The Myrosinases TGG1 and TGG2 Function Redundantly in Reactive Carbonyl Species Signaling in Arabidopsis Guard Cells

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Myrosinase (β-thioglucoside glucohydrolase, enzyme nomenclature, EC 3.2.1.147, TGG) is a highly abundant protein in Arabidopsis guard cells, of which TGG1 and TGG2 function redundantly in abscisic acid (ABA)- and methyl jasmonate-induced stomatal closure. Reactive carbonyl species (RCS) are α,β-unsaturated aldehydes and ketones, which function downstream of reactive oxygen species (ROS) production in the ABA signalling pathway in guard cells. Among the RCS, acrolein is the most highly reactive, which is significantly produced in ABA-treated guard cells. To clarify the ABA signal pathway downstream of ROS production, we investigated the responses of tgg mutants (tgg1-3, tgg2-1 and tgg1-3 tgg2-1) to acrolein. Acrolein induced stomatal closure and triggered cytosolic alkalization in wild type (WT), tgg1-3 single mutants and in tgg2-1 single mutants, but not in tgg1-3 tgg2-1 double mutants. Exogenous Ca^{2+} induced stomatal closure and cytosolic alkalization not only in WT but also in all of the mutants. Acrolein- and Ca^{2+}-induced stomatal closures were inhibited by an intracellular acidifying agent, butyrate, a Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA) and a Ca^{2+} channel blocker, $LaCl_3$. Acrolein induced cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) elevation in guard cells of WT plants but not in the tgg1-3 tgg2-1 double mutants. Exogenous Ca^{2+} elicited $[Ca^{2+}]_{cyt}$ elevation in guard cells of WT and tgg1-3 tgg2-1. Our results suggest that TGG1 and TGG2 function redundantly, not between ROS production and RCS production, but downstream of RCS production in the ABA signal pathway in Arabidopsis guard cells.

Keywords: Arabidopsis • Cytosolic calcium • Cytosolic pH • Reactive carbonyl species • Stomatal closure.

Introduction

Stomatal pores, which are surrounded by pairs of specialized guard cells, serve as major gateways for regulating gas exchange and transpirational water loss. Guard cells respond to a variety of biotic and abiotic stimuli, such as plant hormones, light, drought and pathogen attack (Shimazaki et al. 2007, Murata et al. 2015, Ye et al. 2015). The phytohormone abscisic acid (ABA) induces stomatal closure, which reduces transpirational water loss (Schroeder et al. 2001, Munemasa et al. 2015).

ABA- and methyl jasmonate (MeJA)-induced stomatal closures are accompanied by cytosolic alkalization and cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) elevation in Arabidopsis guard cells (Islam et al. 2010). In Arabidopsis thaliana, ABA-induced reactive oxygen species (ROS) production is catalyzed by the plasma membrane NADPH oxidases AtrbohD and AtrbohF (Murata et al. 2001, Kwak et al. 2003). ABA-induced ROS production plays a vital role in the activation of Ca^{2+}-permeable cation (I_{Ca}) channels in the plasma membrane of guard cells (Pei et al. 2000, Murata et al. 2001, Kwak et al. 2003).

ROS are constitutively produced in plants under a variety of stresses, and the accumulating ROS can oxidize lipids, especially polyunsaturated fatty acids. Oxidized lipids decompose or degrade spontaneously to form various aldehydes and ketones (Mano 2012, Farmer and Mueller 2013). Alpha,beta-unsaturated carbonyls, such as acrolein and 4-hydroxy-E-2-nonenal, are termed as reactive carbonyl species (RCS) because of their high reactivity and high cytotoxicity (Mano 2012). In plants, there is a positive correlation between RCS accumulation and damage under stress conditions (Mano et al. 2010, Yin et al. 2010). RCS act as downstream components of ROS in the nuclear factor-κB-induced inflammatory response in animal cells (Yadav and Ramana 2013). RCS function downstream of ROS production in the ABA signaling pathway in guard cells using transgenic tobacco (Nicotiana tabacum) plants overexpressing the RCS-scavenging enzyme, 2-alkenal reductase (Islam et al. 2016). RCS act as signal mediators downstream of ROS production and regulate $[Ca^{2+}]_{cyt}$ elevation in the ABA signaling pathway in Arabidopsis guard cells (Islam et al. 2019).

Cytosolic alkalization in guard cells is a common phenomenon in both ABA- and MeJA-induced stomatal closure in A. thaliana, Pisum sativum, Paphiopedilum supersuk (Royal Horticulture Society, 1973) and Paphiopedilum tonsum (Rchb. f) Stein (Irving et al. 1992, Gehring et al.1997, Suhita et al. 2004, Gonugunta et al. 2008, Islam et al. 2010). In Vicia faba guard cells, cytosolic alkalization activates outward K^+ currents and inactivates inward K^+ currents to promote the net efflux of K^+ (Blatt and Armstrong 1993). These results suggest that cytosolic pH plays a role as an important regulator of stomatal movements.

Myrosinase (EC 3.2.1.147) is the β-thioglucohydrolase (TGG) enzyme abundantly present in crucifer plants and

catalyzes the degradation of glucosinolates to produce isothiocyanates and so on. The glucosinolate–myrosinase system plays a major chemical defense mechanism in plants against bacteria, insects and herbivores (Bones and Rossiter 1996, Wittstock and Halkier 2002, Barth and Jander 2006). In the Arabidopsis genome, six myrosinase (TGG) genes have been identified and two functional myrosinase genes, *TGG1* and *TGG2*, have been found (Xue et al. 1995, Husebye et al. 2002, Barth and Jander 2006). TGG1 is a strikingly abundant guard cell protein (Zhao et al. 2008). *TGG1* is significantly expressed in guard cells and phloem cells, while *TGG2* is strongly expressed in phloem associated cells (Husebye et al. 2002, Barth and Jander 2006). *TGG3* is a pseudogene that is expressed in stamens and petals (Zhang et al. 2002). *TGG4* and *TGG5* are expressed in root tissues, and *TGG6* is expressed in flowers, specifically in the stamens (Toufighi et al. 2005). Islam et al. (2009) showed that the myrosinases, TGG1 and TGG2, redundantly function downstream of ROS production and upstream of cytosolic Ca^{2+} elevation in ABA and MeJA signaling in Arabidopsis guard cells. However, to our knowledge, there is no report on whether the myrosinases, TGG1 and TGG2, function downstream or upstream of RCS production in ABA signaling in Arabidopsis guard cells. Therefore, we investigated stomatal movement, cytosolic alkalization and $[Ca^{2+}]_{cyt}$ elevation in guard cells of the *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1* mutants in response to RCS.

