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Reactive Carbonyl Species Function as Signal Mediators Downstream of H_2O_2 Production and Regulate $[Ca^{2+}]_{cyt}$ Elevation in ABA Signal Pathway in Arabidopsis Guard Cells

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We have demonstrated that reactive carbonyl species (RCS) function as an intermediate downstream of hydrogen peroxide (H_2O_2) production in abscisic acid (ABA) signaling for stomatal closure in guard cells using transgenic tobacco plants overexpressing alkenal reductase. We investigated the conversion of the RCS production into downstream signaling events in the guard cells. Both ABA and H_2O_2 induced production of the RCS, such as acrolein and 4-hydroxy-(*E*)-2-nonenal (HNE), in epidermal tissues of wild-type *Arabidopsis thaliana* plants. Application of the RCS scavengers, carnosine and pyridoxamine, did not affect the ABA-induced H_2O_2 production but inhibited the ABA- and H_2O_2-induced stomatal closure. Both acrolein and HNE induced stomatal closure in a plasma membrane NAD(P)H oxidase mutant *atrbohD atrbohF* as well as in the wild type, but not in a calcium-dependent kinase mutant *cpk6*. Acrolein activated plasma membrane Ca^{2+}-permeable cation channels, triggered cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) elevation, and induced stomatal closure accompanied by depletion of glutathione in the guard cells. These results suggest that RCS production is a signaling event between the ROS production and $[Ca^{2+}]_{cyt}$ elevation during guard cell ABA signaling.

Keywords: Abscisic acid • Arabidopsis • Cytosolic calcium • Reactive carbonyl species • Reactive oxygen species • Stomatal closure.

Abbreviations: AER, alkenal reductase; BY-2, Bright Yellow-2; $[Ca^{2+}]_{cyt}$, cytosolic free Ca^{2+} concentration; CDPK, calcium-dependent protein kinase; CFP, cyan fluorescent protein; DNPH, 2,4-dinitrophenylhydrazine; EGTA, ethylene glycol tetraacetic acid; FDA, fluorescein diacetate; GSH, glutathione; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HHE, 4-hydroxy-(E)-2-hexenal; HNE, 4-hydroxy-(E)-2-nonenal; HPLC, high performance liquid chromatography; ICa channel, plasma membrane Ca^{2+}-permeable cation channels; MCB, monochlorobimane; RCS, reactive carbonyl species; ROS, reactive oxygen species; SEM, standard error of mean; YC, Yellow Cameleon; YFP, yellow fluorescent protein.

Introduction

Plants regulate gas exchange and water loss and suppress invasion of microorganisms and pests via stomatal movements (Schroeder et al. 2001, Melotto et al. 2008). Guard cells forming stomatal pores can respond to numerous biotic and abiotic stimuli, such as light, drought and pathogen attack (Shimazaki et al. 2007, Murata et al. 2015, Ye et al. 2015). Abscisic acid (ABA) is synthesized in drought-stressed plants, triggering the signal transduction in guard cells that results in stomatal closure to reduce transpirational water loss (Assmann and Shimazaki 1999, Schroeder et al. 2001).

ABA induces the production of reactive oxygen species (ROS) that is mediated by the plasma membrane NAD(P)H oxidases, AtrbohD and AtrbohF, in *Arabidopsis thaliana* guard cells and then ROS activates the Ca^{2+}-permeable cation (I_{Ca}) channels in the plasma membrane of the guard cells (Pei et al. 2000, Murata et al. 2001, Kwak et al. 2003). Activation of the I_{Ca} channels causes cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) elevation (Schroeder and Hagiwara 1990, Hamilton et al. 2000), which is essential for ABA-induced stomatal closure (McAinsh et al. 1990, McAinsh et al. 1992, Staxen et al. 1999). These findings suggest that $[Ca^{2+}]_{cyt}$ plays the role as a second messenger in ABA signaling in the guard cells. Calcium-dependent protein kinases (CDPKs) are regulators of the Ca^{2+}-dependent guard cell ABA signaling (Mori et al. 2006, Zhu et al. 2007, Zou et al. 2010, Geiger et al. 2011, Munemasa et al. 2011) and the CDPK isozyme CPK6 is responsible for the regulation of I_{Ca} channels in ABA-induced stomatal closure (Mori et al. 2006, Munemasa et al. 2011). Furthermore, ABA-induced stomatal closure is accompanied by depletion of intracellular glutathione (GSH) in Arabidopsis (Akter et al. 2012) and GSH depletion increases the sensitivity to ROS in the plasma membrane

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I_{Ca} channels (Munemasa et al. 2013). However, the conversion mechanism of the ROS production into downstream signaling events in the guard cells needs to be clarified.

ROS are constitutively formed in cells and can oxidize lipids to their peroxides (Mène-Saffrané et al. 2007). Lipid peroxides decompose to form various aldehydes and ketones in the presence of redox catalysts such as transition metal ions or free radicals (i.e. carbonyls; Farmer and Mueller 2013). Among the lipid peroxide-derived carbonyls, the α,β-unsaturated carbonyls, such as acrolein and 4-hydroxy-(*E*)-2-nonenal (HNE), are termed reactive carbonyl species (RCS) because of their high reactivity (Mano 2012). There is a close relationship between the RCS level and damage in plants under stress conditions (Mano et al. 2010, Yin et al. 2010). The application of RCS scavengers, carnosine and pyridoxamine, and the overexpression of RCS-scavenging enzymes improve the stress tolerance in plants (Oberschall et al. 2000, Mano et al. 2002, Mano et al. 2005, Huang et al. 2008, Papdi et al. 2008, Yin et al. 2010, Turóczy et al. 2011, Stiti et al. 2011, Yamauchi et al. 2011, Zhu et al. 2011, Biswas and Mano 2016).

Recently, we demonstrated that RCS functions as an intermediate downstream of the ROS production during ABA-induced stomatal closure using transgenic tobacco (*Nicotiana tabacum*) plants overexpressing the RCS-scavenging enzyme 2-alkenal reductase (AER-OE plants) (Islam et al. 2016). When epidermal tissues were treated with ABA, the in situ RCS levels were elevated within 30 min and remained constant up to 120 min (Islam et al. 2016). Thus, it is evident that the RCS plays the role as a signaling component downstream of ROS.

To investigate the function of the RCS in guard cell ABA signaling, we scrutinized the RCS-induced stomatal closure using the *cpk6* mutant; that is stomatal movements, production of hydrogen peroxide (H_2O_2) and RCS, activation of I_{Ca} channels, and elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in the guard cells of *A. thaliana*. Based on our results, we propose a new model of the ABA signaling pathway in the guard cells.

Results

ABA- and H_2O_2-induced production of RCS in Arabidopsis

To clarify that ROS production is transduced to the RCS production, we quantified the RCS production in epidermal tissues of Arabidopsis rosette leaves treated with ABA or H_2O_2 for 30 min using reverse-phase HPLC after derivatization with 2,4-dinitrophenylhydrazine (DNPH). Typical chromatograms for the epidermal tissues treated with or without ABA and H_2O_2 are shown in **Figs. 1A**, 2A. Treatment with either 50 μM ABA or 1 mM H_2O_2 significantly increased the contents of acrolein and HNE (**Figs. 1B**, 2B). The changes in concentrations of acrolein and HNE in epidermal tissues treated with ABA and H_2O_2 were approximately 1.5 μM and 2.5 μM, respectively, assuming that 1 g of fresh weight is equivalent to 1 mL. The measured values are average concentrations in the epidermal tissues. ABA induces detectable ROS production only in the guard cells, but not in other epidermal cells (**Fig. 4A**), and the guard cells occupy less than 10% (v/v) of the epidermal tissue, suggesting that RCS production through the lipid is localized in the guard cells. Hence, actual changes in the levels of these aldehydes in the guard cells should be greater than 10 μM. ABA or H_2O_2 also significantly increased the contents of 4-hydroxy-(*E*)-2-hexenal (HHE), (*E*)-2-pentenal, (*E*)-2-heptenal, (*Z*)-3-hexenal, acetaldehyde and propionaldehyde (**Figs. 1B**, 2B). Both ABA and H_2O_2 induced the RCS production in the epidermal tissues of Arabidopsis rosette leaves like the tobacco leaves, and the RCS production is likely to be downstream events of the ROS production in ABA signaling in the guard cells.

Impairment of ABA-induced stomatal closure in Arabidopsis by application of RCS scavengers

To confirm that RCS mediates the ABA-induced stomatal closure, we examined the effects of the RCS scavengers, carnosine and pyridoxamine, on the RCS accumulation. Both carnosine at 1 mM and pyridoxamine at 0.5 mM inhibited the ABA- and H_2O_2-induced RCS accumulation (**Figs. 1**, **2**) and inhibited the ABA-induced stomatal closure (carnosine: $P < 0.01$ at 1 μM ABA, $P < 0.02$ at 10 μM ABA, $P < 0.01$ at 50 μM ABA; pyridoxamine: $P < 0.04$ at 1 μM ABA, $P < 0.03$ at 10 μM ABA, $P < 0.02$ at 50 μM ABA) (**Fig. 3A**, **B**, Supplementary Fig. S1). It should be noted that neither of these scavengers suppressed the ABA-induced ROS accumulation in the guard cells of Arabidopsis under the conditions used in this study (**Fig. 4A**). These results suggest that the RCS is involved in the ABA-induced stomatal closure of *A. thaliana*.

