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Regulation of reactive oxygen species-mediated abscisic acid signaling in guard cells and drought tolerance by glutathione

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The phytohormone abscisic acid (ABA) induces stomatal closure in response to drought stress, leading to reduction of transpirational water loss. A thiol tripeptide glutathione (GSH) is an important regulator of cellular redox homeostasis in plants. Although it has been shown that cellular redox state of guard cells controls ABA-mediated stomatal closure, roles of GSH in guard cell ABA signaling were largely unknown. Recently we demonstrated that GSH functions as a negative regulator of ABA signaling in guard cells. In this study we performed more detailed analyses to reveal how GSH regulates guard cell ABA signaling using the GSH-deficient Arabidopsis mutant *cad2-1*. The *cad2-1* mutant exhibited reduced water loss from rosette leaves. Whole-cell current recording using patch clamp technique revealed that the *cad2-1* mutation did not affect ABA regulation of S-type anion channels. We found enhanced activation of Ca^{2+} permeable channels by hydrogen peroxide (H_2O_2) in *cad2-1* guard cells. The *cad2-1* mutant showed enhanced H_2O_2-induced stomatal closure and significant increase of ROS accumulation in whole leaves in response to ABA. Our findings provide a new understanding of guard cell ABA signaling and a new strategy to improve plant drought tolerance.

Keywords: abscisic acid, glutathione, reactive oxygen species, guard cell, stomata

INTRODUCTION

A phytohormone abscisic acid (ABA) induces closing of stomatal pores on leaf epidermis, resulting in reduction of transpirational water loss. The central ABA signaling module is composed of ABA receptors PYR/PYL/RCAR, clade A type 2C protein phosphatases (PP2Cs), and subclass 2 of Snf1-related kinases (SnRK2s) and regulates downstream targets (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009) including ion channels (Geiger et al., 2009; Lee et al., 2009).

Activation of slow type (S-type) anion channels is a key step for ABA signaling in guard cells and drives depolarization of plasma membrane of guard cells, which in turn evokes K^+ extrusion (Linder and Raschke, 1992; Schroeder and Keller, 1992). A guard cell plasma membrane protein SLAC1 represents the S-type anion channel activity (Negi et al., 2008; Vahisalu et al., 2008). It has been demonstrated that ABA activation of S-type anion channels is mediated via a cytosolic Ca^{2+}-dependent pathway (Marten et al., 2007; Siegel et al., 2009). ABA activates hyperpolarization-activated Ca^{2+}-permeable cation (I_{Ca}) channels in the plasma membrane of guard cells (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Pei et al., 2000; Kwak et al., 2003) and induces elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in guard cells (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Allan et al., 1994; Grabov and Blatt, 1998; Allen et al., 1999; Marten et al., 2007). The $[Ca^{2+}]_{cyt}$ signals are decoded by Ca^{2+} sensor proteins such as calcium dependent protein kinases (CDPKs). Electrophysiology experiments using *Xenopus* oocyte demonstrated that Arabidopsis CDPKs, CPK6, CPK21, and CPK23, directly phosphorylate and activate SLAC1 channel (Geiger et al., 2010; Brandt et al., 2012).

It has been suggested that guard cell ABA signaling involves redox regulation. Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) serve as a key mediator of ABA activation of I_{Ca} channels (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). Exogenous application of H_2O_2 activates I_{Ca} channels and evokes guard cell $[Ca^{2+}]_{cyt}$ increases (Pei et al., 2000). Plasma membrane NAD(P)H oxidases are responsible for ABA-induced ROS production in guard cells (Kwak et al., 2003). Arabidopsis glutathione peroxidase 3 (AtGPX3) was shown to function as both a ROS scavenger and a ROS signal transducer in ABA signaling (Miao et al., 2006). Emerging evidences suggest that ROS production by apoplastic enzymes such as cell-wall bound peroxidases is also involved in induction of stomatal closure (An et al., 2008; Khokon et al., 2011; Hossain et al., 2013).