Results

TGG1 and TGG2 are involved in acrolein-induced stomatal closure

Acrolein inhibits light-induced stomatal opening (Islam et al. 2015) and induces stomatal closure in Arabidopsis (Islam et al. 2019). To clarify the involvement of TGG1 and TGG2 in the RCS-induced stomatal closure in Arabidopsis guard cells, we examined stomatal responses to acrolein of *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1* mutants. Application of 10 μM acrolein significantly induced stomatal closure in WT ($P < 0.03$) in agreement with the previous result (Islam et al. 2019). Acrolein at 10 μM significantly induced stomatal closure in the *tgg1-3* mutant ($P < 0.02$) and *tgg2-1* mutant ($P < 0.001$) but not in the *tgg1-3 tgg2-1* double mutant ($P > 0.79$) (Fig. 1A). These results suggest that TGG1 and TGG2 are redundantly involved in RCS-induced stomatal closure.

Acrolein-induced cytosolic alkalization in Arabidopsis guard cells

Both ABA and MeJA induce cytosolic alkalization, which leads to induced stomatal closure (Irving et al. 1992, Gehring et al. 1997, Suhita et al. 2004, Gonugunta et al. 2008, Islam et al. 2010). To examine the involvement of TGG1 and TGG2 in RCS signaling in Arabidopsis guard cells, we monitored cytosolic pH in guard cells of Arabidopsis *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1* mutants using a pH-sensitive fluorescent dye, 2,7-*bis*-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Islam et al. 2010).

Acrolein at 10 μM significantly increased BCECF fluorescence intensity in the WT guard cells ($P < 0.002$) (Fig. 1C), indicating that acrolein increases cytosolic pH in WT plants. Acrolein at 10 μM also increased guard cell BCECF fluorescence intensity in both *tgg1-3* ($P < 0.01$) and *tgg2-1* ($P < 0.02$) but not in *tgg1-3 tgg2-1* ($P > 0.74$) (Fig. 1C), which is consistent with the results of stomatal response to acrolein (Fig. 1A). These results indicate that RCS-induced stomatal closure is accompanied by cytosolic alkalization in guard cells and suggests that the myrosinases, TGG1 and TGG2, function upstream of cytosolic alkalization in ABA signaling.

Ca^{2+}-induced stomatal closure in Arabidopsis guard cells

We investigated the effects of exogenous Ca^{2+} on stomatal apertures of *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1* mutants. Application of 1 mM $CaCl_2$ significantly induced stomatal closure in all mutants (*tgg1-3*: $P < 0.002$; *tgg2-1*: $P < 0.001$; *tgg1-3 tgg2-1*: $P < 0.01$) as well as in WT ($P < 0.0004$) (Fig. 2A), which is consistent with previous results (Islam et al. 2009).

Ca^{2+}-induced cytosolic alkalization in Arabidopsis guard cells

To analyze the role of TGG on Ca^{2+}-induced cytosolic alkalization in guard cells, we examined cytosolic pH in WT and in the *tgg* mutants. Exogenous $CaCl_2$ at 1 mM significantly increased BCECF fluorescence intensity in guard cells of *tgg1-3* ($P < 0.03$), *tgg2-1* ($P < 0.002$) and *tgg1-3 tgg2-1* ($P < 0.01$) mutants as well as in WT ($P < 0.03$) (Fig. 2C). These results are consistent with the results that Ca^{2+} induced stomatal closure in WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1* mutants (Fig. 2A). These results indicate that exogenous Ca^{2+} triggers cytosolic alkalization in WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1* mutants and induces stomatal closure.

Acrolein- and Ca^{2+}-induced stomatal closure were repressed by EGTA and $LaCl_3$