RCS-induced stomatal closure in Arabidopsis

We previously showed that RCS added exogenously to epidermal tissues induced stomatal closure in tobacco (Islam et al. 2016). We now tested whether the *A. thaliana* guard cells respond to RCS in a similar way. We employed acrolein and HNE, which were increased by the ABA treatment in *A. thaliana* epidermal tissues (**Fig. 1**). Application of 10 μM and 100 μM acrolein induced stomatal closure in the wild type (WT) ($P < 0.006$ for 10 μM, $P < 0.0003$ for 100 μM) (**Fig. 4B**, **C**) and so did that of HNE at 10 μM and 100 μM ($P < 0.02$ for 10 μM, $P < 0.0004$ for 100 μM) (**Fig. 4D**, **E**). The acrolein- and HNE-induced stomatal closure was inhibited by 1 mM carnosine and 0.5 mM pyridoxamine (carnosine: $P < 0.01$ at 10 μM acrolein, $P < 0.003$ at 100 μM acrolein, $P < 0.03$ at 10 μM HNE, $P < 0.01$ at 100 μM HNE; pyridoxamine: $P < 0.02$ at 10 μM acrolein, $P < 0.001$ at 100 μM acrolein, $P < 0.01$ at 10 μM HNE, $P < 0.002$ at 100 μM HNE) (**Fig. 4B–E**). These results also suggest that guard cells in *A. thaliana* responded to RCS and exerted stomatal closure similar to those in tobacco.

Reversibility of RCS-induced stomatal closure in Arabidopsis

As RCS at higher concentrations can be toxic and may cause irreversible changes in plants, we examined the reversibility of the acrolein- and HNE-induced stomatal closure. Treatment with 100 μM acrolein or 100 μM HNE for 2 h and 4 h induced stomatal closure and stomatal apertures at 4 h were slightly

narrower than those at 2 h (Supplementary Fig. S2; closed square). When the bathing solution supplemented with acrolein or HNE was replaced with the bathing solution without acrolein or HNE, the stomatal apertures were significantly increased within 2 h (Supplementary Fig. S2; open square). These results indicated that acrolein and HNE reversibly induce stomatal closure.

We then tested the effects of RCS on the viability of the guard cells using Evans blue and fluorescein diacetate (FDA). Guard cells treated with acrolein or HNE at 100 μM for 2 h were stained with Evans blue for 30 min or with FDA for 5 min. During the Evans blue staining, 84.3% of the guard cells did not accumulate the Evans blue dye under the control conditions, and when treated with acrolein and HNE, 77.9% ($P = 0.17$) and 74.4% ($P = 0.08$) of the guard cells did not accumulate the Evans blue dye (Supplementary Fig. S3A). During FDA staining, 81.3% of the guard cells showed a green fluorescence under the control conditions, and when treated with acrolein and HNE, 78.4% ($P = 0.23$) and 77.1% ($P = 0.19$) of the guard cells emitted a fluorescence (Supplementary Fig. S3B). Thus, acrolein and HNE at up to 100 μM do not significantly affect the viability of the guard cells. The tolerance of the cells to acrolein and HNE

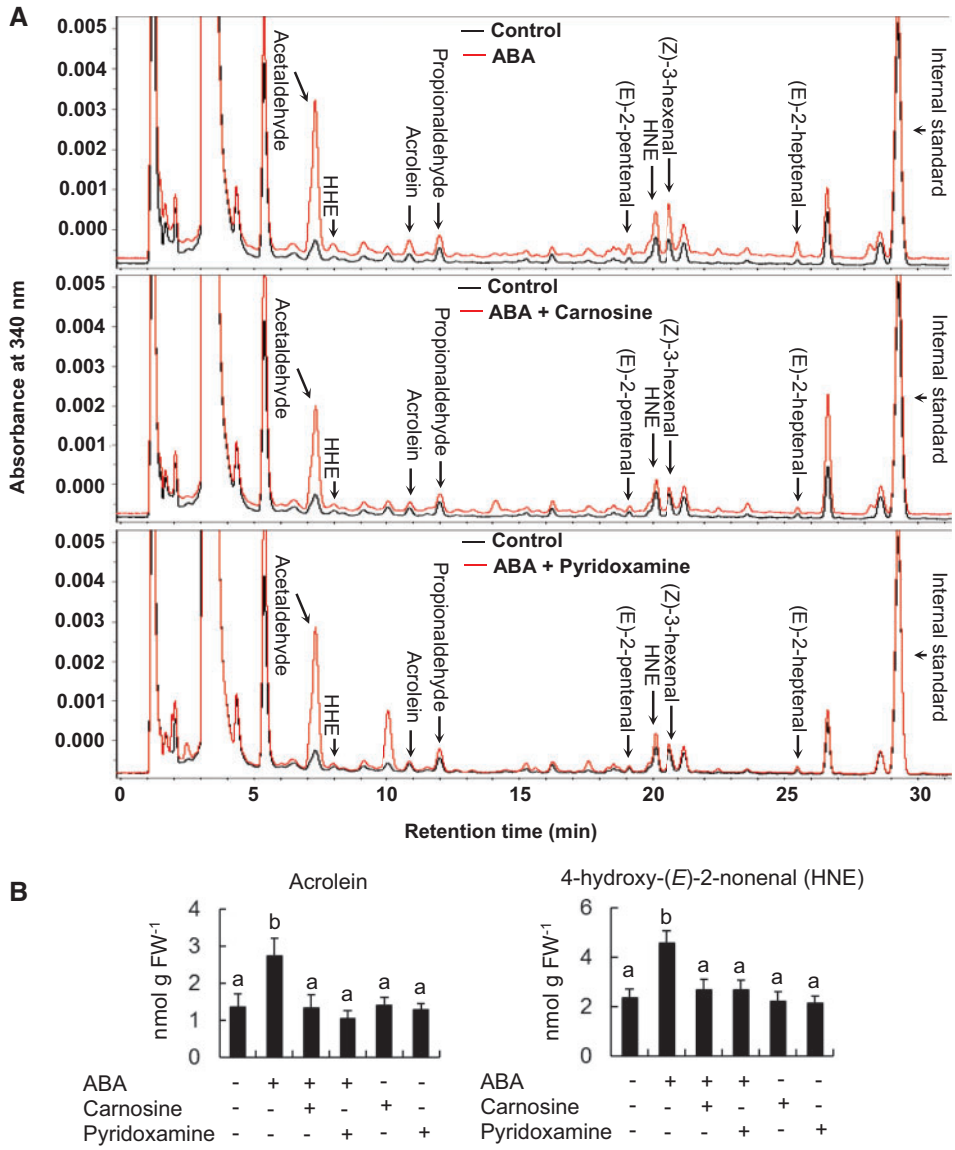
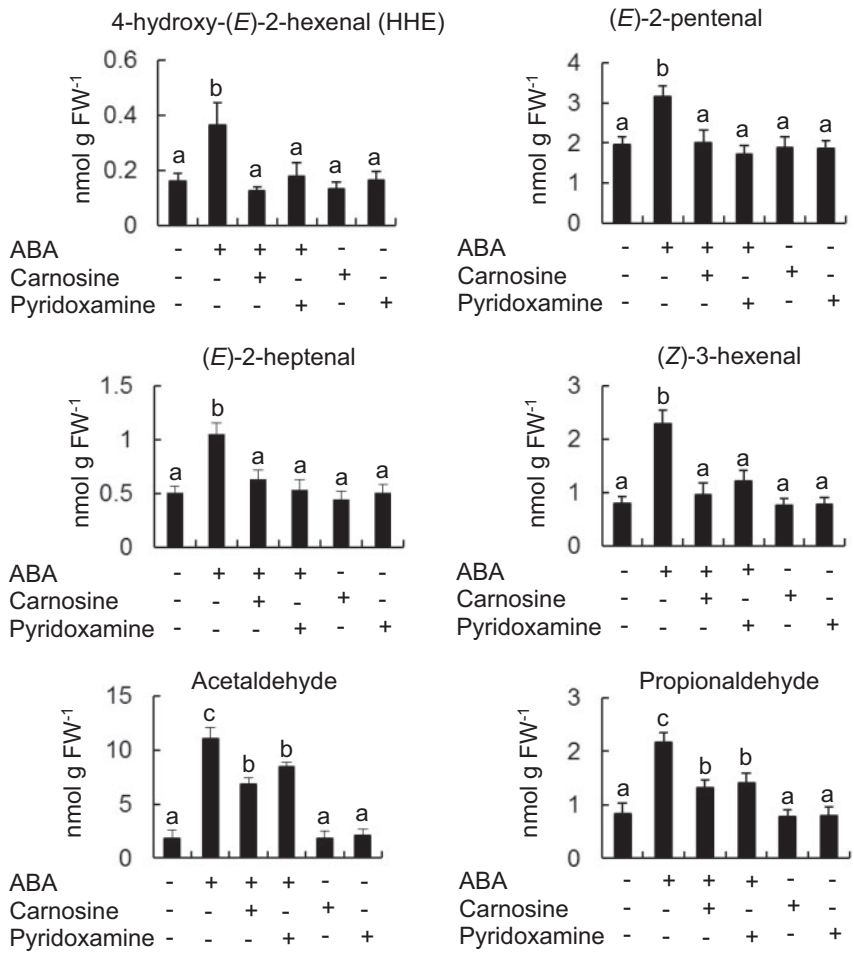