Glutathione (GSH) is the most abundant non-protein thiol compound in plants and a key regulator of cellular redox homeostasis. GSH is involved in various physiological processes including growth, development, and defense response to biotic and abiotic stresses (May et al., 1998; Noctor and Foyer, 1998). Previously we reported that ABA as well as methyl jasmonate

(MeJA) decreases the GSH contents of guard cells (Akter et al., 2010; Okuma et al., 2011). Arabidopsis GSH-deficient mutants, *chl-1* and *cad2-1* exhibit enhanced ABA-induced and MeJA-induced stomatal closure and a membrane permeable derivative of GSH, GSH monoethyl ester (GSHmee) restored the stomatal phenotype of *chl-1* and *cad2-1* mutants (An et al., 2008; Akter et al., 2010, 2012, 2013; Okuma et al., 2011), demonstrating that GSH functions as a negative regulator of ABA and MeJA signaling in guard cells. However, the detailed mechanism of how GSH modulates the guard cell responses is still unclear.

In this study, we analyzed GSH regulation of ABA signaling in guard cells using the Arabidopsis GSH-deficient mutant *cad2-1*. The *cad2-1* mutant is deficient in the first GSH biosynthesis enzyme, γ-glutamylcysteine synthetase. We found that the *cad2-1* mutation causes enhanced ROS activation of I_{Ca} channel and ABA-induced ROS accumulation in apoplast. A new signal model for regulation of ROS-mediated ABA signaling by GSH in guard cells is also proposed.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH

The Arabidopsis ecotype Columbia (Col) and *cad2-1* mutant plants were grown on soil mixture of 70% (v/v) vermiculite (Asahi-kogyo, Okayama, Japan) and 30% (v/v) Sakata Supermix-A (Sakata Seed Corporation, Yokohama, Japan) in growth chambers at 21°C under a 16-h-light/8-h-dark photoperiod with photon flux density of 80 μmol m^{-2} s^{-1}. Four- to six-week-old plants were used in all experiments.

WATER LOSS ASSAY

Three detached rosette leaves were placed on a plastic tray and the loss in fresh weight was monitored at the indicated times.

STOMATAL APERTURE MEASUREMENTS

Stomatal aperture measurements were performed as described previously (Munemasa et al., 2007; Okuma et al., 2011). Detached rosette leaves were floated on stomatal assay buffer containing 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 5.6) for 2 h in the light to induce stomatal opening, followed by the addition of H_2O_2. After 2-h incubation in the light, the leaves were shredded and epidermal tissues were collected. At least 20 stomatal apertures were measured on each individual experiment.

ELECTROPHYSIOLOGY

Guard cell protoplasts (GCPs) were prepared from Arabidopsis rosette leaves by the enzymatic method, as described previously (Pei et al., 1997). Whole-cell currents were recorded using patch clamp technique, as described previously (Munemasa et al., 2007, 2011). For S-type anion current measurements, the pipette solution contained 150 mM CsCl, 2 mM $MgCl_2$, 6.7 mM EGTA, 5.58 mM $CaCl_2$ (free Ca^{2+} concentration: 2 μM), and 10 mM HEPES-Tris (pH 7.1). The bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM MES-Tris (pH 5.6). 5 mM Mg-ATP was freshly added to the pipette solution before experiments. For I_{Ca} current measurements, the pipette solution contained 10 mM $BaCl_2$, 4 mM EGTA, and 10 mM HEPES-Tris (pH 7.1). The bath solution contained 100 mM $BaCl_2$, and 10 mM MES-Tris (pH 5.6). 0.1 mM DTT was added to both pipette and bath solutions freshly before experiments. In both cases, osmolarity was adjusted to 500 mmol/kg (pipette solutions) and 485 mmol/kg (bath solutions) with D-sorbitol.

DETECTION OF ROS ACCUMULATION IN WHOLE LEAVES

Accumulation of H_2O_2 in whole leaves was monitored using 3,3′-diaminobenzidine (DAB) according to Maruta et al. (2010) with slight modifications. Detached rosette leaves were vacuum infiltrated with DAB assay buffer containing 1 mg mL^{-1} DAB, 5 mM KCl, 50 μM $CaCl_2$, and 10 mM MES-Tris (pH 5.6). Infiltrated leaves are incubated in DAB assay buffer with or without ABA for 4 h in the light. The leaves were then decolorized by boiling in ethanol. Apoplastic ROS were visualized as a reddish-brown color and quantified using Image-J software (National Institutes of Health, USA).

STATISTICAL ANALYSIS

All statistical significance was analyzed by double-tailed Student's *t*-test. We regarded differences at the level of $P < 0.05$ as significant.