We examined the effects of EGTA (Ca^{2+} chelator) and $LaCl_3$ (Ca^{2+} channel blocker) on acrolein- and Ca^{2+}-induced stomatal closure. The acrolein-induced stomatal closure was significantly inhibited by EGTA and $LaCl_3$ in WT (EGTA: $P < 0.02$; $LaCl_3$: $P < 0.01$), *tgg1-3* (EGTA: $P < 0.02$; $LaCl_3$: $P < 0.03$) and *tgg2-1* (EGTA: $P < 0.002$; $LaCl_3$: $P < 0.002$) mutants (Fig. 3A, C, E). Acrolein did not induce stomatal closure in the *tgg1-3 tgg2-1* double mutant in the presence or absence of EGTA and $LaCl_3$ (EGTA: $P < 0.92$; $LaCl_3$: $P < 0.98$) (Fig. 3G). By contrast, the Ca^{2+}-induced stomatal closure was also significantly suppressed by EGTA and $LaCl_3$ in WT (EGTA: $P < 0.002$; $LaCl_3$: $P < 0.009$) and all mutants (EGTA: $P < 0.003$; $LaCl_3$: $P < 0.0007$ in *tgg1-3*, EGTA: $P < 0.003$; $LaCl_3$: $P < 0.0006$ in *tgg2-1*, EGTA: $P < 0.002$; $LaCl_3$: $P < 0.007$ in *tgg1-3 tgg2-1*) (Fig. 3A, C, E, G). These results suggest that $[Ca^{2+}]_{cyt}$ functions downstream of myrosinase in the ABA signal pathway in guard cells and that the myrosinases, TGG1 and TGG2, function redundantly between the RCS production and $[Ca^{2+}]_{cyt}$ elevation stages.
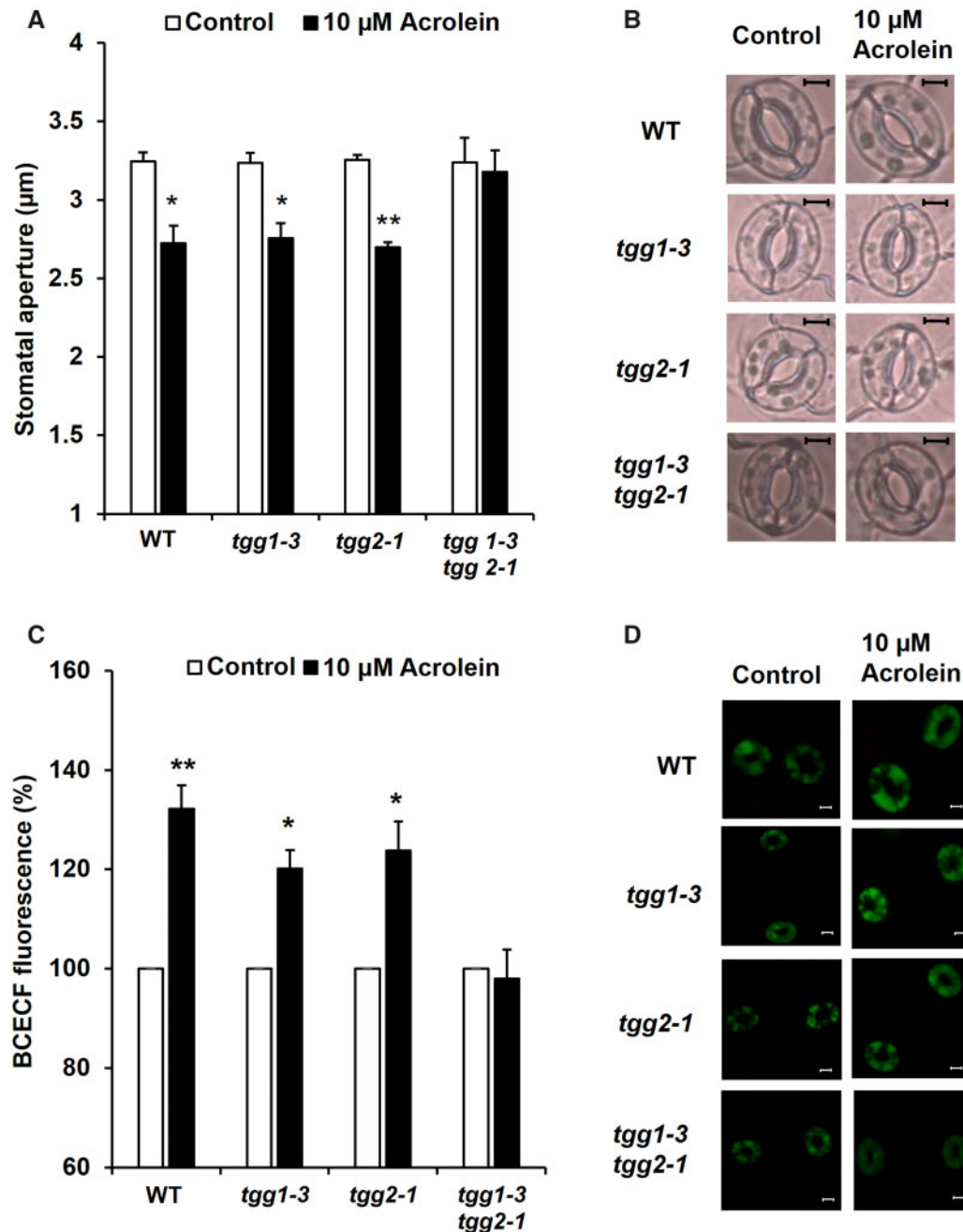
Fig. 1 Effects of acrolein on stomatal aperture and cytosolic alkalization in Arabidopsis guard cells. (A) Acrolein-induced stomatal closure in WT and *tgg* mutants, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. (B) Representative images of the epidermal tissues containing guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Excised rosette leaves were treated with acrolein for 2 h. Averages from three ($n = 3$) independent experiments (60 total stomata per bar) are shown. Scale bar $= 5$ μm. (C) Effects of acrolein on BCECF fluorescence in guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. The vertical axis shows the percentage of BCECF fluorescence when fluorescent intensities of acrolein-treated cells are normalized to the control value taken as 100% for each experiment. Each data was obtained from at least 90 guard cells. (D) Representative BCECF fluorescence images of the epidermal tissues containing guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Scale bar $= 20$ μm. The error bar represents the standard error. Differences between treatments were analyzed by Student's *t*-test: $P < 0.05$ and $P < 0.01$.