Fig. 1 Effects of ABA and RCS scavengers, carnosine and pyridoxamine, on RCS contents in the epidermal tissues of Arabidopsis. Typical chromatograms for the epidermal tissues treated with or without ABA for 30 min. (A) Typical chromatograms of the dinitrophenylhydrazone (DNP) derivatives of RCS extracted from control (black lines) and ABA-, ABA plus carnosine- and ABA plus pyridoxamine-treated (red lines) epidermal tissues of Arabidopsis. The identified RCS are labeled at the top of each peak. The DNP derivatives of RCS were detected at 340 nm. (B) Contents of RCS in the epidermal tissues of Arabidopsis. Rosette leaves of 4- to 6-weeks-old were blended for 30 s and the epidermal tissues were collected and floated on an assay solution containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris, pH 6.15, for 2 h in the light followed by the addition of ABA. Carnosine and pyridoxamine were added 30 min prior to the ABA application. Error bars represent SEM ($n = 5$). Differences among the treatments were analyzed by the Tukey's test: $P < 0.05$.



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and the reversibility of the effect of these agents satisfy the condition that RCS is a physiological signaling mediator in the guard cells.

NADPH oxidases-mediated ROS production

While ABA-induced stomatal closure is partially impaired in the *atrbohF* single mutant plants but not in the *atrbohD* single mutant plants, ABA-induced stomatal closure is significantly impaired in the *atrbohD atrbohF* double mutant plants (Kwak et al. 2003). To confirm that RCS is produced downstream of the H_2O_2 production during guard cell ABA signaling, we tested whether acrolein or HNE induces stomatal closure in the *atrbohD atrbohF* double mutant plants. Application of ABA did not induce stomatal closure and H_2O_2 production in the guard cells of the *atrbohD atrbohF* double mutant plants (**Fig. 5A**, Supplementary Fig. S4), which is consistent with the previous result (Kwak et al. 2003). In contrast, application of 100 μM acrolein or 100 μM HNE induced stomatal closure in the *atrbohD atrbohF* double mutant plants (**Fig. 5A**). Moreover, the H_2O_2-induced stomatal closure was inhibited by 1 mM carnosine and 0.5 mM pyridoxamine (**Figs. 5B**, **C**), which did not affect the ABA-induced ROS production (**Fig. 4A**). It was reported that the RbohD and RbohF are localized in the plasma membrane (Kwak et al. 2003). Given the localization of RbohD and RbohF, plasma membrane lipids are likely the major RCS source for the guard cell ABA signaling, but other possibilities (organelle membranes, etc.) cannot be eliminated. These results taken altogether indicate that during ABA signaling in the Arabidopsis guard cells, RCS is produced downstream of the ROS that are enzymatically generated by NADPH oxidases and they mediate the ROS signal to the further downstream components and cause stomatal closure. However, there remains a possibility that ROS production catalyzed by RbohD and RbohF triggers activation of other ROS-producing enzymes to account for total ROS production in the guard cells.

The RCS scavengers almost completely inhibited the accumulation of RCS induced by ABA and H_2O_2 (**Figs. 1**, **2**) but not completely inhibited the ABA-induced stomatal closure (**Fig. 3**), RCS-induced stomatal closure (**Fig. 4**) or H_2O_2-induced stomatal closure (**Fig. 5**). These observations imply that there is another signaling pathway in which the RCS are not involved in the ABA signaling and H_2O_2 signaling. Another possibility is that the scavengers can take a certain time to react with the RCS and a trace of the unreacted and remaining RCS can be attributed to signal transmission to the downstream signal components before being scavenged. The

existence of ROS-independent pathway in the ABA signaling was proposed in previous reports (Pei et al. 2000, Kwak et al. 2003). It has been reported that H_2O_2 targets protein phosphatase 2Cs ABI1 and ABI2 (Meinhard and Grill 2001, Meinhard et al. 2002), GPX3 (Miao et al. 2006), GHR1 (Hua et al. 2012) and cytosolic glyceraldehyde-3-phosphate dehydrogenases (GAPCs) (Guo et al. 2012) in guard cell ABA signaling. It remains to be clarified whether these ROS-targeted proteins are regulated in a RCS-dependent or -independent manner.

CPK6 is required for the RCS-mediated stomatal closure

It is well known that the CPKs are master regulators for the Ca^{2+}-dependent ABA signaling (Mori et al. 2006, Zhu et al. 2007, Zou et al. 2010, Geiger et al. 2011) and CPK6 regulates the ABA-induced stomatal closure without affecting the H_2O_2 production (Mori et al. 2006, Munemasa et al. 2011). We examined the roles of CPK6 in the RCS-induced stomatal closure. The acrolein- and HNE-induced stomatal closure was impaired in the *cpk6–1* ($P = 0.23$ for 10 μM acrolein, $P < 0.02$

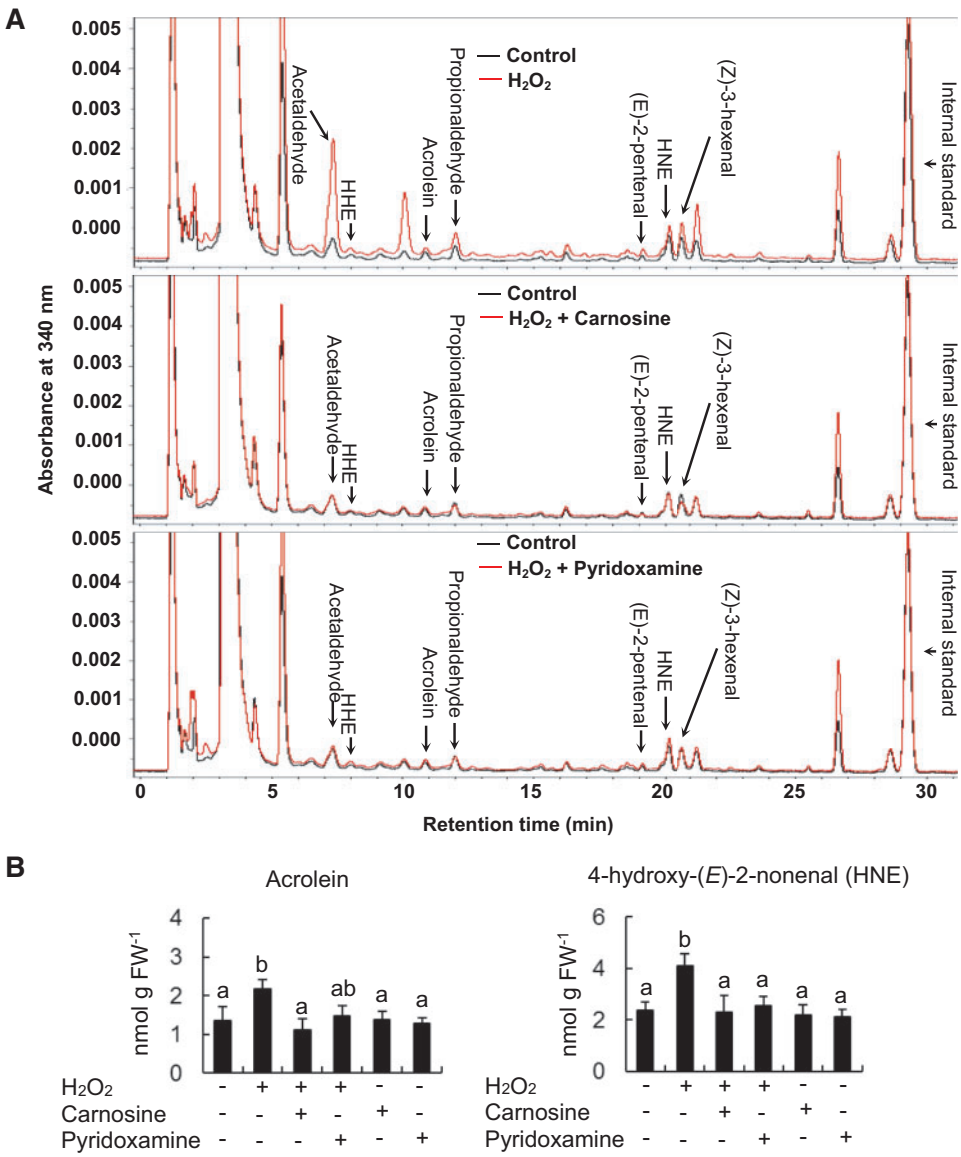
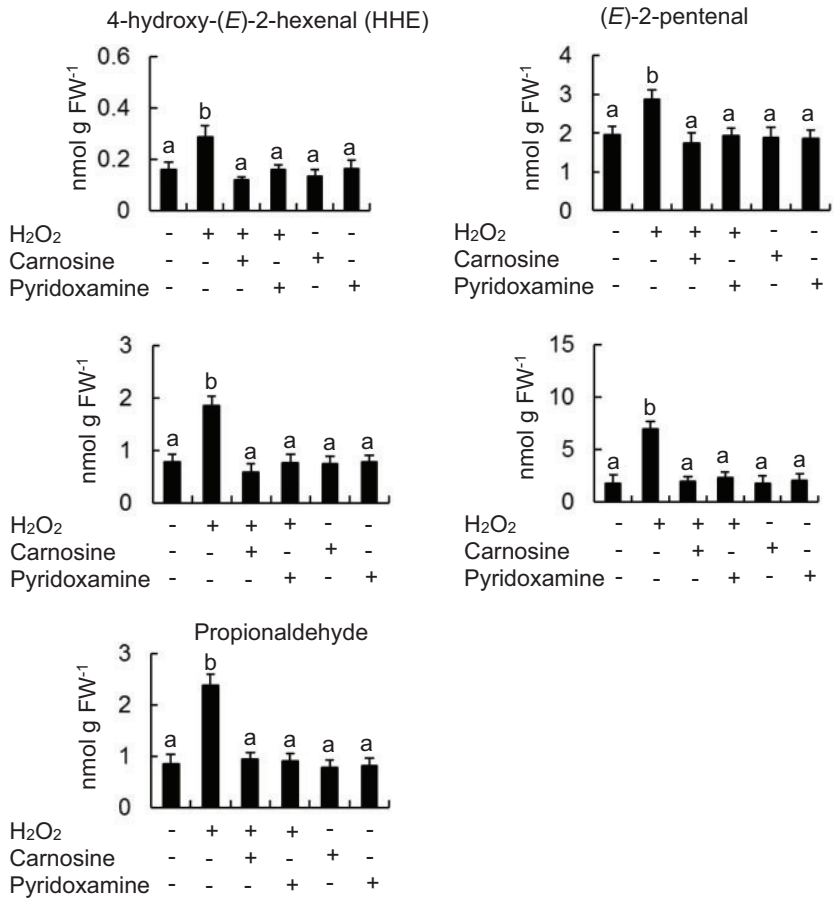