RESULTS

THE *cad2-1* MUTANT SHOWED ENHANCED DROUGHT TOLERANCE

Previously we reported that GSH depletion by the *cad2-1* mutation enhances ABA-induced stomatal closure in Arabidopsis (Okuma et al., 2011). To assess effect of the *cad2-1* mutation on drought tolerance, we monitored water loss from detached rosette leaves. As shown in **Figure 1**, compared to wild type, the *cad2-1* mutant exhibited reduced water loss from detached rosette leaves ($P < 0.018$ for Col *vs*. *cad2-1* at 120 min after leaf detachment). This result suggests that the *cad2-1* mutation improves drought tolerance.

EFFECT OF THE *cad2-1* MUTATION ON ABA REGULATION OF S-TYPE ANION CHANNELS.

The *cad2-1* mutation does not affect ABA activation of I_{Ca} channels, suggesting that GSH functions downstream of $[Ca^{2+}]_{cyt}$

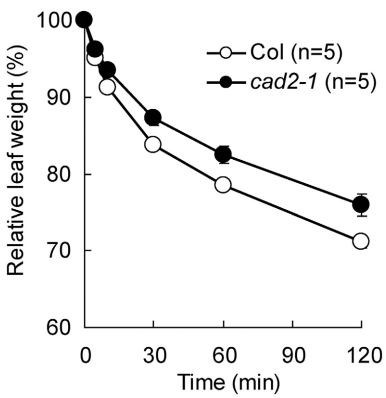

FIGURE 1 | The *cad2-1* mutant shows reduced transpirational water loss. Fresh weight loss of detached rosette leaves. The bars represent the mean ± *SE* values of five independent replicates.

elevation in guard cell ABA signaling (Okuma et al., 2011). Activation of S-type anion channel is mediated by $[Ca^{2+}]_{cyt}$ elevation and considered as a crucial step for ABA-, MeJA-, and CO_2-induced stomatal closure (Schroeder and Hagiwara, 1989; Pei et al., 1997; Munemasa et al., 2007; Hu et al., 2010). To confirm whether GSH depletion in the *cad2-1* mutant affects ABA regulation of S-type anion channel activity, whole-cell patch-clamp analysis was performed. S-type anion currents were observed in wild-type GCPs pretreated with 10 μM ABA ($P < 0.023$ for Col Control *vs.* Col ABA at −135 mV; **Figure 2**). We also found that ABA evoked S-type anion currents in *cad2-1* GCPs to the same extent as in wild-type GCPs ($P < 0.045$ for *cad2-1* Control *vs.* *cad2-1* ABA at −135 mV; **Figure 2**). Note that in our experimental condition, free Ca^{2+} concentration in the pipette solution was buffered to 2 μM and no obvious S-type anion currents were observed in both wild-type guard cells and *cad2-1* guard cells without ABA pretreatment.

THE *cad2-1* MUTATION ENHANCED H_2O_2 ACTIVATION OF I_{Ca} CHANNELS

ABA activation of I_{Ca} channels involves ROS as a second messenger (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). Depletion of the major redox regulator, GSH, in the *cad2-1* mutant might affect ROS-mediated ABA signaling in guard cells. Previously we demonstrated that ABA activation of I_{Ca} channels is not enhanced in *cad2-1* guard cells (Okuma et al., 2011). However, ROS activation of I_{Ca} channels in *cad2-1* guard cells was not yet analyzed. We examined effects of the *cad2-1* mutation on ROS regulation of I_{Ca} channels in guard cells. H_2O_2 at 1 mM activates hyperpolarization-activated currents in both wild-type GCPs and *cad2-1* GCPs (**Figure 3**). The H_2O_2 activation of I_{Ca} currents was enhanced in *cad2-1* GCPs compared to wild-type GCPs ($P < 0.018$ for Col H_2O_2 *vs.* *cad2-1* H_2O_2 at −180 mV; **Figure 3**). To examine effects of the enhanced H_2O_2 activation of *cad2-1* I_{Ca} channels on H_2O_2-induced stomatal closure, we performed stomatal bioassay. Significant reduction of stomatal apertures was observed in 100 μM H_2O_2-treated *cad2-1* mutant ($P < 0.038$ for *cad2-1* Control *vs.* *cad2-1* H_2O_2; **Figure 4**) but not wild type ($P = 0.13$ for Col Control *vs.* Col H_2O_2; **Figure 4**). Moreover, we found that H_2O_2-induced stomatal closure in the *cad2-1* mutant was significantly attenuated by a membrane permeable derivative of GSH, GSHmee ($P < 0.047$ for H_2O_2 without GSHmee *vs.* H_2O_2 with GSHmee; **Figure S1**). These results suggest that decreased GSH contents in *cad2-1* guard cells confer enhanced stomatal response to H_2O_2.