Effects of butyrate on acrolein- and Ca²⁺-induced stomatal closure

We examined the effects of butyrate on acrolein- and Ca²⁺-induced stomatal closure to clarify whether they required cytosolic alkalization. Acrolein-induced stomatal closure was significantly inhibited by butyrate in WT ($P < 0.002$), *tgg1-3* ($P < 0.02$) and *tgg2-1* ($P < 0.003$) mutants (**Fig. 4A, C, E**). Acrolein did not induce stomatal closure in the *tgg1-3 tgg2-1* ($P < 0.89$) double mutant in the presence or absence of butyrate (**Fig. 4G**). The Ca²⁺-induced stomatal closure was also significantly inhibited by butyrate in WT ($P < 0.003$) and in all mutants (*tgg1-3*: $P < 0.003$; *tgg2-1*: $P < 0.01$; *tgg1-3 tgg2-1*: $P < 0.0003$) (**Fig. 4A, C, E, G**).
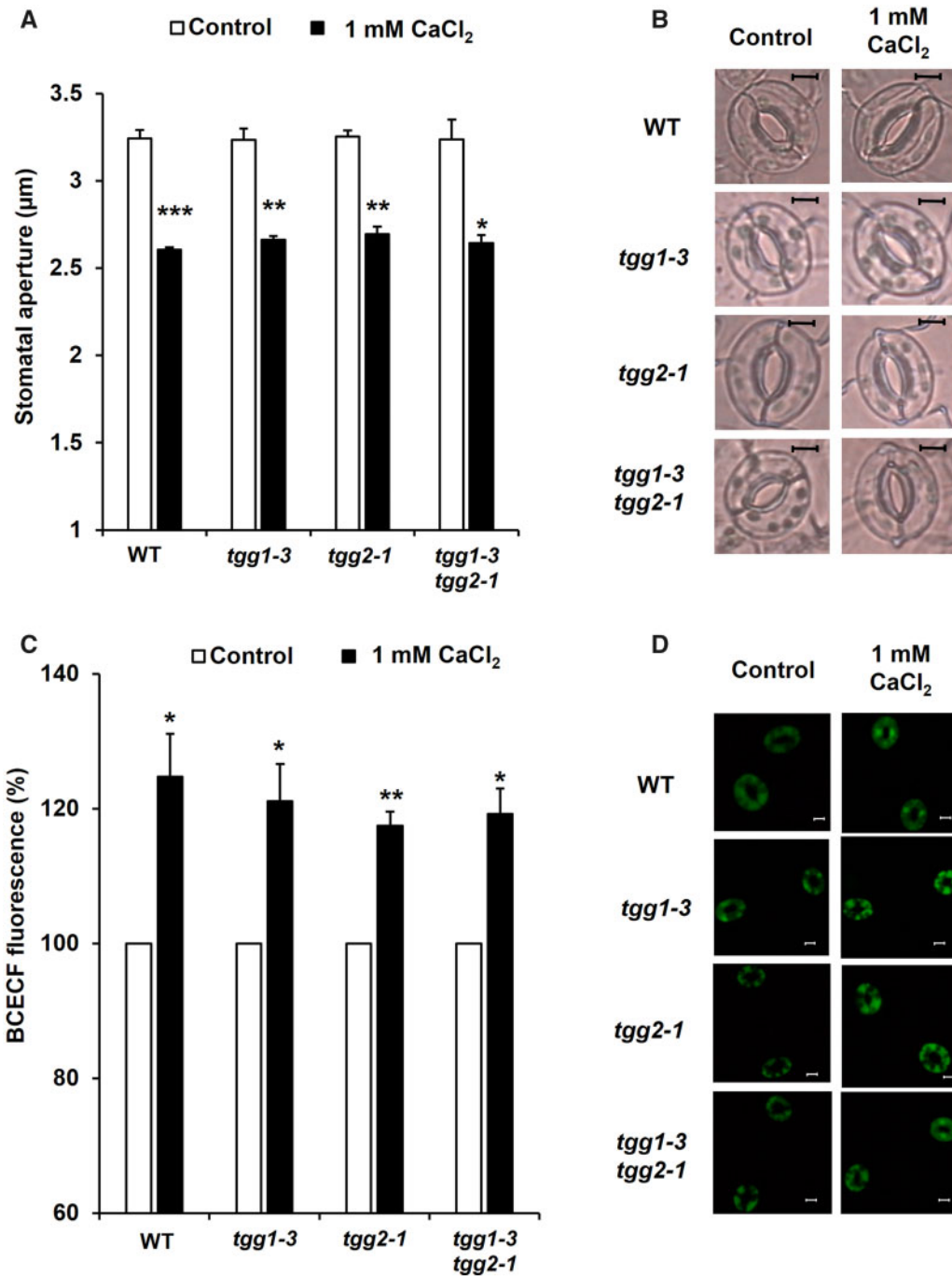
Fig. 2 Exogenous Ca^{2+}-induced stomatal closure and cytosolic alkalization in Arabidopsis guard cells. (A) Exogenous Ca^{2+} at 1 mM reduces stomatal aperture in WT and *tgg* mutants, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. (B) Representative images of the epidermal tissues containing guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Averages from three ($n = 3$) independent experiments (60 total stomata per bar) are shown. Scale bar = 5 μm. (C) Effects of exogenous Ca^{2+} at 1 mM on BCECF fluorescence in guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. The vertical axis shows the percentage of BCECF fluorescence when fluorescent intensities of Ca^{2+}-treated cells are normalized to the control value taken as 100% for each experiment. Each data was obtained from at least 90 guard cells. (D) Representative BCECF fluorescence images of the epidermal tissues containing guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Scale bar = 20 μm. The error bar represents standard error. Differences between treatments were analyzed by Student's *t*-test: $P < 0.05$, $P < 0.01$ and $P < 0.001$.

Application of butyrate did not affect stomatal apertures of either WT or mutants untreated with acrolein or Ca^{2+}. These results indicate that the acrolein- and Ca^{2+}-induced stomatal closure are mediated by cytosolic alkalization. Taken together