Fig. 2 Effects of H_2O_2 and RCS scavengers, carnosine and pyridoxamine, on RCS contents in the epidermal tissues of Arabidopsis. Typical chromatograms for the epidermal tissues treated with or without H_2O_2 for 30 min. (A) Typical chromatograms of the DNP derivatives of RCS extracted from control (black lines) and H_2O_2-, H_2O_2 plus carnosine- and H_2O_2 plus pyridoxamine-treated (red lines) epidermal tissues of Arabidopsis. The identified RCS are labeled at the top of each peak. The DNP derivatives of the RCS were detected at 340 nm. (B) Contents of RCS in the epidermal tissues of Arabidopsis. Rosette leaves of 4- to 6-weeks-old were blended for 30 s and the epidermal tissues were collected and floated on an assay solution containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES-Tris, pH 6.15, for 2 h in the light followed by the addition of H_2O_2. Carnosine and pyridoxamine were added 30 min prior to the H_2O_2 application. Error bars represent SEM ($n = 5$). Differences among the treatments were analyzed by the Tukey's test: $P < 0.05$.



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for 100 μM acrolein, $P = 0.25$ for 10 μM HNE and $P < 0.01$ for 100 μM HNE) and *cpk6–2* ($P = 0.22$ for 10 μM acrolein, $P < 0.03$ for 100 μM acrolein, $P = 0.27$ for 10 μM HNE and $P = 0.05$ for 100 μM HNE) mutants (**Fig. 6A**, **B**). These results suggest that the RCS functions upstream of the activation of the calcium signaling in the Arabidopsis guard cells. The disruption of *CPK6* diminished the RCS effect but not completely (**Figs. 6A**, **B**), suggesting that other Ca^{2+} signaling mechanisms, for example, the one mediated by CBL-CIPK, contributed to the RCS-induced stomatal closure (Maierhofer et al. 2014).

Acrolein-induced $[Ca^{2+}]_{cyt}$ oscillations in guard cells

The generation of ROS is proposed to trigger the $[Ca^{2+}]_{cyt}$ elevation in the guard cells (Pei et al. 2000, Murata et al. 2001). We examined whether or not the RCS can trigger the $[Ca^{2+}]_{cyt}$ elevation in Yellow Cameleon 3.6 (YC 3.6)-expressing Arabidopsis guard cells. Acrolein at 100 μM triggered the $[Ca^{2+}]_{cyt}$ elevation in 96.4% of the guard cells and H_2O_2 at 100 μM also induced the $[Ca^{2+}]_{cyt}$ elevation in 69.2% of the guard cells (**Fig. 7A–C**). The frequency of the acrolein-induced $[Ca^{2+}]_{cyt}$ elevation was higher than that of the H_2O_2-induced $[Ca^{2+}]_{cyt}$ elevation ($P < 0.01$) (**Fig. 7C**). Moreover, the duration time from onset of the acrolein treatment to the time to reach the first peak of $[Ca^{2+}]_{cyt}$ was significantly shorter than that from onset of the H_2O_2 treatment ($P < 0.01$) (**Fig. 7D**).

Activation of I_{ca} currents by acrolein in the guard cells

Activation of the I_{Ca} causes $[Ca^{2+}]_{cyt}$ elevation (Schroeder and Hagiwara 1990, Hamilton et al. 2000). We tested whether the exogenous acrolein activates the Ca^{2+}-permeable channels. Application of 100 μM acrolein activated the I_{Ca} channel currents in the Arabidopsis guard cell protoplasts (GCPs) ($P < 0.01$ at −180 mV) (**Fig. 8A**, **B**), suggesting that the RCS acts as signal mediators at a point upstream of the activation of I_{Ca} in the guard cell ABA signal pathway. We tested the effect of the RCS scavenger pyridoxamine on activation of the I_{Ca} channels by H_2O_2 in the Arabidopsis GCPs. The guard cell I_{Ca} channels were activated by H_2O_2 (**Fig. 8C**, **D**) as previously shown (Pei et al. 2000) and the RCS scavenger pyridoxamine suppressed activation of the I_{Ca} channels by H_2O_2 ($P < 0.05$ at −180 mV) (**Fig. 8E**, **F**). Taken together, RCS may mediate the activation of I_{Ca} induced by H_2O_2 and may be an activator of I_{Ca} downstream of the ROS production in the guard cells.

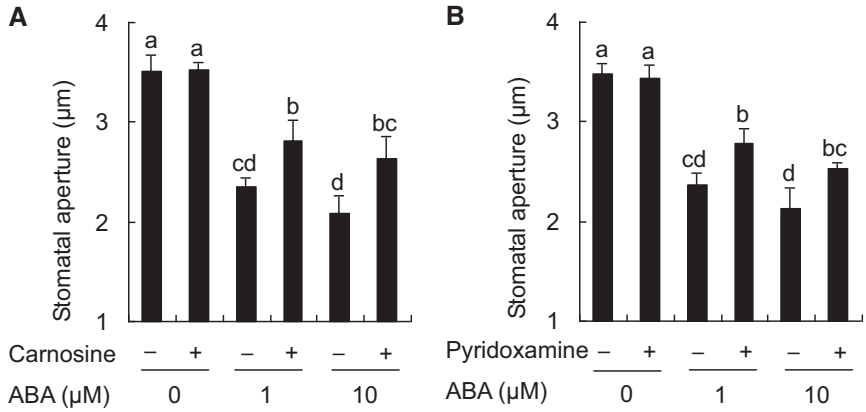


Fig. 3 Effects of RCS scavengers on ABA-induced stomatal closure in Arabidopsis. (A) Effects of RCS scavenger, carnosine, on the ABA-induced stomatal closure in Arabidopsis. (B) Effects of RCS scavenger, pyridoxamine, on the ABA-induced stomatal closure in Arabidopsis. Rosette leaves were treated with ABA for 2 h. RCS scavengers, carnosine and pyridoxamine, were added 30 min prior to the ABA application. Averages from three independent experiments (60 stomata per bar) are shown. Error bars represent SEM. Difference among the treatments were analyzed by the Tukey's test: $P < 0.05$.