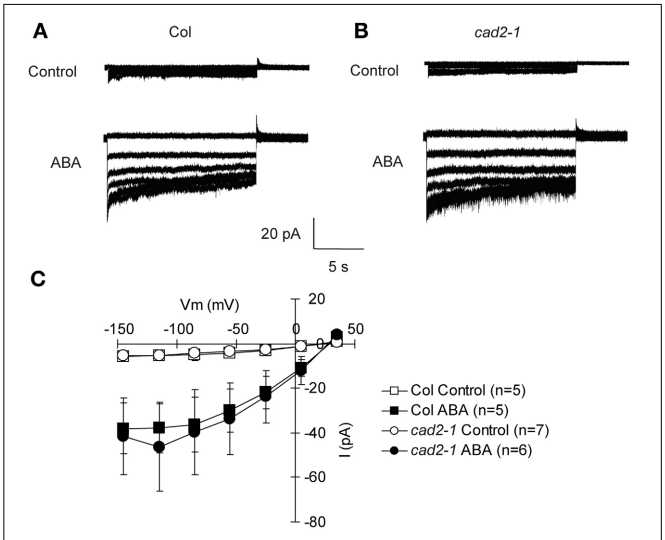

FIGURE 2 | Identical ABA activation of S-type anion channel currents in wild-type GCPs and *cad2-1* GCPs. (A) Representative current traces of wild-type GCPs without ABA (upper trace) or with 10 μM ABA (Lower trace). **(B)** Representative current traces of *cad2-1* GCPs without ABA (upper trace) or with 10 μM ABA (Lower trace). **(C)** Average current-voltage curves of wild-type GCPs and *cad2-1* GCPs as recorded in **(A)** and **(B)**. The voltage protocol was stepped-up from +35 to −145 mV in 30-mV decrements (holding potential: +30 mV). GCPs were treated with 10 μM ABA for 30 min before recordings. The bars represent the mean ± *SE* values of at least five independent replicates.

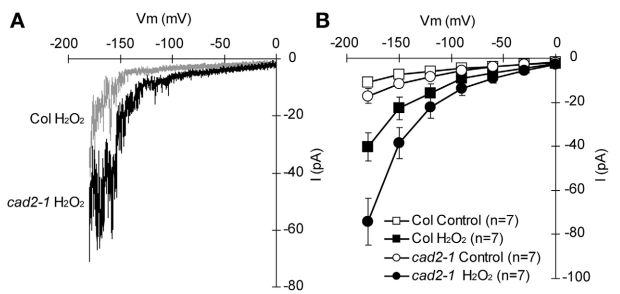

FIGURE 3 | Enhanced activation of *cad2-1* I_{Ca} channel currents by H_2O_2. (A) Representative current traces of 1 mM H_2O_2-activated I_{Ca} currents of wild-type GCPs (gray) and *cad2-1* GCPs (black). **(B)** Average current-voltage curves of wild-type GCPs and *cad2-1* GCPs as recorded in **(A)**. A voltage ramp protocol from 0 to −180 mV was used (holding potential, 0 mV; ramp speed, 200 mV sec^{-1}). GCPs were treated with 1 mM H_2O_2 for 3 min before recordings. The bars represent the mean ± *SE* values of seven independent replicates.

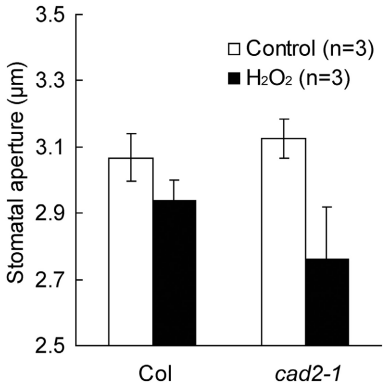

FIGURE 4 | Stomata of the *cad2-1* mutant are more sensitive to exogenous H_2O_2 than those of wild type. Stomatal apertures of wild type and the *cad2-1* mutant were measured 2 h after 100 μM H_2O_2 application. Twenty averages from three independent experiments (60 total stomata per bar) are shown. The bars represent the mean ± *SE* values.