with other results in this study, it appears that cytosolic alkalization is the downstream component of $[Ca^{2+}]_{cyt}$ elevation in ABA-induced stomatal closure and that the myrosinases, TGG1 and TGG2, function redundantly between the RCS production,
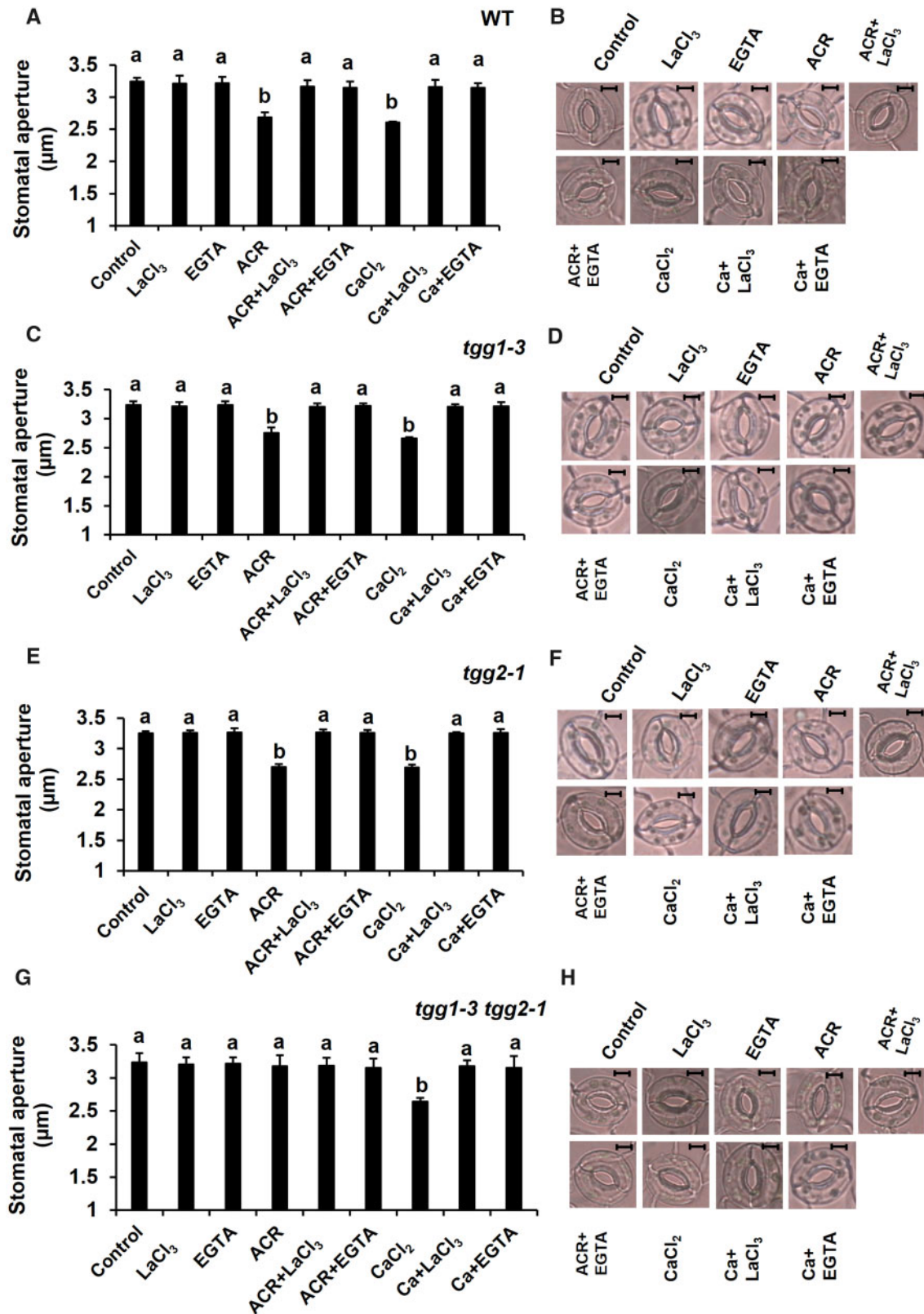
Fig. 3 Effects of EGTA (Ca^{2+} chelator) and $LaCl_3$ (Ca^{2+} channel blocker) on acrolein (ACR)- and Ca^{2+} ($CaCl_2$)-induced stomatal closure. (A, C, E, G) Effects of EGTA and $LaCl_3$ on ACR- and Ca^{2+}-induced stomatal closure in WT and *tgg* mutants, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Excised rosette leaves were incubated for 2 h in light. Light-treated leaves were further treated with ACR and Ca^{2+}. EGTA and $LaCl_3$ were applied 30 min before the application of ACR and Ca^{2+}. Averages from three ($n = 3$) independent experiments (60 total stomata per bar) are shown. (B, D, F, H) Representative images of the epidermal tissues containing guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Scale bar = 5 μm. The error bar represents standard error. Differences among treatments were analyzed by Tukey's test: $P < 0.05$.
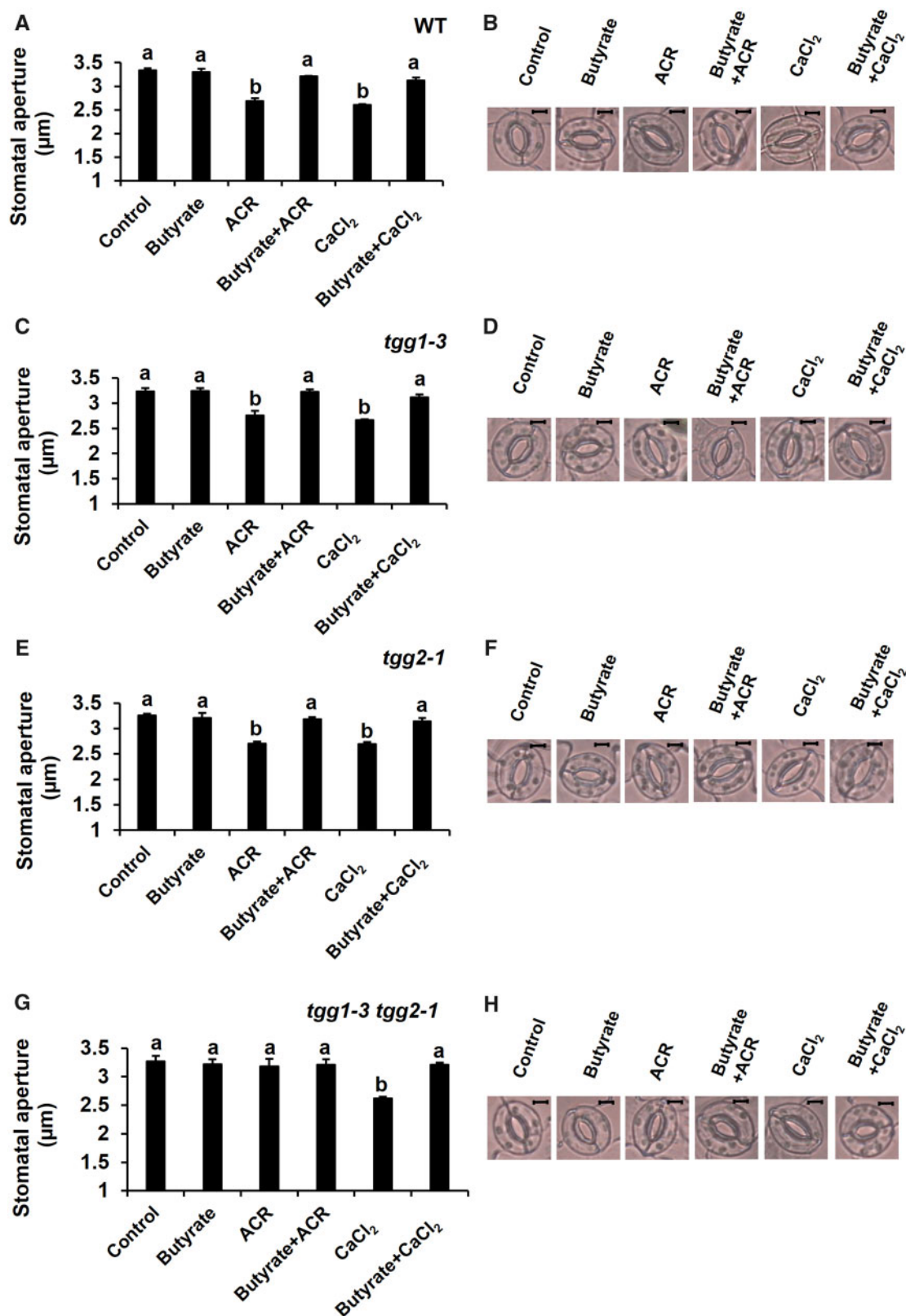
Fig. 4 Effects of butyrate on the inhibition of acrolein (ACR)- and Ca^{2+}-induced stomatal closure. (A, C, E, G) Effects of butyrate on ACR- and Ca^{2+}-induced stomatal closure in WT and *tgg* mutants, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Excised rosette leaves were incubated for 2 h in light. Light-treated leaves were further treated with ACR and Ca^{2+}. Butyrate was applied 30 min before the application of ACR and Ca^{2+}. Averages from three ($n = 3$) independent experiments (60 total stomata per bar) are shown. (B, D, F, H) Representative images of the epidermal tissues containing guard cells of WT, *tgg1-3*, *tgg2-1* and *tgg1-3 tgg2-1*. Scale bar = 5 μm. The error bar represents standard error. Differences among treatments were analyzed by Tukey's test: $P < 0.05$.

$[Ca^{2+}]_{cyt}$ elevation and cytosolic alkalization stages in the ABA signaling pathway.