Glutathione modulation of RCS-induced stomatal closure

ABA-induced stomatal closure is accompanied by the depletion of intracellular glutathione (GSH) in Arabidopsis (Akter et al. 2012). The depletion of GSH increases the sensitivity to ROS in the I_{Ca} (Munemasa et al. 2013). To determine whether GSH modulates the RCS signal in the ABA-induced stomatal closure, we tested the stomatal responses to acrolein and HNE in GSH-deficient Arabidopsis mutant *cad2–1* plants. Application of 100 μM acrolein or 100 μM HNE narrowed the stomatal apertures by 35% ($P < 0.002$) and 26% ($P < 0.0005$) in the wild-type plants and by 50% ($P < 0.0001$) and 40% ($P < 0.0001$) in the GSH-deficient Arabidopsis mutant *cad2–1* plants (**Fig. 9A**), respectively. These results indicated that GSH attenuates the RCS-induced stomatal closure.

In the tobacco BY-2 cells, RCS decreased the GSH levels (Biswas and Mano 2016). We can thus expect that acrolein and HNE, of which their production in epidermal tissues is elicited by ABA, decrease the GSH levels in the guard cells. To test this hypothesis, we histochemically examined the effects of acrolein and HNE on the GSH levels in the wild-type and the *cad2–1* guard cells using monochlorobimane (MCB) staining. MCB reacts with the intracellular GSH to form fluorescent GSH *S*-bimane (GSB) in the guard cells. Acrolein and HNE decreased the level of GSH in the guard cells of the wild-type plants (acrolein: $P < 0.006$ and HNE: $P < 0.008$) and the *cad2–1* plants (acrolein: $P < 0.004$ and HNE: $P < 0.01$) (**Fig. 9B**). The GSH level in the *cad2–1* mutant guard cells was lower than that in the wild-type ($P < 0.009$) (**Fig. 9B**). These results showed that RCS depletes GSH in the Arabidopsis guard cells and suggest that depletion of GSH by ABA is in part due to the depletion of GSH by the RCS.

Discussion

Abscisic acid-induced stomatal closure is accompanied by the production of ROS mediated by plasma membrane NAD(P)H oxidases (Pei et al. 2000, Murata et al. 2001, Kwak et al. 2003). Our previous study using transgenic tobacco plants overexpressing Arabidopsis AER (At5g16970) (AER-OE plants; Mano et al. 2005) has demonstrated that RCS mediates the ABA-induced stomatal closure and functions downstream of the H_2O_2 production in the ABA signaling pathway in the guard cells. In this study, we found that Arabidopsis also employs RCS as a second messenger in the guard cell ABA signaling.

Scavengers of RCS, carnosine and pyridoxamine, inhibited the ABA- and H_2O_2-induced stomatal closure whereas they impaired the ABA-induced RCS accumulation but not the ABA-induced H_2O_2 production in the Arabidopsis guard cells (**Figs. 1–5**). Moreover, this study has shown that acrolein and HNE induce stomatal closure in the *atrbohD atrbohF* double mutant plants (**Fig. 5A**). These results indicated that RCS functions downstream of the H_2O_2 production in the ABA signaling pathway in the guard cells.

ABA induces RCS production and depletes GSH in the Arabidopsis guard cells along with stomatal closure (**Fig. 1**; Akter et al. 2012) and RCS induces stomatal closure and GSH depletion in the Arabidopsis wild-type and *cad2–1* mutant plants (**Fig. 9**). Biswas and Mano (2016) have shown that RCS depletes GSH in the tobacco BY-2 cells and Davoine et al. (2006) have shown that RCS depletes GSH through conjugation during the tobacco hypersensitive response. Hence, ABA is likely to trigger the depletion of GSH through conjugation of RCS with GSH in the guard cells, which is consistent with the result that the intracellular GSH level does not affect ABA-induced ROS production (Akter et al. 2012) because the ROS production is followed by RCS production.

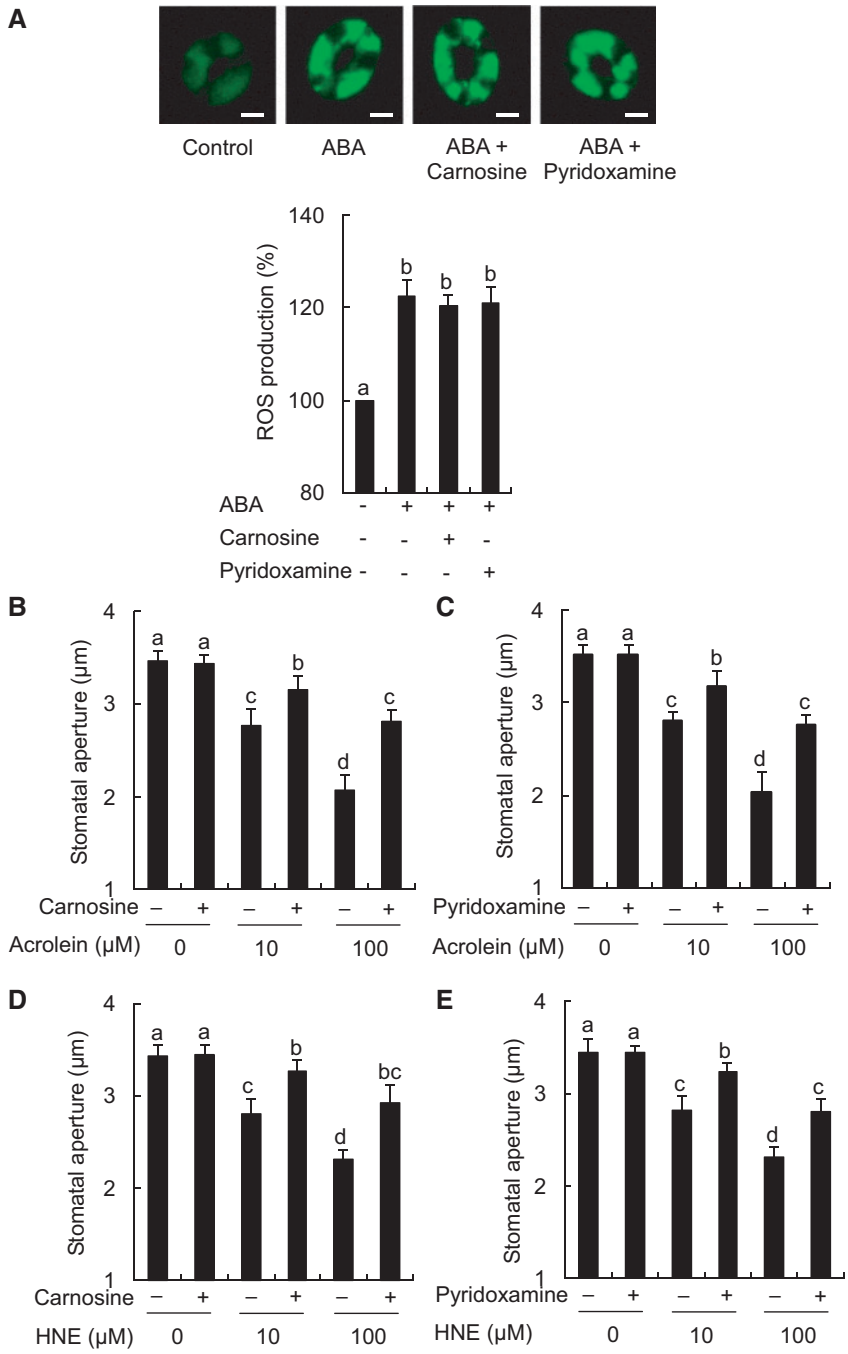


Fig. 4 Effects of RCS scavengers on ABA-induced H_2O_2 production and acrolein- and HNE-induced stomatal closure in Arabidopsis. (A) Effects of RCS scavengers, carnosine and pyridoxamine, on the ABA-induced H_2O_2 production in Arabidopsis guard cells. The vertical scale represents the percentage of H_2DCF-DA fluorescent levels when the fluorescent intensities of the ABA-treated cells are normalized to the control value taken as 100%. Bars indicate averages of three independent experiments (at least 60 guard cells). Scale bars = 5 μm. (B, C) Effects of RCS scavengers, carnosine and pyridoxamine, on acrolein-induced stomatal closure in Arabidopsis. (D, E) Effects of RCS scavengers, carnosine and pyridoxamine, on HNE-induced stomatal closure in Arabidopsis. Rosette leaves were treated with acrolein or HNE for 2 h. The RCS scavengers, carnosine and pyridoxamine, were added 30 min prior to the ABA, acrolein and HNE application. Averages from three independent experiments (60 stomata per bar) are shown. Error bars represent SEM. Differences among the treatments were analyzed by the Tukey's test: $P < 0.05$.