THE *cad2-1* MUTANT SHOWED ABA-INDUCED APOPLASTIC ROS ACCUMULATION IN LEAVES

Accumulation of ROS in guard cell cytosol occurs during ABA-induced stomatal closure (Pei et al., 2000; Kwak et al., 2003). Previously we revealed that ABA-induced ROS accumulation in guard cell cytosol was not altered by GSH depletion (Jahan et al., 2008; Okuma et al., 2011; Akter et al., 2012). In addition to ROS produced by plasma membrane NAD(P)H oxidases, ROS produced by apoplastic enzymes such as cell-wall bound peroxidases participate in regulation of stomatal movement (An et al., 2008; Khokon et al., 2011; Hossain et al., 2013). To examine whether apoplastic ROS accumulation contributes to guard cell ABA signaling, we performed DAB staining experiments, which allows us to monitor ROS produced by apoplastic enzymes as well as ROS produced by NAD(P)H oxidases (Bindschedler et al., 2006; Khokon et al., 2011; Hossain et al., 2013). Wild-type leaves did not exhibit apoplastic ROS accumulation even when treated with high concentration of ABA (50 μM) ($P = 0.72$ for Col Control *vs*. Col ABA; **Figure 5**). However, apoplastic ROS accumulation was significantly increased by ABA in *cad2-1* leaves ($P < 0.04$ for *cad2-1* Control *vs*. *cad2-1* ABA; **Figure 5**). This result suggests that the accumulation of apoplastic ROS contributes to enhanced ABA response of *cad2-1* guard cells.

DISCUSSION

Depletion of GSH enhances ABA-induced stomatal closure (Jahan et al., 2008; Okuma et al., 2011; Akter et al., 2012). However, the mechanism of how GSH regulates guard cell ABA signaling and involvement of GSH in controlling transpirational water loss have been unclear. In this manuscript, we tested involvement of GSH in controlling water loss from leaves and performed the detailed analysis of GSH regulation of guard cell ABA signaling using the GSH-deficient *cad2-1* mutant. We confirmed that the *cad2-1* mutation caused not only enhanced ABA-induced stomatal closure (Okuma et al., 2011) but also reduction of water loss from leaves (**Figure 1**). Hence manipulation of GSH level might provide a new strategy to improve plant drought tolerance.

ABA activation of S-type anion channels is not altered in the *cad2-1* guard cells (**Figure 2**). It has been suggested that the elevated $[Ca^{2+}]_{cyt}$ is required for ABA activation of S-type anion channels and ABA "primes" Ca^{2+} sensitivity of S-type anion channels (Siegel et al., 2009). In this study, we used the pipette solution where free $[Ca^{2+}]$ was buffered to 2 μM (See Materials and Methods). No obvious S-type anion current was observed in *cad2-1* guard cells as well as wild-type guard cells without ABA pretreatment (**Figure 2**), suggesting that the *cad2-1* mutation does not affect the priming state of Ca^{2+} sensitivity of S-type anion channels.

ABA induces guard cell $[Ca^{2+}]_{cyt}$ elevation via activation of plasma membrane I_{Ca} channels (Hamilton et al., 2000; Pei et al., 2000). Previously we found identical ABA activation of I_{Ca} channels in wild-type and *cad2-1* guard cells (Okuma et al., 2011). ROS mediates ABA activation of I_{Ca} channels (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). In this study, we found enhanced response of *cad2-1* I_{Ca} channels to exogenous H_2O_2 (**Figure 3**). It was reported that ozone, an elicitor of ROS, induces biphasic $[Ca^{2+}]_{cyt}$ elevation in seedlings and depletion of GSH by the *cad2-1* mutation and buthionine sulphoximine, an inhibitor of γ-glutamylcysteine synthetase, enhances first peak of the ozone-induced biphasic $[Ca^{2+}]_{cyt}$ response (Evans et al., 2005). These results suggest that GSH pools control sensitivity of plasma membrane Ca^{2+} permeable channels to ROS and downstream Ca^{2+} signals in plant cells.