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

Application of ABA, MeJA and Ca^{2+} leads to stomatal closure through $[Ca^{2+}]_{cyt}$ elevation in Arabidopsis guard cells (McAinsh et al. 1990, Allen et al. 2000, Pei et al. 2000, Allen et al. 2001, Allen et al. 2002, Suhita et al. 2004, Munemasa et al. 2011). We examined the effects of acrolein and Ca^{2+} on $[Ca^{2+}]_{cyt}$ elevation in guard cells using the Yellow Cameleon (YC) technique (Nagai et al. 2004, Mori et al. 2006, Islam et al. 2010, Hossain et al. 2011, Munemasa et al. 2011). When WT guard cells were treated with 10 µM acrolein, 82.8% ($n = 30$) of the guard cells showed $[Ca^{2+}]_{cyt}$ elevations (Fig. 5A, C). When *tgg1-3 tgg2-1* guard cells were treated with 10 µM acrolein, 22.7% ($n = 22$) of the guard cells showed $[Ca^{2+}]_{cyt}$ elevations (Fig. 5B, C). There was a significant difference in $[Ca^{2+}]_{cyt}$ elevations between WT and *tgg1-3 tgg2-1* ($P = 0.0001$). When WT and *tgg1-3 tgg2-1* double mutant guard cells were treated with 1 mM Ca^{2+}, 88.3% ($n = 18$) and 87.5% ($n = 16$) of the guard cells showed $[Ca^{2+}]_{cyt}$ elevations (Fig. 5D–F). There was no significant difference in Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations between WT and *tgg1-3 tgg2-1* ($P = 0.65$). These results indicate that acrolein-induced $[Ca^{2+}]_{cyt}$ elevation was impaired by the *tgg1-3 tgg2-1* double mutation and that the myrosinases, TGG1 and TGG2, function downstream of RCS production and upstream of $[Ca^{2+}]_{cyt}$ elevation in the ABA signal pathway in Arabidopsis guard cells.

Discussion

Zhao et al. (2008) reported that TGG1 is a strikingly abundant protein in Arabidopsis guard cells and that inhibition of light-induced stomatal opening by ABA was disrupted in *tgg1* mutants. However, ABA-induced stomatal closure functions in the *tgg1* single mutant, while not only ABA-induced stomatal closure but also MeJA-induced stomatal closure were impaired in the *tgg1-3 tgg2-1* double mutant (Islam et al. 2009). In our study, we found that acrolein induced stomatal closure in WT, *tgg1-3* and *tgg2-1*, but not in the *tgg1-3 tgg2-1* double mutant (Fig. 1A). These results suggest a redundant function of TGG1 and TGG2 in the response to RCS in guard cells.

Both ABA and MeJA induce stomatal closure, which is accompanied by cytosolic alkalization in Arabidopsis guard cells (Irving et al. 1992, Gehring et al. 1997, Suhita et al. 2004, Gonugunta et al. 2008, Islam et al. 2010). Acrolein induced cytosolic alkalization and stomatal closure in WT, *tgg1-3* and *tgg2-1* (Fig. 1A, C) while acrolein did not induce cytosolic alkalization or stomatal closure in the *tgg1-3 tgg2-1* double mutant (Fig. 1A, C). Butyrate inhibited the acrolein-induced stomatal closure in WT, *tgg1-3* and *tgg2-1* (Fig. 4A, C, E), which indicates that acrolein-induced stomatal closure is accompanied by cytosolic alkalization. However, acrolein did not induce stomatal closure in *tgg1-3 tgg2-1* double mutants in the presence or absence of butyrate (Fig. 4G). These results indicate that

cytosolic alkalization is required for RCS-induced stomatal closure and suggests that the myrosinases, TGG1 and TGG2, are involved in the regulation of cytosolic pH downstream of RCS production in the ABA signal pathway in guard cells. These results also strengthen the notion that TGG1 and TGG2 function redundantly downstream of RCS production and upstream of cytosolic alkalization in guard cell ABA signaling.

Both ABA and MeJA induce $[Ca^{2+}]_{cyt}$ elevation in guard cells, leading to stomatal closure (Allen et al. 2000, Pei et al. 2000, Allen et al. 2001, Allen et al. 2002, Suhita et al. 2004, Munemasa et al. 2011). In this study, acrolein elicited $[Ca^{2+}]_{cyt}$ elevations in the WT guard cells but not in the *tgg1-3 tgg2-1* double mutant guard cells (Fig. 5A–C). Exogenous Ca^{2+} elicited $[Ca^{2+}]_{cyt}$ elevations in the guard cells of WT plants and *tgg1-3 tgg2-1* (Fig. 5D–F), suggesting that TGG1 and TGG2 function downstream of RCS production and upstream of $[Ca^{2+}]_{cyt}$ elevations in the ABA signal pathway in guard cells.