The GSH level in the *cad2–1* guard cells is lower than that in the wild-type guard cells regardless of the ABA treatment (**Fig. 9B**), and stomatal closure induced by ABA is enhanced in the GSH-deficient mutant *cad2–1* (**Fig. 9A**). Given the formation of conjugates of RCS with GSH, larger amounts of RCS would remain unconjugated in the *cad2–1* mutants than in the wild-type and consequently, the ABA-induced stomatal closure is enhanced in the *cad2–1* mutant plants rather than in the wild-type. Stomatal closure induced by ABA is also enhanced in the GSH-deficient mutant *ch1–1*, suggesting that

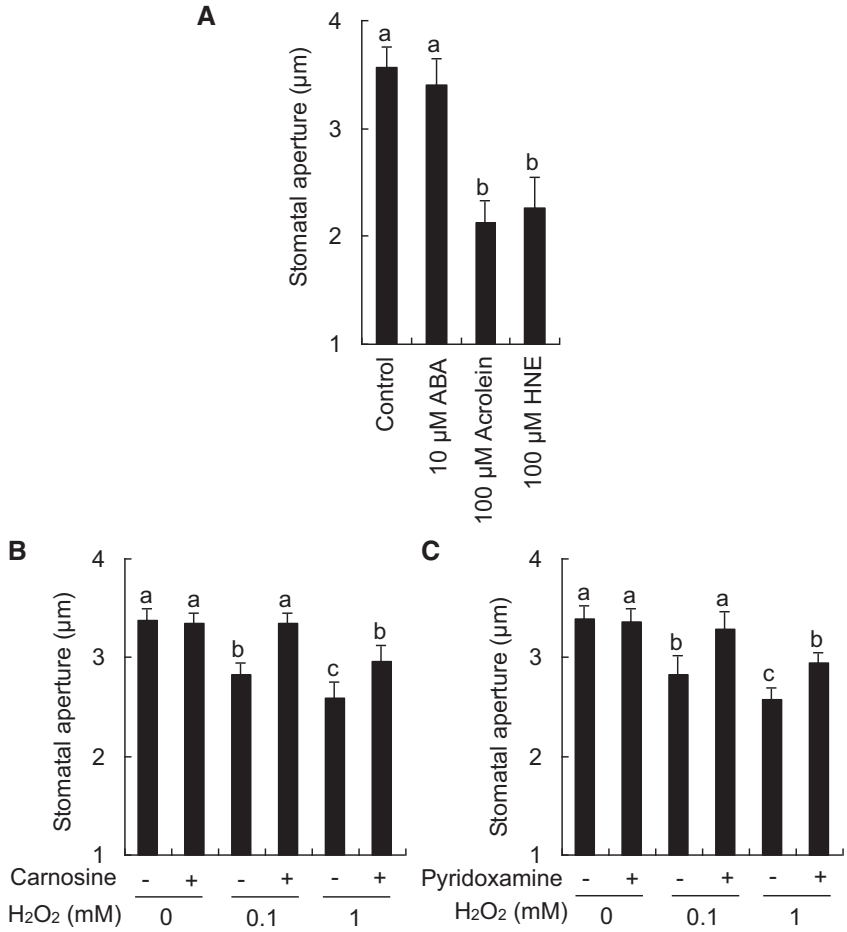


Fig. 5 Stomatal movement in Arabidopsis. (A) ABA-, acrolein- and HNE-induced stomatal closure in *atrbohD atrbohF* double mutant plants. Rosette leaves were treated with ABA or acrolein or HNE for 2 h. (B) Effects of RCS scavenger, carnosine, on H_2O_2-induced stomatal closure in Arabidopsis. (C) Effects of RCS scavenger, pyridoxamine, on H_2O_2-induced stomatal closure in Arabidopsis. Rosette leaves were treated with H_2O_2 for 2 h. RCS scavengers, carnosine and pyridoxamine, were added 30 min prior to the H_2O_2 application. Averages from three independent experiments (60 total stomata per bar) are shown. Error bars represent SEM. Difference among the treatments were analyzed by the Tukey's test: $P < 0.05$.

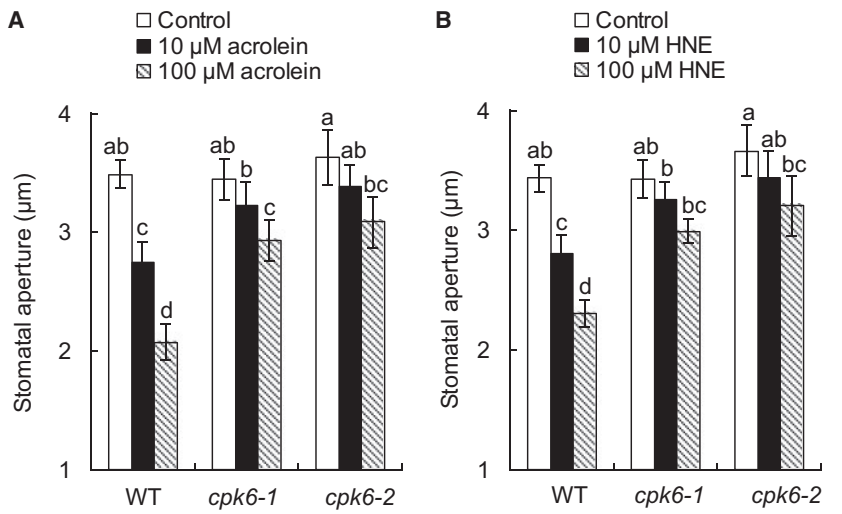
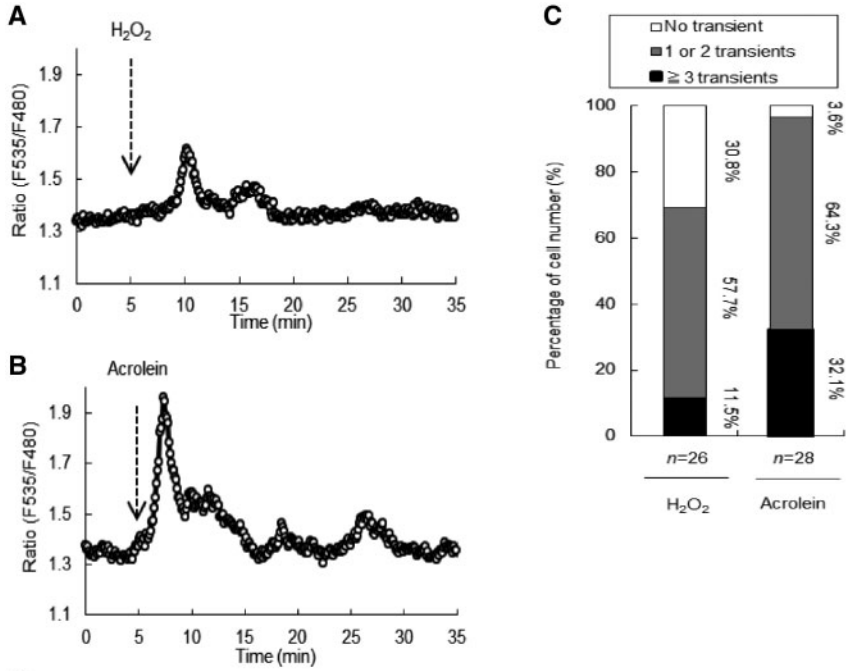


Fig. 6 Acrolein- and HNE-induced stomatal closure in Arabidopsis. (A) Acrolein-induced stomatal closure in wild type and *cpk6–1* and *cpk6–2* mutants. (B) HNE-induced stomatal closure in wild type and *cpk6–1* and *cpk6–2* mutants. Rosette leaves were treated with acrolein or HNE for 2 h. Averages from three independent experiments (60 stomata per bar) are shown. Error bars represent SEM. Difference among the treatments were analyzed by the Tukey's test: $P < 0.05$.



D

	Time of first peak	Area of transients	No. of Cell
H_2O_2	5.55±0.55 min	4908±948	18
Acrolein	1.69±0.14 min	8749±1132	27

Fig. 7 H_2O_2- and acrolein-induced transient elevations of $[Ca^{2+}]_{cyt}$ in Arabidopsis guard cells. (A) A representative trace of fluorescence emission ratios (535/480 nm) showing 100 μM H_2O_2-induced transient $[Ca^{2+}]_{cyt}$ elevations. (B) A representative trace of fluorescence emission ratios (535/480 nm) showing 100 μM acrolein-induced transient $[Ca^{2+}]_{cyt}$ elevations. (C) Percentage of the number of guard cells showing a different number of transient $[Ca^{2+}]_{cyt}$ elevations. $[Ca^{2+}]_{cyt}$ elevations were counted when changes in the fluorescence emission ratios were more than or equal to 0.1 from the baseline. (D) Table showing H_2O_2- and acrolein-induced transient $[Ca^{2+}]_{cyt}$ elevations in Arabidopsis guard cells expressing YC 3.6. Data were obtained during the first 30 min after the application of H_2O_2 and acrolein. The total area of the transients was considered proportional to the amount of the involved Ca^{2+}.

the same mechanism functions in the *ch1–1* mutant (Jahan et al. 2008).