Consistent with the enhancement of I_{Ca} channel response to ROS, the *cad2-1* mutant exhibits enhanced H_2O_2-induced stomatal closure (**Figure 4**). Note that depletion of GSH in guard cell cytosol is induced by ABA (Okuma et al., 2011), but not by H_2O_2 (Akter et al., 2013). These results imply that ABA sensitizes guard cells to ROS by decreasing GSH content via a pathway distinct from the ABA-mediated ROS production pathway (**Figure 6**).

Previously we reported identical cytosolic ROS accumulation induced by ABA in cytosol of wild-type and *cad2-1* guard cells using 2′,7′-dichlorodihydrofluorescein diacetate (Okuma et al., 2011). It has been suggested that apoplastic ROS signals are also

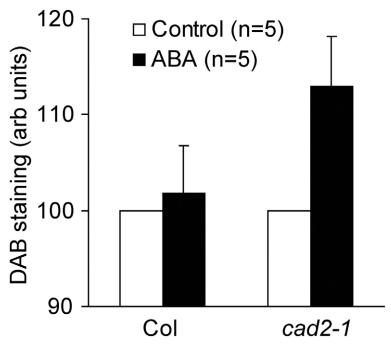

FIGURE 5 | ABA induces ROS accumulation in whole leaves of the *cad2-1* mutant but not in whole leaves of wild type. Accumulation of ROS in whole leaves was monitored using DAB. The vertical scale represents the relative values of pixel intensity of DAB brown color when the values of 50 μM ABA treated leaves are normalized to control value taken as 100 for each experiment. Each datum was obtained from at least four leaves. The bars represent the mean ± *SE* values of five independent replicates.

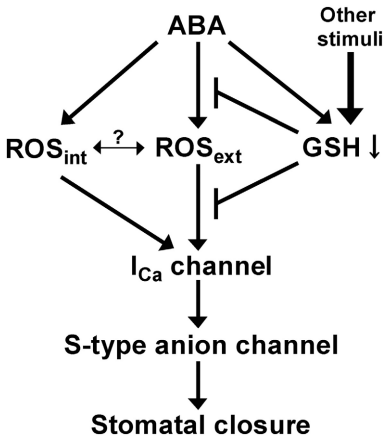

FIGURE 6 | A simplified model of GSH regulation of guard cell ABA signaling.

involved in regulation of stomatal movement (An et al., 2008; Khokon et al., 2011; Hossain et al., 2013). In this study we monitored ROS accumulation in whole leaves using DAB. ABA induced ROS accumulation in whole leaves of the *cad2-1* mutant but not in leaves of wild type (**Figure 5**). These results suggest that GSH depletion by the *cad2-1* mutation affects ROS homeostasis in apoplastic space rather than that in guard cells during ABA-induced stomatal closure.

Based on the findings obtained in this study, we present one simplified signal model shown in **Figure 6**. ABA decreases guard cell GSH content via ROS-independent pathway (Akter et al., 2013). The decreased GSH content causes significant ROS accumulation in apoplast (**Figure 5**) and also sensitizes guard cell I_{Ca} channels to apoplastic ROS (**Figure 3**) by unknown mechanism, resulting in enhanced stomatal response to ABA. In wild-type leaves, ABA decreases GSH content (Okuma et al., 2011) but does not induce significant apoplastic ROS accumulation (**Figure 5**), suggesting that the apoplastic ROS signal is employed to modulate ABA responsiveness of guard cells by other stimuli rather than by ABA signaling itself. Molecular mechanisms of how GSH regulates ROS sensitivity to plasma membrane Ca^{2+} permeable channels and apoplastic ROS homeostasis during ABA-induced stomatal closure would be investigated in the future.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00472/abstract

Figure S1 | GSHmee attenuates enhanced stomatal response to H_2O_2 of the *cad2-1* mutant. Detached rosette leaves of the *cad2-1* mutant were incubated on stomatal assay buffer (see Materials and Methods) with or without 10 μM GSHmee for 2 h in the light, followed by the addition of 100 μM H_2O_2. Stomatal apertures were measured 2 h after H_2O_2 application. Twenty averages from three independent experiments (60 total stomata per bar) are shown. The bars represent the mean ± *SE* values.

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