Zhao et al. (2008) proposed that isothiocyanate might modify plasma membrane inward-rectifying K^+ channels in the guard cells, leading to the inhibition of stomatal opening through inactivation of the inward-rectifying K^+ channels. This study shows that acrolein induces stomatal closure in WT, *tgg1-3* and *tgg2-1*, but not in the *tgg1-3 tgg2-1* double mutant, and that exogenous Ca^{2+} induces stomatal closure (Fig. 2A) and elicits $[Ca^{2+}]_{cyt}$ elevation (Fig. 5D–F) in all lines. Both EGTA and $LaCl_3$ significantly inhibited the acrolein- and Ca^{2+}-induced stomatal closure (Fig. 3). This difference in stomatal responses in the *tgg1-3 tgg2-1* double mutant between acrolein and exogenous Ca^{2+} may be accounted for by the difference in the concentrations of extracellular Ca^{2+}. In other words, an extracellular substrate of the plasma membrane Ca^{2+}-permeable channels, Ca^{2+}, at higher concentrations may overcome the reduction in the activities of Ca^{2+}-permeable channels because TGG1 and TGG2 are unlikely to totally inhibit the channels. Butyrate inhibited acrolein- and Ca^{2+}-induced stomatal closure (Fig. 4). This result indicates that acrolein- and Ca^{2+}-induced stomatal closures require cytosolic alkalization and that the cytosolic alkalization is the downstream signaling event of $[Ca^{2+}]_{cyt}$ in Arabidopsis guard cells (Fig. 2A, C), which is consistent with previous results (Islam et al. 2010). Islam et al. (2009) reported that TGG1 and TGG2 can function downstream of ROS production in ABA signaling in Arabidopsis guard cells. ABA induced stomatal closure in WT, *tgg1-3* and *tgg2-1*, but not in the *tgg1-3 tgg2-1* double mutant (Islam et al. 2009), while ABA induced ROS production in all lines (Supplementary Fig. S1; Islam et al. 2009). This study clearly indicates that TGG1 and TGG2 function downstream of RCS and upstream of cytosolic Ca^{2+} elevation and cytosolic alkalization in the ABA signaling in Arabidopsis guard cells. Taken together with the previous results, these findings allow us to propose a simple model for the position of TGG1, TGG2 and RCS production in the ABA signaling pathway in Arabidopsis guard cells (Fig. 6), where TGG1 and TGG2 function downstream of RCS and upstream of both cytosolic Ca^{2+} elevation and cytosolic alkalization.
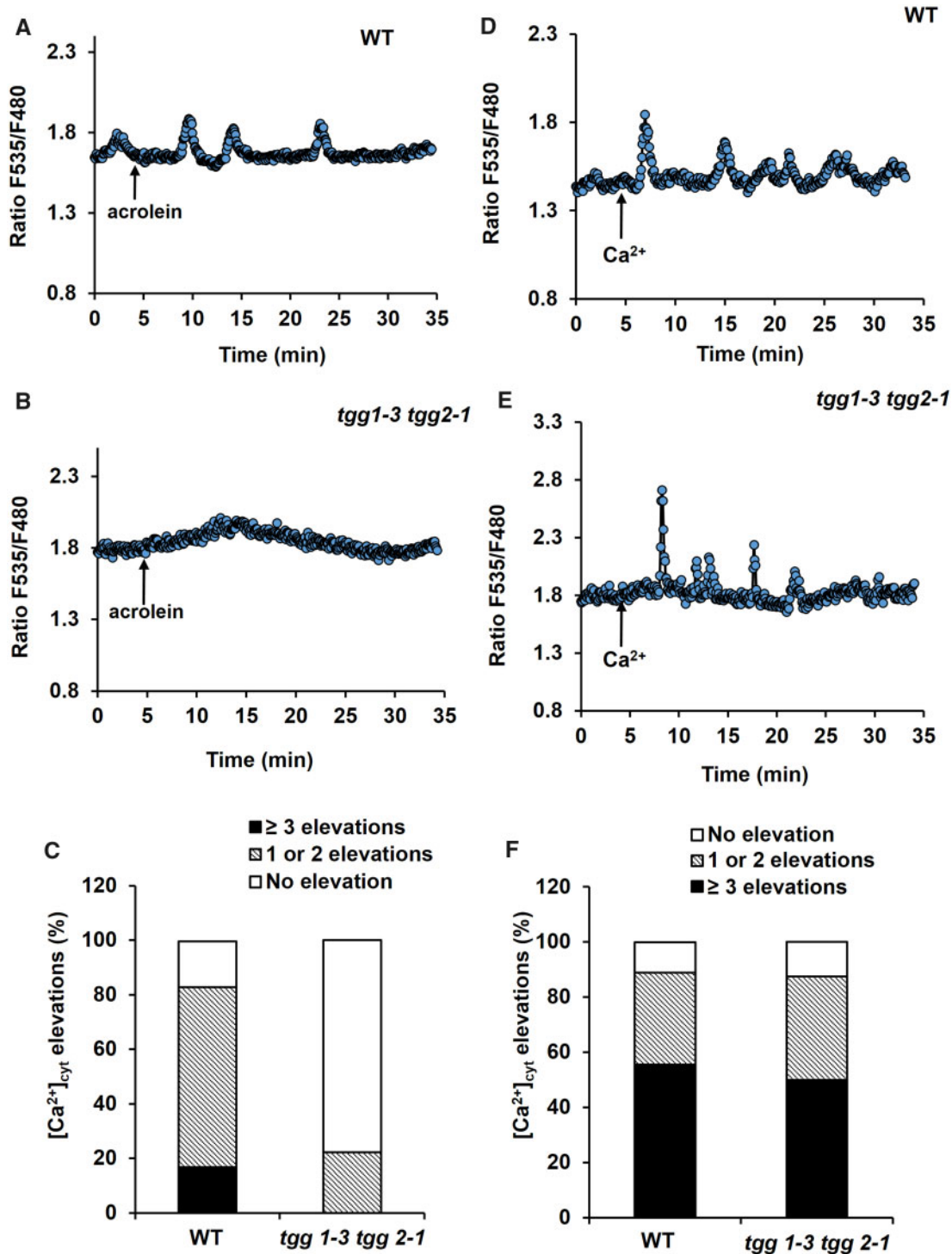
Fig. 5 Effects of acrolein and exogenous Ca^{2+} on $[Ca^{2+}]_{cyt}$ elevations in WT and *tgg1-3 tgg2-1* double mutant guard cells. The fluorescence ratio (535/480 nm) was measured in guard cells expressing YC3.6. (A) Representative fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ elevations in 10 μM acrolein-treated WT guard cells ($n = 30$). (B) Representative fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ elevations in 10 μM acrolein-treated *tgg1-3 tgg2-1* double mutant guard cells ($n = 22$). (C) Percentage bar chart showing the number of acrolein-induced $[Ca^{2+}]_{cyt}$ elevations in the WT and the *tgg1-3 tgg2-1* double mutant guard cells. (D) Representative fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ elevations in 1 mM Ca^{2+}-treated WT guard cells ($n = 18$). (E) Representative fluorescence emission ratio (535/480 nm) showing $[Ca^{2+}]_{cyt}$ elevations in 1 mM Ca^{2+}-treated *tgg1-3 tgg2-1* double mutant guard cells ($n = 16$). (F) Percentage bar chart showing the number of Ca^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations in the WT and the *tgg1-3 tgg2-1* double mutant guard cells. $[Ca^{2+}]_{cyt}$ transient elevations were counted when changes in $[Ca^{2+}]_{cyt}$ ratios were ≥ 0.1 units.
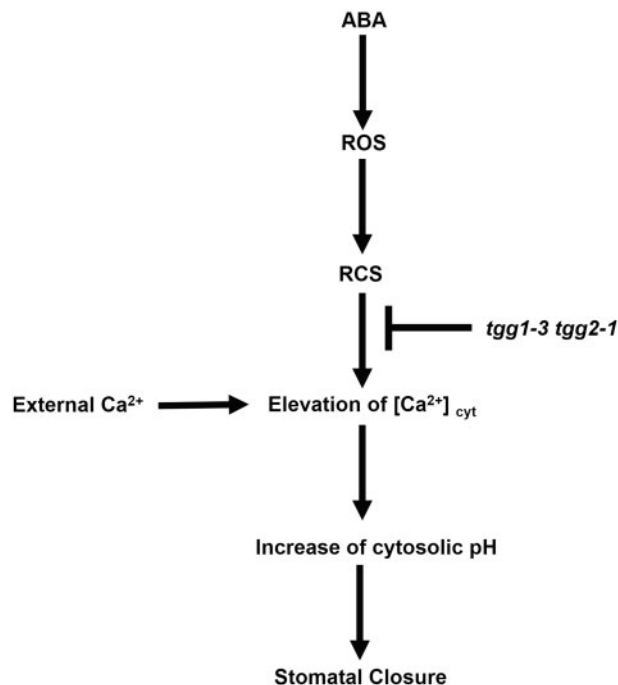
Fig. 6 Simplified model for the ABA signaling pathway in Arabidopsis guard cells. The myrosinases TGG1 and TGG2 function downstream of RCS and upstream of cytosolic Ca^{2+} elevation and cytosolic alkalization in ABA signaling in Arabidopsis guard cells.