Moreover, genetical and chemical depletion of GSH in the guard cells enhances the ABA-induced stomatal closure and supplement with cytosolic GSH using GSH monoethyl ester (GSHmee) suppressed the enhancement (Okuma et al. 2011). Thus, RCS rather than ROS is likely to be closely involved in the GSH depletion induced by ABA in the guard cells and to dominantly regulate the ABA-induced stomatal closure.

We previously reported that CPK6 is a positive regulator of the ABA-induced stomatal closure but not involved in the ABA-induced H_2O_2 accumulation in the guard cells (Mori et al. 2006, Munemasa et al. 2011). In the current study, we revealed that CPK6 functions as a positive regulator in the acrolein- and HNE-induced stomatal closure (**Fig. 6**). These results suggest that RCS functions at a point between the ROS production and CPK6 activation in the ABA signaling pathway in the guard cells. A recent study has shown that activation by H_2O_2 of the I_{Ca} was enhanced in a GSH-depletion mutant, *cad2–1* (Munemasa et al. 2013), and the current study revealed that $[Ca^{2+}]_{cyt}$ in the guard cells was more significantly affected by the exogenous RCS than by the exogenous H_2O_2 (**Fig. 7**). Taken together, these findings suggest that RCS efficiently potentiates the downstream signal transduction events like the elevation of $[Ca^{2+}]_{cyt}$ in the guard cell ABA signaling pathway, which is modulated by GSH.

Stomatal closure was more strongly induced by acrolein and HNE at 100 μM than by H_2O_2 at 100 μM (**Figs. 4**, **5**). In addition, stomatal closure was significantly induced by 10 μM acrolein but not by 10 μM H_2O_2 (Supplementary Fig. S5). We also found that 100 μM acrolein elicited a higher and more frequent $[Ca^{2+}]_{cyt}$ elevation than did H_2O_2 (**Fig. 7C**) and the peak time of the initial $[Ca^{2+}]_{cyt}$ change from onset of the acrolein treatment was significantly shorter than that from onset of the H_2O_2 treatment (**Fig. 7D**). These results suggest that RCS more effectively induces stomatal closure and $[Ca^{2+}]_{cyt}$ elevation than H_2O_2 does. However, in our experiment, intracellular concentrations of RCS may be higher than that of H_2O_2 in the guard cells because of the difference in membrane permeability. Our previous study report that ABA, even at 10 μM, induces $[Ca^{2+}]_{cyt}$ elevation in 83% of the guard cells (Hossain et al. 2011), which is comparable to the response to acrolein

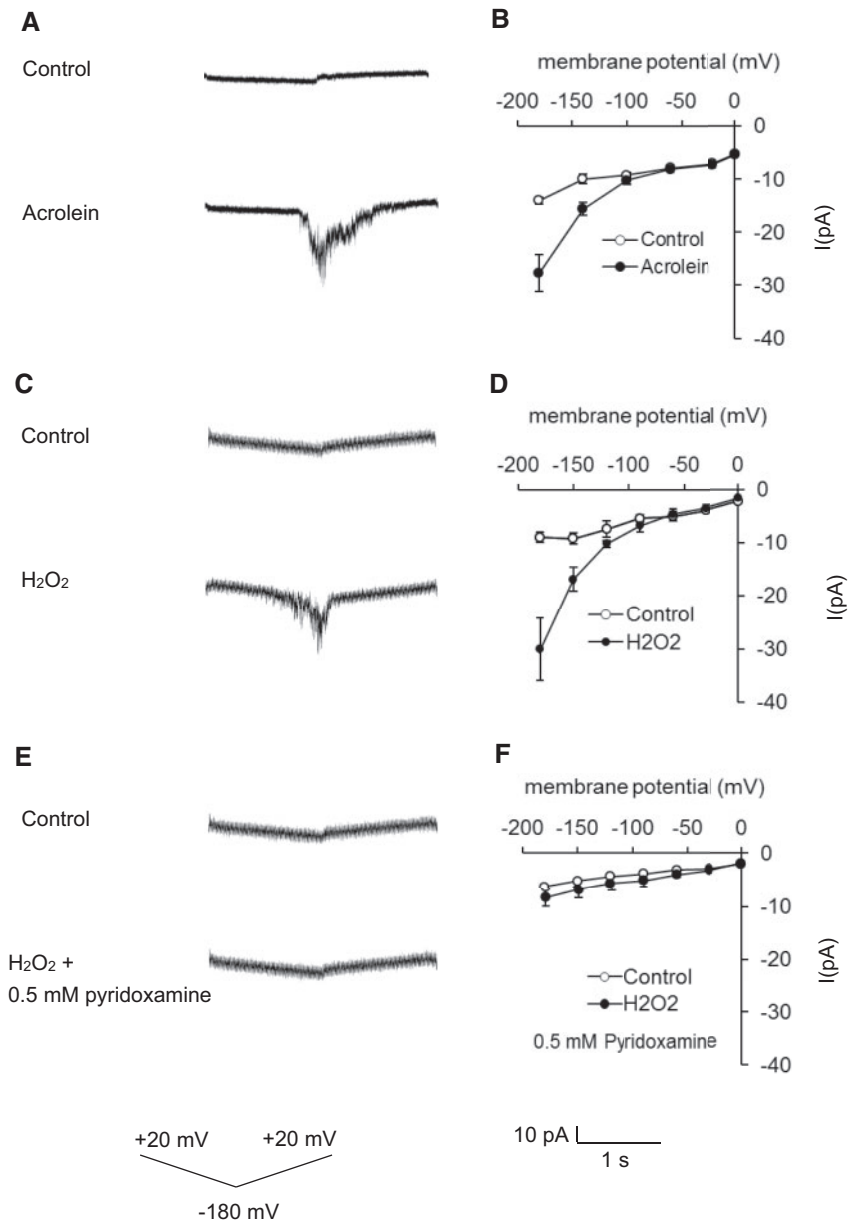


Fig. 8 Acrolein activation of I_{Ca} currents in Arabidopsis GCPs. (A) I_{Ca} currents in Arabidopsis GCPs treated without acrolein (top trace) or with 100 μM acrolein (bottom trace). (B) Current-voltage relationship for acrolein activation of I_{Ca} currents in Arabidopsis GCPs ($n = 6$) as recorded in A (white circles, control; black circles, 100 μM acrolein). (C) I_{Ca} currents in Arabidopsis GCPs treated without H_2O_2 (top trace) or with 100 μM H_2O_2 (bottom trace). (D) Current-voltage relationship for H_2O_2 activation of I_{Ca} currents in Arabidopsis GCPs ($n = 6$) as recorded in C (white circles, control; black circles, 100 μM H_2O_2). (E) I_{Ca} currents in Arabidopsis GCPs treated without H_2O_2 (top trace) or with 100 μM H_2O_2 + 0.5 mM pyridoxamine (bottom trace). (F) Current-voltage relationship for H_2O_2 activation of I_{Ca} currents in Arabidopsis GCPs ($n = 6$) as recorded in E (white circles, control; black circles, 100 μM H_2O_2 + 0.5 mM pyridoxamine). A ramp voltage protocol from +20 to −180 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was used. After making the whole-cell configuration, control currents were recorded 16 times for each GCP as the control data. Acrolein was then applied to the bath solution and I_{Ca} currents were recorded another 16 times to obtain averages for the acrolein treatment. The interpulse period was 1 min.

(**Fig. 7C**). These observations imply that the $[Ca^{2+}]_{cyt}$ elevation in the ABA signal transduction is also modulated by signal components other than ROS and RCS. Taken together, these results suggest that ROS production induced by ABA causes RCS production, more effectively leading to $[Ca^{2+}]_{cyt}$ elevation although we cannot exclude the possibility that ROS and RCS collaboratively elicit $[Ca^{2+}]_{cyt}$ elevation in the ABA signal cascade.

