed to increase intracellular GSH contents[14].

When the guard cells of *ch1-1* mutants were treated with GSHmee, the GSH content increased (Fig. 2). In addition, when *ch1-1* mutant plants were treated with 100 μM of CDNB, the GSB fluorescence was almost completely abolished (data not shown) as was the case in wild types (Fig. 1j). These results suggest that GSH level in guard cells can be quantified using MCB fluorescence dye.

DISCUSSION

GSH has many functions in sulphur metabolism, growth, development, cell defence, redox signalling, and regulation of gene expression. In particular, GSH is closely concerned with ROS homoeostasis and redox status. In guard cells, ROS is one of the most important signalling components in abscisic acid (ABA) signal cascades[15–17]. GSH level can be also regulated by the ascorbate/GSH/NADPH cycle (Halliwell and Asada pathway). Environmental stresses induce oxidative damages via ROS production in plants[18]. Hydrogen peroxide plays a role as a signal transducer, for example, in ABA signalling of guard cells, that is, ABA induces H_2O_2 production and H_2O_2 induces intracellular Ca^{2+} increment mediated by activation of plasma membrane Ca^{2+} channels, resulting in stomata closure[15]. GSH can be involved in H_2O_2-related and redox-sensitive signal transduction[19,20]. Plants open stomata to photosynthesize in response to blue light and accumulate GSH via photosynthesis under light condition[5,21,22]. This study presents that GSH accumulates highly in guard cells and trichome cell rather than other cells of leaves, e.g., mesophyll cells and epidermal cells (Fig. 1k). The ability to retain different GSH levels in different cells of leaves might due to the effects of a cell-specific function.

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