Myrosinases are spatially separated from their substrates, glucosinolates, in plants. Upon herbivore attack, plants use myrosinases to convert glucosinolates to isothiocyanates and so on. However, it is difficult for ABA to induce the breakdown of glucosinolate mediated by TGG1 and TGG2 because ABA is unlikely to release the separation of TGG1 and TGG2 from glucosinolates. Hence, the myrosinase activities of TGG1 and TGG2 may not be essential, but TGG1 and TGG2 may directly interact with ABA signal component(s), such as Ca^{2+} permeable channels, and may regulate the component(s) by an unknown mechanism.

Conclusion

It is concluded that two myrosinases, TGG1 and TGG2, function redundantly downstream of RCS and upstream of cytosolic Ca^{2+} elevation along with cytosolic alkalization in ABA signaling in Arabidopsis guard cells.

Materials and Methods

Plant materials and growth conditions

Arabidopsis ecotype Columbia-0 was used as the WT plant. In controlled growth conditions, WT, *tgg1-3* (SAIL_786_B08), *tgg2-1* (SALK_038730) and *tgg1-3 tgg2-1* mutant plants (Barth and Jander 2006) were grown in plastic pots filled with soil:vermiculite (v/v) 1:1 mixture under a 16-h light period with 80 μmol m^{-2} s^{-1} photon flux density and a 8-h dark period. The temperature and relative humidity in the growth chamber were 21 ± 2°C and 70%, respectively. Water containing 0.1% Hyponex (Hyponex Japan, Osaka, Japan)

nutrient solution was applied to the plant growth tray one time per 2 weeks. The mutant lines were confirmed by genomic PCR (**Supplementary Fig. S2**). The left genomic primer, right genomic primer and T-DNA border primer were as follows: *tgg1-3*: CTCTCACATGTACCACTCTATT, CCACTCACTTGATGACTTTACT and TAGCAT CTGA ATTTCATAACCAATCTCGATACAC; *tgg2-1*: GGAGATTCTT CCACCGAACCC, CGTTTAACATCCCTCTTGGGTGG and ATTTTGCC GATTTCGGAAC.

Acrolein preparation

Acrolein was prepared from acrolein diethyl acetal (Tokyo Chemical Industry Co. Ltd.). Acrolein diethyl acetal was dissolved in 0.1 M HCl, and the solution was incubated at 40°C for 40 min. Then, NaHCO$_3$ was added to the solution until bubbles are no longer produced. The neutralized solution was used for experiments in this study.

Measurement of stomatal aperture

Stomatal apertures were measured as reported by Munemasa et al. (2007) with modifications. Excised leaves of 4- to 6-week-old plants were floated on an assay solution containing 5 mM KCl, 50 μM CaCl$_2$ and 10 mM MES-Tris (pH 6.15). The leaves were incubated for 2 h under light, acrolein was added to the pre-incubated leaves and then again leaves were incubated in the light for 2 h. For the measurement of stomatal apertures, the leaves were shredded in a commercial blender for 25 s and epidermal tissues were collected using a nylon mesh (pore size, 60 μm). Twenty stomatal apertures were measured for each individual experiment.

Measurement of cytosolic pH

Changes in pH in guard cells were analyzed by using a pH-sensitive fluorescent dye, BCECF-AM (Islam et al. 2010), with modifications. Five or six leaves from 4- to 6-week-old plants were shredded in a commercial blender for 40 s, and epidermal tissues were collected using a nylon mesh (pore size, 60 μm). The collected epidermal tissues were incubated for 2 h in the medium containing 5 mM KCl, 50 μM CaCl$_2$ and 10 mM MES-Tris (pH 6.15) and then 20 μM BCECF-AM was added to this medium. Then, epidermal tissues were again incubated for 30 min in the dark at room temperature to load the dye. After 30 min from the dye loading, the excess dye was washed out with distilled deionized water and then 10 μM acrolein was added. Twenty minutes later, fluorescence was observed and imaged under a fluorescence microscope and analyzed using ImageJ software.

Measurement of $[Ca^{2+}]_{cyt}$ elevations

Four- to six-week-old leaves of Arabidopsis YC3.6-expressing plants were employed as a Ca^{2+} indicator to monitor $[Ca^{2+}]_{cyt}$ in Arabidopsis guard cells as described previously (Munemasa et al. 2011). The excised leaves were gently mounted on a glass slide by a medical adhesive to remove the adaxial epidermis and the mesophyll tissue. The mounted abaxial epidermal peels were placed in a 6-wall plate containing 5 mM KCl, 50 μM CaCl$_2$ and 10 mM MES-Tris (pH 6.15) for 2 h under light incubation. The turgid guard cells were treated with 10 μM acrolein using a pipette at 5 min from the start of measurement. We selected 6–8 guard cells from one sample leaf for each experiment and repeated the experiment using different sample leaves on different days. Monitoring of YC3.6 was done by dual-emission ratio imaging. For the imaging of dual-emission ratio, we used a 440AF21 excitation filter, a 445DRLP dichroic mirror and two emission filters 480DF30 for cyan fluorescent protein (CFP) and 535DF25 for yellow fluorescent protein (YFP). The CFP and YFP fluorescent intensities of guard cells were imaged and analyzed using AQUA COSMOS software (Hamamatsu Photonics).

Statistical analysis

The significance of differences between mean values was assessed using Student's *t*-test and Tukey's test. Differences in the frequency of $[Ca^{2+}]_{cyt}$ elevations in the WT and *tgg1-3 tgg2-1* mutants induced by acrolein and Ca^{2+} were determined by χ^2 test. We regarded differences at the level of $P < 0.05$ as statistically 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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