Conclusion

The presented results suggest that RCS functions as a signal mediator downstream of the H_2O_2 production and regulates

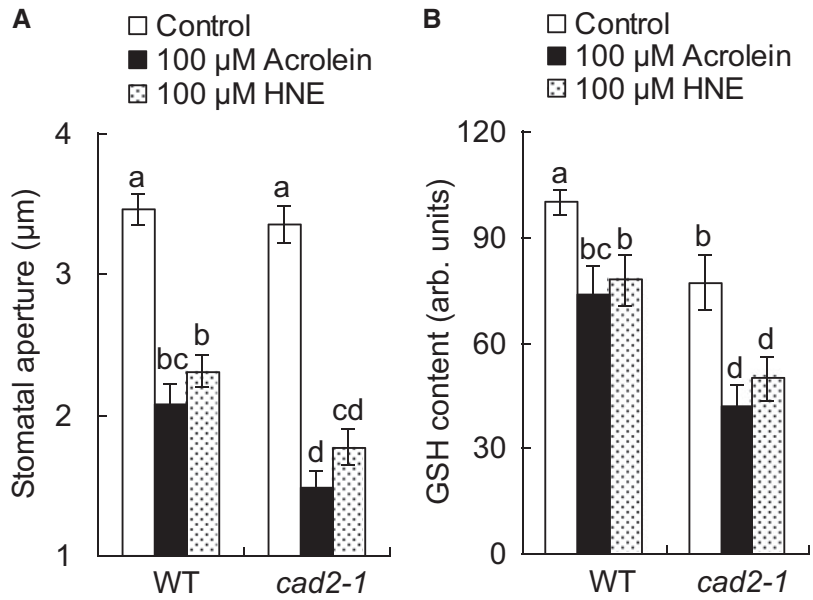


Fig. 9 Effects of acrolein and HNE on stomatal aperture (A) and GSH content (B) in the guard cells of the wild-type plants and cad2–1 plants. Rosette leaves (A) and abaxial epidermal leaf tissues (B) were treated with acrolein and HNE for 2 h. Averages from three independent experiments (60 stomata per bar) are shown. GSH content is expressed as GSB fluorescence intensity. The data in each panel were obtained from at least 60 guard cells. Error bars represent SEM. Difference among the treatments were analyzed by the Tukey's test: $P < 0.05$.

$[Ca^{2+}]_{cyt}$ elevation in the ABA signal pathway in Arabidopsis guard cells.

Materials and Methods

Plant materials and growth conditions

Arabidopsis (*A. thaliana*) wild type (Columbia-0), *cpk6–1*, *cpk6–2*, *cad2–1* and *atrbohD atrbohF* mutant plants were grown on soil containing 70% (v/v) vermiculite (Asahi-kogyo, Okayama, Japan) and 30% (v/v) Kureha soil (Kureha Chemical, Tokyo, Japan) in a growth chamber at 21 ± 2°C and 60% relative humidity with a 16-h light period with 80 μmol m^{-2} s^{-1} photon flux density and 8 h of dark. Water containing 0.1% Hyponex (Hyponex Japan, Osaka, Japan) was applied to the plant growth tray two to three times a week. $[Ca^{2+}]_{cyt}$ in guard cells was measured using a Ca^{2+}-sensing fluorescent protein, YC3.6 (Nagai et al. 2004). To obtain YC3.6-expressing plants, wild-type plants were crossed with a Columbia-0 plant that had previously been transformed with YC3.6. These plants were grown under the same conditions as described above.

Measurement of stomatal aperture

Stomatal apertures were measured as described previously (Ye et al. 2015). Excised rosette leaves of 4- to 6-week-old were floated on an assay solution containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) [pH 6.15 adjusted with tris(hydroxymethyl)aminomethane (Tris)]. The rosette leaves were incubated in the light for 2 h to open the stomata. Then ABA (Sigma) or H_2O_2 or acrolein (Tokyo Chemical Industry) or HNE (Enzo Life Science) was added, and the leaves were kept in the light for 2 h before measurement. RCS scavengers, carnosine and pyridoxamine, were added 30 min prior to ABA or H_2O_2 or acrolein or HNE application. For measurement of stomatal apertures, the leaves were shredded in a commercial blender for 30 s, and epidermal tissues were collected using nylon mesh.

Identification and quantification of RCS by HPLC

Excised rosette leaves of 4- to 6-week-old were blended for 30 s and epidermal tissues were collected and floated on an assay solution containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES, pH 6.15 (adjusted with Tris), for 2 h in the light followed by the addition of 50 μM ABA or 1 mM H_2O_2 for 30 min. RCS scavengers, carnosine and pyridoxamine, were added 30 min prior to ABA or H_2O_2 application. Then, epidermal tissues were used for RCS analysis. RCS was extracted from the epidermal tissues and derivatized with 2,4-dinitrophenylhydrazine (DNPH) and then identified and quantified by reverse-phase HPLC according to the method (Islam et al. 2016). We identified dinitrophenylhydrazone derivatives of RCS by their retention times and determined their contents by a comparison with authentic compounds (Matsui et al. 2009).

Detection of H_2O_2 in guard cells

H_2O_2 production in guard cells was analyzed by using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma, St. Louis, MO, USA) as described previously (Islam et al. 2016). The epidermal peels were incubated for 3 h in the assay solution containing 5 mM KCl, 50 μM $CaCl_2$ and 10 mM MES (pH 6.15 adjusted with Tris), and then 50 μM H_2DCF-DA was added to the sample. The epidermal tissues were incubated for 30 min at room temperature, and then the excess dye was washed out with the solution. Collected tissues were again incubated with solution and 50 μM ABA for 20 min in the dark condition. The image was captured using a fluorescence microscope (Bio Zero BZ-8000; KEYENCE), and the pixel intensity of the fluorescence in guard cells was measured using ImageJ 1.42q (National Institutes of Health, Bethesda, MD, USA).

Viability of guard cells

Viability of guard cells was investigated using Evans blue (Sigma) and FDA (Sigma) as described previously (Kim et al. 2003, Islam et al. 2016) with modification. Just prior to stomatal aperture measurement after 2 h acrolein and HNE treatment, the epidermal tissues were stained with 1% (w/v) Evans blue dye for 30 min or with 10 μM FDA for 5 min. Guard cells were observed under a fluorescence microscope (Biozero BZ-8000; KEYENCE).

Imaging of $[Ca^{2+}]_{cyt}$ in guard cells

Four- to six-week-old Arabidopsis expressing YC3.6 were used for the measurement of $[Ca^{2+}]_{cyt}$ in guard cells as described (Ye et al. 2015). The abaxial side of an excised leaf was gently mounted on a glass slide with a medical adhesive (stock no. 7730; Hollister), followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade to keep the lower epidermis intact on the

slide. The remaining abaxial epidermis was incubated in solution containing 5 mM KCl, 50 mM $CaCl_2$ and 10 mM MES-Tris (pH 6.15) in the light for 2 h at 22°C to promote stomatal opening. Turgid guard cells were used to measure $[Ca^{2+}]_{cyt}$. Guard cells were treated with 100 μM acrolein and 100 μM H_2O_2 using a peristaltic pump at 5 min after monitoring. For dual-emission ratio imaging of YC3.6, we used a 440AF21 excitation filter, a 445DRLP dichroic mirror, a 480DF30 emission filter for cyan fluorescent protein (CFP) and a 535DF25 emission filter for yellow fluorescent protein (YFP). The CFP and YFP fluorescence intensity of guard cells were imaged and analyzed using the W-View system and AQUA COSMOS software (Hamamatsu Photonics). CFP and YFP fluorescence were simultaneously monitored.

Patch-clamp measurement

Current measurements of I_{Ca} channels in Arabidopsis guard cells were performed as described previously (Ye et al. 2013). Arabidopsis GCPs were prepared from rosette leaves of 4- to 6-week-old plants. Pipette solution contained 10 mM $BaCl_2$, 0.1 mM dithiothreitol (DTT), 5 mM NADPH, 4 mM EGTA, and 10 mM HEPES-Tris (pH 7.1) Bath solution contained 100 mM $BaCl_2$, 0.1 mM DTT and 10 mM MES-Tris, pH 5.6. A ramp voltage protocol from +20 to −180 mV (holding potential, 0 mV; ramp speed, 200 mV s^{-1}) was used. Osmolarity was adjusted to 500 mmol kg^{-1} (pipette solutions) and 485 mmol kg^{-1} (bath solutions) with D-sorbitol.

Measurement of GSH in guard cells

The contents of GSH in guard cells were examined using MCB as described previously (Akter et al. 2013). The abaxial side of the excised leaf was gently mounted on a glass slide with a medical adhesive (Hollister Inc., Libertyville, IL, USA), followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade to keep the abaxial epidermis intact on the slide. The abaxial epidermis was incubated in a staining solution containing 100 μM of MCB for 2 h at room temperature. After incubation, the excess dye was washed out with deionized water and covered with a cover slip. The fluorescence intensity of GSB in guard cells was observed under a fluorescence microscope (Biozero BZ-8000, Keyence, Osaka, Japan) with a filter [OP-66834 BZ filter DAPI-BP (excitation wavelength = 360/40 nm, emission wavelength = 460/50 nm, and dichroic mirror wavelength = 400 nm)]. The fluorescence intensity of guard cells was quantified using ImageJ 1.42q software (NIH, Bethesda, MD, USA).

Statistical analysis

The significance of differences between mean values was assessed by the Tukey's test in all parts of this article. We regarded differences at the level of $P < 0.05$ as significant.

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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